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Linoleic and α-linolenic acid both prevent insulin resistance but have divergent impacts on skeletal muscle mitochondrial bioenergetics in obese Zucker rats

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ABSTRACT

The therapeutic use of polyunsaturated fatty acids (PUFA) in preserving insulin sensitivity has gained interest in recent decades; however, the roles of linoleic acid (LA) and α-linolenic acid (ALA) remain poorly understood. We investigated the efficacy of diets enriched with either LA or ALA on attenuating the development of insulin resistance (IR) in obesity. Following a twelve-week intervention, LA and ALA both prevented the shift towards an IR phenotype and maintained muscle-specific insulin sensitivity otherwise lost in obese control animals. The beneficial effects of ALA were independent of changes in skeletal muscle mitochondrial content and oxidative capacity, as obese control and ALA treated rats showed similar increases in these parameters. However, ALA increased the propensity for mitochondrial H_2O_2 emission and catalase content within whole-muscle, and reduced markers of oxidative stress (4-HNE and carbonyl content). In contrast, LA prevented changes in markers of mitochondrial content, respiratory function, H_2O_2 emission and oxidative stress in obese animals, thereby resembling levels seen in lean animals. Together, our data suggests that LA and ALA are efficacious in preventing IR but have divergent impacts on skeletal muscle mitochondrial content and function. Moreover, we propose that LA has value in preserving insulin sensitivity in the development of obesity; thereby challenging the classical view that n-6 PUFAs are detrimental.

KEYWORDS: Polyunsaturated fatty acids, insulin resistance, mitochondria

INTRODUCTION

Skeletal muscle, given its mass and capacity for insulin-stimulated glucose uptake, has been implicated in the development of IR in obesity. The use of PUFA as a treatment modality has gained considerable interest, with a particular emphasis on the insulin-sensitizing effects of long-chain n-3 PUFA [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] (33, 39, 50). EPA and DHA are proposed to improve insulin sensitivity through remodeling of mitochondrial membrane phospholipid composition (28), reduced intramuscular accumulation of reactive lipid intermediates (33), and increased transcription of gene targets involved in mitochondrial biogenesis and fatty acid oxidation (FAO) (24, 33, 39). Together, the prominent theory surrounding n-3 PUFA and insulin sensitivity suggests improvements in mitochondrial bioenergetics.

Mitochondrial dysfunction is a central hypothesis in the progression of skeletal muscle IR and is traditionally characterized by reduced content or impairment of function affecting rates of FAO (26). However, given that increased mitochondrial content can parallel the development of IR (15, 19, 53) and that the capacity of ATP production far exceeds reductions in content (16, 21), the relationship between mitochondrial dysfunction and IR remains largely unresolved. Recently, alterations in mitochondrial bioenergetics have also been associated with increased reactive oxygen species (ROS) emission-induced IR (2), while pharmacological and genetic approaches that increase antioxidants prevent diet-induced IR (6, 35). Therefore, therapies that improve mitochondrial oxidative phosphorylation or reduce mitochondrial ROS emission may be particularly advantageous. While n-3 PUFA appear ideal in mitigating IR, improved insulin sensitivity with EPA and DHA occurs independent of improvements in mitochondrial content or function (33), but is known to increase mitochondrial susceptibility to oxidative damage (i.e.

lipid peroxidation) and propensity to emit ROS (33). Clearly the mechanistic relationship between long-chain n-3 PUFA and insulin sensitivity remains to be fully delineated.

In comparison to EPA and DHA, little is known about the relationship between IR and α-linolenic acid (ALA). Although ALA can be endogenously converted into EPA/DHA, tracer studies have revealed that the conversion efficiency is low (< 8%) (44, 54). Therefore, it is conceivable that ALA and EPA/DHA have divergent effects on insulin sensitivity, although this remains to be shown. In contrast, n-6 PUFA have traditionally been viewed as detrimental to insulin sensitivity, in part, because they serve as precursors for the production of pro-inflammatory eicosanoids (14); however, this view has been challenged as accumulating evidence suggests not all n-6 PUFA are pro-inflammatory (25). Interestingly, LA may also influence mitochondrial function as it is the predominant fatty acyl moiety in the mitochondrial-specific phospholipid species cardiolipin (28). Current estimates suggest that n-6 PUFA are consumed in 5 to 20-fold greater amounts than n-3 PUFA (7); however, the health benefits of LA supplementation remain ambiguous. This highlights the need to study LA and the mechanisms by which it may influence IR in obesity.

We therefore investigated in young obese Zucker rats if LA and ALA enriched diets could prevent the expected age-related decline in glucose homeostasis. Skeletal muscle mitochondria exist in two spatially distinct subpopulations known as subsarcolemmal (SS) and the predominant intermyofibrillar (IMF) mitochondria. These subpopulations possess unique characteristics (5, 17, 38, 41) and respond differently to various metabolic perturbations in obesity and type 2 diabetes (T2D) (9, 19, 45), as well as changes in diet composition (8, 36). We therefore also determined subpopulation-specific responses of SS and IMF mitochondria to LA and ALA enriched diets, and the necessity of adaptations within these mitochondria in mitigating

IR. Altogether our data suggest that both LA and ALA prevented impairments in whole-body glucose homeostasis consistently seen with obese Zucker rats, and have differential effects on SS mitochondrial content and function.

MATERIALS AND METHODS

Animals: Five-week old male lean (n=48) and obese (n=48) Zucker rats were purchased from Charles River. Animals were housed in a temperature-regulated room on a 12:12 hr light-dark cycle with water available *ad libitum*. Control animals were given unrestricted access to control diet while treated animals within each genotype were pair-fed to match for caloric content. After twelve-weeks animals were randomly assigned to either determine whole body and muscle specific insulin sensitivity (n=6) or for assessments of mitochondrial bioenergetics (n=10). Anesthetia (60 mg/kg sodium pentobarbital injection), animal care, and housing procedures were approved by the University of Guelph Animal Care Committee.

Diets and Feeding: All diets used in the present study were purchased through Research Diets (New Brunswick, NJ, US). Daily food consumption of lean and obese rats fed the control diet (#AIN-93G; 20% protein, 64% carbohydrate and 16% fat) was recorded by weight in order to pair-feed rats given LA (#AIN-93G + 10% safflower oil; 20% protein, 54% carbohydrate and 26% fat) and ALA (#AIN-93G + 10% flaxseed oil; 20% protein, 54% carbohydrate and 26% fat) supplemented diets. Diet fatty acid composition was confirmed by gas-chromatography.

Whole-body glucose and insulin tolerance: Four-hour fasted animals underwent an intraperitoneal glucose (IPGTT, 2 g/kg) and insulin (IPITT, 1.0 U/kg) tolerance test separated by 48 hours, as previously described (23).

Muscle specific insulin signaling: To determine the phosphorylation of proteins involved in insulin-mediated signaling by Western blotting (described below) muscle was excised before and 15 minutes after an intraperitoneal insulin injection (1.0 U/kg), and rapidly frozen in liquid nitrogen.

Skeletal muscle mitochondrial isolation: Isolation of SS and IMF mitochondria was achieved by differential centrifugation. The respective speeds of centrifugation at each step were adapted from previous work (11), as well as the chemical composition of isolation buffer (52). The exact protocols used in the present study were previously reported (32).

Mitochondrial Bioenergetics: Rates of mitochondrial oxygen consumption and mitochondrial hydrogen peroxide (H_2O_2) emission were measured, as previously reported (32). In addition, separate experiments were performed to measure rates of oxygen consumption in the presence of 25 μM palmitoyl-CoA (P-CoA) + 2 mM malate + 750 μM L-carnitine. A submaximal (100 μM) ADP concentration was used to determine P/O ratios and a saturating ADP concentration (5 mM) to determine maximal P-CoA driven respiration.

Western blotting: Whole-muscle homogenate (n=6) as well as isolated SS and IMF mitochondrial samples were separated by electrophoresis using SDS-PAGE, transferred to polyvinylidene difluoride membranes, and quantified, as previously reported (32). The following commercially available antibodies were used: total and phosphorylated (Thr308 and Ser473) Akt (Cell Signalling), total and phosphorylated (Thr642) AS160 (Cell Signalling), MitoProfile Total OXPHOS antibody cocktail (MitoSciences), adenine nucleotide translocase 1 (ANT1, MitoSciences), ANT2 (Abcam, Cambridge, MA), manganese-superoxide dismutase (SOD2; Abcam), uncoupling protein 3 (UCP3, Abcam), and 4-hydroxynonenal (Alpha Diagnostics). All

147 samples were detected from the same Western blot by cutting gels and transferring onto a single
148 membrane to limit variability. Equal loading of protein was verified using Ponceau staining.
149 **Protein Carbonylation:** The commercially available Oxyblot Protein Oxidation Detection Kit
150 (Millipore; Billerica, MA) was used to assess protein carbonylation, as previously described
151 (40).
152 **Statistics:** A one-way ANOVA, followed by a Newman-Keuls Multiple Comparison post-hoc
153 analysis was used to determine the effects of LA and ALA supplementation within genotypes. It
154 was determined that diets did not affect markers of interest in lean animals, thus permitting the
155 use of an unpaired Student's t-test to compare diet-matched lean and obese Zucker rats for
156 subsequent analyses (Figures 1-7). A $p \leq 0.05$ was considered statistically significant.
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158

RESULTS

LA and ALA maintain whole-body glucose homeostasis

LA and ALA did not alter glucose or insulin tolerance in lean animals (Figure 1A, C). In contrast, obese control rats had elevated fasting blood glucose compared to control lean rats (13.9±2.1 mM vs. 5.0±0.2 mM), which resulted in an increased AUC during both glucose and insulin intolerance tests. However, when the baseline values where adjusted to take into consideration the obesity related increase in fasting blood glucose, consumption of both ALA and LA prevented glucose and insulin intolerance in obese animals (Figure 1C-F). Specifically, the baseline value during the IPGTT was constrained as the lowest individual glucose concentration within each genotype (lean = 3.9 mM and obese = 5.5 mM) (Figure 1E), while during the IPITT individual baseline values were set as the lowest blood glucose value of each animal. This method adjusts for the elevated basal glycaemia of obese control rats, allowing for a more concrete assessment of glucose and insulin action independent of fasting blood glucose levels. Accordingly, we report that obese control rats exhibit a substantially greater AUC during both glucose (+70%) and insulin challenges (+84%) relative to lean controls, while no differences were observed between diet-match animals fed LA and ALA (Figure 1E and F). Altogether, these data suggest that both LA and ALA prevented the development of insulin resistance in obese Zucker rats.

LA and ALA preserve skeletal muscle insulin signaling

Several parameters can influence whole body glucose and insulin tolerance independent of skeletal muscle insulin sensitivity (e.g. glucose/insulin actions within adipose tissue, liver and pancreas). Therefore it was important to specifically determine skeletal muscle insulin sensitivity in obese animals following LA and ALA supplementation. To determine this we next

investigated the ability of insulin to induce phosphorylation of proteins involved in the conical insulin-signaling cascade. Within lean and obese animals there were no differences in total content of Akt and AS160 protein (Figure 2A). In obese control animals, insulin failed to stimulate phosphorylation of Akt at serine 473 (Figure 2B) and threonine 308 (Figure 2C), as well as AS160 at threonine 642 (Figure 2D), above basal levels (Figure 2A). In contrast, obese rats supplemented with ALA maintained insulin-induced phosphorylation of Akt Ser473 (+100%) and Thr308 (+75%), as well as AS160 Thr642 (+40%) (Figures 2B-D). While LA evoked similar improvements in Akt phosphorylation at both sites, no changes were seen with AS160 (Thr642). These data, in combination with the IPITT results, suggest both LA and ALA maintain skeletal muscle insulin signaling in obese Zucker rats.

ALA preferentially increases ETC proteins in SS mitochondria

The accumulation of OXPHOS proteins in whole-muscle extracts was not different following ALA or LA supplementation compared to the control (Figure 3 A-F). We therefore re-examined OXPHOS protein content in purified SS and IMF mitochondrial fractions. Compared to lean controls, obese control rats showed a significant increase (+100%) in ATP synthase content in SS mitochondria only (Figure 4A, F). Interestingly, SS mitochondria from obese ALA rats showed significant increases in complex I subunit NUDFB8 (+100%), complex III subunit core 2 (+80%), and ATP synthase (+150%) as observed in obese control animals (Figures 4A, B, D, F). In contrast, the content of electron transport chain (ETC) markers in mitochondria isolated from LA supplemented rats appeared identical to diet-matched leans (Figures 4A-F), suggesting an absence of compensatory adaptations. Finally, IMF mitochondria remained constant for all OXPHOS protein targets measured across all groups (Figure 4A-F), likely accounting for the inability to detect SS mitochondrial adaptations at the whole-muscle level.

LA prevents compensatory bioenergetic adaptations in obesity

We next determined if mitochondrial bioenergetics were altered by measuring rates of oxygen consumption and H_2O_2 emission in isolated SS and IMF mitochondria. To confirm the integrity of our isolation protocol, mitochondrial ADP:O and respiratory control ratios (RCR), as well as absolute values of state 3 and 4 respiration, are presented in Table 1. Following analysis it was determined that mean values for all lean animals did not vary significantly; therefore, for simplicity, we present subsequent analyses as a percent change corresponding to diet-matched lean animals.

We measured mitochondrial pyruvate and palmitoyl-CoA supported respiration as a primary assessment of respiratory function. In comparison to lean animals, obese controls exhibited significant increases in maximal ETC capacity (complex I and complex I+II) of SS mitochondria only (Figure 5B-F), analogous to that observed in ATP synthase protein content (Figure 4). Similar adaptations were seen in obese ALA rats compared to their lean counterparts, including a significant increase in pyruvate-supported state 4 respiration (Figure 5A). In contrast, respiration of SS mitochondria from obese LA rats was identical to lean animals; thus fitting with the observed expression of OXPHOS proteins. Rates of oxygen consumption in IMF mitochondria were similar in all groups and diets. Altogether, it appears that the improvements in whole-body and muscle-specific insulin sensitivity conferred by ALA and LA are associated with distinct impacts on mitochondrial content and function.

Mitochondrial H_2O_2 emission and markers of oxidative stress

We next determined if PUFA supplementation was associated with a reduced mitochondrial H_2O_2 emission and oxidative stress. Obese control rats did not display increased SS or IMF mitochondrial H_2O_2 emission (Figure 6A), while ALA increased maximal H_2O_2 emission rates in

SS mitochondria by 80% (Figure 6A). When expressed relative to absolute state 4 respiration values (Table 1), diet-specific differences in H_2O_2 emission were abolished and resembled that of lean animals. Interestingly, a recent study showed that EPA/DHA supplementation in mice on a high fat diet increased ROS emission in isolated mitochondria using a similar approach (33). Therefore, we sought to rule out the contribution EPA/DHA-derived lipid radicals, which could potentially interact with amplex red to artificially increase background fluorescence. Using purified EPA and DHA at concentrations known to exist in rat mitochondria, we showed that increased H_2O_2 emission following ALA supplementation was not a methodological artifact (data not shown). In addition, LA supplementation did not alter maximal H_2O_2 emission in obese animals (Figure 6A). To assess the implications of the change in maximal H_2O_2 emission, we quantified protein carbonyls and 4-hydroxynonenal (4HNE) content (a marker of lipid peroxidation) in whole muscle extracts and in isolated mitochondria. Protein oxidation was not significantly altered by genotype or by diet in whole muscle extracts (Figure 6B) or in isolated mitochondrial fractions (Figure 6C). In contrast, these analyses revealed that, despite unaltered rates of mitochondrial H_2O_2 emission, obese control animals have a significant increase in total muscle lipid peroxidation (+30%), suggesting the presence of oxidative stress (Figure 6D). Furthermore, 4HNE was increased ~70% within IMF mitochondria of obese controls, but was reduced in SS mitochondria (Figure 6E). Overall, in obesity LA prevented changes in 4HNE content within whole muscle (Figure 6D) and SS/IMF mitochondria (Figure 6E), resembling lean healthy animals. ALA supplementation also prevented increases in 4HNE content within whole muscle (Figure 6D) and IMF mitochondria (Figure 6E) seen in obese control rats.

Mitochondrial ADP transport, uncoupling, and antioxidant proteins

Given the apparent discrepancy between maximal *in vitro* mitochondrial H_2O_2 emission rates and *in vivo* markers of oxidative stress, we next examined the expression of proteins known to influence mitochondrial H_2O_2 emission. ANT1 content did not change in SS mitochondria across all groups and diets (Figure 7A). In contrast, ANT1 content in IMF mitochondria from obese control rats increased significantly (+100%), but were comparable to a lean phenotype in obese LA and ALA groups (Figure 7A). Relative to lean healthy animals, ANT2 was decreased 50% in SS mitochondria of obese controls; however, this change was prevented with LA and ALA supplementation (Figure 7B). In obese rats the abundance of UCP3 (Figure 7C) in SS mitochondria was increased ($p<0.05$) across all diet groups (control +250%; ALA +300% and LA +500%). Finally, SOD2 content in SS mitochondria of obese controls showed a trending increase ($p=0.07$) and was significantly elevated in IMF mitochondria (+50%). These changes were prevented by LA and ALA in obese rats (Figure 7D), as SOD2 content was similar to lean animals. In contrast, catalase content in muscle homogenate was not altered in either obese control or LA groups, but was significantly increased (+60%) in obese rats fed ALA (Figure 7E).

DISCUSSION

The current study shows that the development of IR in obesity can be prevented by dietary supplementation with LA and ALA. Strikingly, a moderate 10% isocaloric increase in either of these PUFA species was efficient in attenuating the impaired glucose homeostasis documented in a common genetic model of obesity and insulin resistance. These findings were associated with the conservation of skeletal muscle insulin signaling and oxidative stress relative to lean healthy animals. Examining aspects of mitochondrial dysfunction revealed that LA and ALA have markedly different impacts on SS mitochondrial ETC content and bioenergetics compared to IMF. This was further supported by ALA-specific increases in maximal H_2O_2 emission in SS mitochondria, as well as the expression of catalase. Overall, the current data supports a beneficial link between ALA and insulin sensitivity, and provides novel evidence that LA can prevent impairments in glucose homeostasis and skeletal muscle insulin sensitivity in a model of severe genetic obesity.

ALA attenuates the development of whole-body glucose homeostasis

Obese Zucker rats display normal glucose and insulin tolerance at 5 weeks of age (56), when the current dietary intervention was initiated, however rapidly display genetic obesity, hyperinsulinemia, hyperlipidemia and peripheral insulin resistance. Therefore, obese Zucker rats represent an attractive model to investigate nutritional approaches that prevent the development of IR (27). While the benefits of fish oil on insulin sensitivity are supported extensively in the literature (31, 33, 39, 46, 50), we provide evidence that ALA, the precursor of EPA and DHA, may also be efficacious in improving insulin sensitivity. Recent work suggests that fish oil supplementation promotes the expression of OXPHOS proteins in ameliorating IR (33). The current data supports this interpretation, as we observed similar increases in H_2O_2 emission,

OXPHOS proteins and catalase with ALA. We extend this model to show responses exclusive to SS mitochondria, which given the proximity to nuclei, may represent a local signal to drive gene transcription. These results are in agreement with our previous work (4, 19) and work from others (30, 36, 51); supporting the notion that significant changes within SS mitochondria can impact lipid homeostasis and insulin sensitivity.

LA prevents the decline of whole-body glucose homeostasis

In comparison to n-3s, n-6 PUFA have garnered a more negative reputation due to their links to oxidative stress, IR, and inflammation. However, multiple studies have challenged this view (3, 13, 14, 43, 47). Indeed, the current data shows that LA prevented the development of whole-body glucose intolerance, maintained muscle specific insulin sensitivity, and 4HNE content in obese animals. In contrast to ALA, these improvements were independent of changes in H_2O_2 emission, OXPHOS proteins and antioxidant enzyme expression; raising the possibility of a divergent mechanism for improving insulin sensitivity. However, our findings do not exclude the possibility that LA remodels the membrane cardiolipin profile within mitochondria (28), which is known to impact mitochondrial function (18, 28). Although previous reports have linked obesity with changes that would likely promote mitochondrial H_2O_2 emission, including increased ETC sensitivity to reducing equivalents (34) and diminished sensitivity to ADP (49), we found no changes in maximal ADP-stimulated respiration following LA supplementation. Therefore, it remains possible that LA may alter the dynamic response of mitochondria to submaximal substrate concentrations. Regardless of the elusive mechanism-of-action, the current data provides convincing evidence that LA prevented IR in obese Zucker rats.

Mitochondrial H_2O_2 emission, uncoupling and antioxidant proteins

The current study only found increased ETC content within SS mitochondria, which represents ~20% of total cellular mitochondrial volume (12, 20), accounting for the absence of changes in whole muscle measurements. The current study cannot explain mechanistically why SS mitochondria preferentially respond, although this appears to be a conserved observation across cellular stresses (19, 36). However, the increased expression of ETC proteins within the SS mitochondria likely contributed to the increase in maximal mitochondrial H_2O_2 emission, as normalization of emission rates to state IV respiration negated all differences. The increase in ETC subunits following ALA supplementation may therefore be construed as a negative adaptation, as mitochondrial lipid uptake and ROS emission has been causally linked to IR (2), possibly through ROS-mediated activation of the NF-κB/IκB/IKKβ pathway, attenuating insulin signaling by serine phosphorylation of the insulin receptor substrate 1 (IRS1) (48, 55). However, this working model remains controversial, as chronic mitochondrial antioxidant treatment that improves cellular redox balance does not improve insulin sensitivity following a high-fat diet (40). Perhaps subtle increases in mitochondrial ROS emission are required for the transcriptional adaptations necessary to regulate metabolic homeostasis during a high fat challenge. Our data indirectly supports this model as ALA supplementation increased maximal H_2O_2 emission and OXPHOS proteins in SS mitochondria, as well as muscle catalase content. Our results are consistent with the suggestion that incorporating n-3 PUFA into mitochondrial membranes increases the propensity for ROS production (1, 33). Furthermore, *in vivo* markers of oxidative stress (4HNE and carbonyl content) suggest that despite an increase in maximal H_2O_2 emission, ALA supplementation conserved redox balance in whole-muscle and mitochondrial samples. Given that ROS have several intracellular functions, including participation in the complex signaling network involved in mitochondrial biogenesis (22, 37), perhaps the increased

OXPHOS expression within SS mitochondria in the obese ALA group is, in part, mediated through tightly regulated ROS signaling.

In contrast, we found no differences in maximal H_2O_2 emission or catalase content in lean and obese animals fed LA. Unlike the changes seen with obese control and ALA groups, mitochondria from the obese LA group were identical to their lean counterparts, suggesting that compensatory bioenergetic adaptations are not necessary to preserve glucose homeostasis and muscle insulin signaling while consuming LA. Previous work showed that arachidonic acid increased mitochondrial ROS emission and was linked to mitochondrial dysfunction (10). Surprisingly, the precursor LA did not alter rates of mitochondrial H_2O_2 emission, and therefore prevention of IR occurs through a mechanism not investigated in the current study. Although speculative, LA was shown to drive a lipoxygenase-mediated eicosanoid response, leading to production of the PPAR-α activating 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) (29). Therefore, if LA evokes a PPAR-mediated improvement in glucose homeostasis, the mechanism by which it acts may differ from that of n-3 PUFA. Alternatively, LA consumption may involve primary adaptations within the liver, pancreas and white adipose tissue and secondary responses within muscle, a possibility that has not been explored in the current study. Regardless, the current study provides evidence that LA is beneficial at preventing the development of insulin resistance.

Perspectives and Limitations

The current study provides insight on the link between ALA and insulin sensitivity, and evidence that LA supplementation represents additional therapeutic potential. Although aspects of mitochondrial dysfunction were very similar between obese control and ALA supplemented rats, the preservation of skeletal muscle insulin signaling and whole-body glucose homeostasis

355 highlights the value of this n-3 PUFA. The precise mechanism(s) by which LA and ALA exert
356 their preserving effects requires further elucidation, and may involve changes in liver given the
357 enhanced glucose tolerance. Indeed, EPA/DHA may augment hepatic IR and lipotoxicity by
358 increasing FAO, inhibiting *de novo* lipogenesis and reducing proinflammatory cytokine
359 production (42). Furthermore, within white adipose tissue, these n-3 PUFAs are known to
360 improve factors influencing IR such as adipocyte morphology, rates of endogenous FAO,
361 adipokine secretory profiles as well as immuno-metabolic status (as recently reviewed (42)).
362 Whether LA and ALA exert their effects through similar mechanisms remains to be shown.
363 In the current study we were unable to uncouple the effects of ALA from EPA and DHA;
364 therefore, it is possible that the effects seen with ALA are due to its conversion (albeit limited)
365 into EPA/DHA. Future work using animal models that prevent the conversion of ALA into
366 EPA/DHA will enable us to more definitively describe the independent role of ALA on skeletal
367 muscle insulin signaling. Also, in the current study the macronutrient composition of the LA and
368 ALA diets were out of necessity different than control diets, having higher fat (26% vs. 16% in
369 control diet), and by default decreased carbohydrate content (54% vs 64% in control diet).
370 Therefore, future studies should also determine if the modest 10% increase/decrease in dietary
371 fat/carbohydrate could over-ride the strong genetic predisposition for an IR phenotype.
372 More importantly, our data challenges the traditional view that LA is harmful, and
373 welcomes the reassessment of its use as a therapeutic strategy for preserving insulin sensitivity.
374 Despite observing no changes in mitochondrial content, function and maximal H_2O_2 emission in
375 isolated mitochondria, LA maintained skeletal muscle insulin signaling similar to ALA. Future
376 investigations should focus on changes in membrane phospholipid composition, as both n-3 and
377 n-6 PUFA are known to compete in the remodeling of membranes including mitochondria, and

may also preferentially accumulate in different tissues. The impacts of LA and ALA on IR may transcend the boundaries of skeletal muscle and mitochondria, but nevertheless, represent valuable therapeutic strategies for preventing the development of an insulin-resistant phenotype in obesity.

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FIGURE LEGENDS

Fig. 1. – Intraperitoneal glucose (IPGTT) and insulin (IPITT) tolerance tests for (A, C, respectively) lean and (B, D, respectively) obese rats. Black circles = control diet; black triangles = ALA diet; black squares = LA diet. Area under the curve (AUC) values for (E) IPGTT and (F) IPITT. Data expressed as means ± SEM. n = 6 for each measure. + significantly different from obese control ($P < 0.05$). * significantly different from diet-matched lean animals ($P < 0.05$).

Fig. 2. – Skeletal muscle insulin-signaling proteins in basal state and following insulin injection. (A) Representative blots of total and phosphorylated Akt (B, serine 473; C, threonine 308) and (D) AS160 threonine 642. Data for insulin-stimulated phosphorylation are expressed as means ± SEM of the percentage change from the basal state. n = 6 for each measure. * significantly different from basal state of same animal ($P < 0.05$).

Fig. 3. – Skeletal muscle homogenate mitochondrial OXPHOS proteins. (A) Representative blots reveal no changes in (B) complex 1 subunit NDUFB8; (C) complex II subunit 30 kDa; (D) complex III subunit Core 2; (E) complex IV subunit 4; (F) ATP synthase subunit α (F) of obese animals in comparison to diet-matched lean animals. n = 6 for each measure. Data expressed as mean ± SEM.

Fig. 4. – Changes in OXPHOS proteins of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. (A) Representative blots for (B) complex 1 subunit NDUFB8; (C) complex II subunit 30 kDa; (D) complex III subunit Core 2; (E) complex IV subunit 4; (F) ATP synthase subunit α (F). n = 10 for each measure. Data are expressed as mean ± SEM. * significantly different from diet-matched lean animals ($P < 0.05$).

Fig. 5. – Isolated subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial respiration. Basal (A) and ADP-stimulated (B) pyruvate supported respiration, (C) maximal complex I with glutamate and (D) maximal ETC respiration with succinate, basal (E) and ADP-stimulated (F) states of palmitoyl-CoA respiration. n = 10 for each measure. Data expressed as mean ± SEM. * significantly different from diet-matched lean animals ($P < 0.05$).

Fig. 6. – Markers of oxidative stress. (A) Maximal succinate H_2O_2 emission in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. (B) Protein oxidation in muscle homogenate and (C) SS and IMF mitochondria. (D) 4-hydroxynonenal (4-HNE) content in muscle homogenate and (E) SS and IMF mitochondria indicates lipid peroxidation. Representative blots shown in respective panels. n = 10 for each measure. Data expressed as mean ± SEM. * significantly different from diet-matched lean animals ($P < 0.05$).

Fig. 7. – Changes in (A) adenine nucleotide translocase 1 (ANT1), (B) ANT2, and (C) mitochondrial uncoupling protein 3 (UCP3), (D) manganese superoxide dismutase (SOD2) in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. For data shown in (A-D), n = 10 for each measure. (E) Catalase content in muscle homogenate; n = 6 for all groups. Data expressed as mean ± SEM. * significantly different from diet-matched lean animals ($P < 0.05$).

TABLE 1 – Mitochondrial respiratory characteristics

	Lean Control	Lean ALA	Lean LA	Obese Control	Obese ALA	Obese LA
SS Mitochondria – Pyruvate Respiration						
P/O Ratio	2.47 ± 0.07	2.56 ± 0.08	2.53 ± 0.06	2.57 ± 0.08	2.49 ± 0.09	2.56 ± 0.05
RCR	10.9 ± 1.21	9.6 ± 0.92	10.5 ± 1.21	13.2 ± 1.27	10.4 ± 1.11	12.9 ± 1.35
State 4	24.5 ± 3.28	21.1 ± 1.68	25.1 ± 3.38	26.6 ± 3.79	30.5 ± 3.21	18.9 ± 1.60*
State 3	247 ± 39.8	184 ± 13.9	262 ± 39.5	327 ± 41.6	297 ± 42.7	234 ± 23.0
IMF Mitochondria – Pyruvate Respiration						
P/O Ratio	2.48 ± 0.08	2.49 ± 0.07	2.35 ± 0.07	2.42 ± 0.07	2.42 ± 0.07	2.32 ± 0.07
RCR	20.5 ± 3.44	16.2 ± 2.62	15.7 ± 3.03	17.8 ± 3.15	20.5 ± 3.78	21.4 ± 2.72
State 4	26.8 ± 55.2	28.9 ± 52.2	25.8 ± 39.6	23.9 ± 33.5	22.8 ± 49.1	18.2 ± 31.7
State 3	426 ± 41.2	389 ± 45.1	348 ± 34.5	389 ± 49.3	364 ± 32.7	352 ± 52.5
SS Mitochondria – Palmitoyl-CoA Respiration						
P/O Ratio	2.27 ± 0.11	2.33 ± 0.10	2.26 ± 0.17	2.49 ± 0.12	2.28 ± 0.12	2.52 ± 0.10
RCR	9.11 ± 1.02	6.30 ± 0.58	5.75 ± 0.55	7.13 ± 0.70	9.34 ± 1.21	8.23 ± 0.72
State 4	14.2 ± 1.99	16.8 ± 1.85	22.9 ± 4.52	24.2 ± 2.97	29.7 ± 4.90	28.1 ± 5.70
State 3	116 ± 11.8	101 ± 13.6	142 ± 30.9	196 ± 34.4	238 ± 31.9	185 ± 33.9
IMF Mitochondria – Palmitoyl-CoA Respiration						
P/O Ratio	2.18 ± 0.22	2.26 ± 0.13	2.16 ± 0.14	2.65 ± 0.13	2.32 ± 0.13	2.33 ± 0.18
RCR	12.8 ± 2.47	11.8 ± 2.29	8.65 ± 1.75	10.2 ± 1.86	14.6 ± 3.11	10.5 ± 1.10
State 4	19.6 ± 6.91	25.5 ± 3.01	20.0 ± 16.1	21.7 ± 2.42	20.1 ± 3.94	16.9 ± 2.17
State 3	187 ± 28.5	158 ± 28.9	173 ± 21.4	220 ± 42.4	229 ± 33.7	178 ± 32.2

Absolute rates of oxygen consumption in the presence (state 3) and absence (state 4) of ADP for isolated subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria. Diets groups are control, α-linolenic acid (ALA) or linoleic acid (LA). State 3 and 4 values are expressed as nmol/min/mg mitochondrial protein. Respiratory control ratios (RCR; state 3/state 4) and ADP consumed per unit oxygen (P/O ratio) reflect mitochondrial integrity and coupling. Data are presented as the mean ± SEM. n = 10 for each measure. * Significantly different than obese ALA ($p<0.05$)

Figure 1

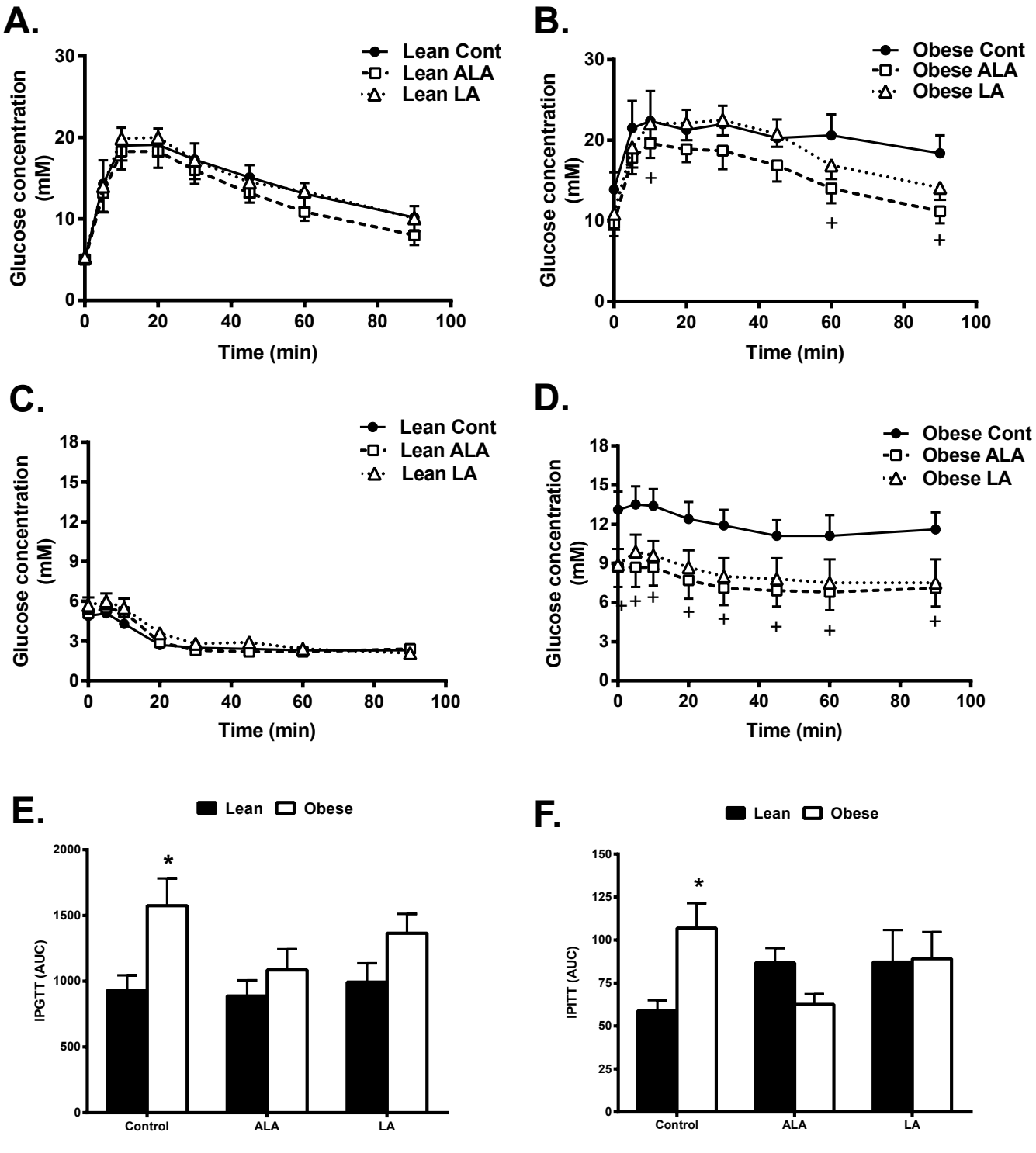

A.
Lean Cont
Lean ALA
Lean LA
Glucose concentration (mM)
Time (min)
B.
Obese Cont
Obese ALA
Obese LA
Glucose concentration (mM)
Time (min)
C.
Lean Cont
Lean ALA
Lean LA
Glucose concentration (mM)
Time (min)
D.
Obese Cont
Obese ALA
Obese LA
Glucose concentration (mM)
Time (min)
E.
Lean
Obese
IPGTT (AUC)
Control
ALA
LA
F.
Lean
Obese
IPITT (AUC)
Control
ALA
LA

Figure 2

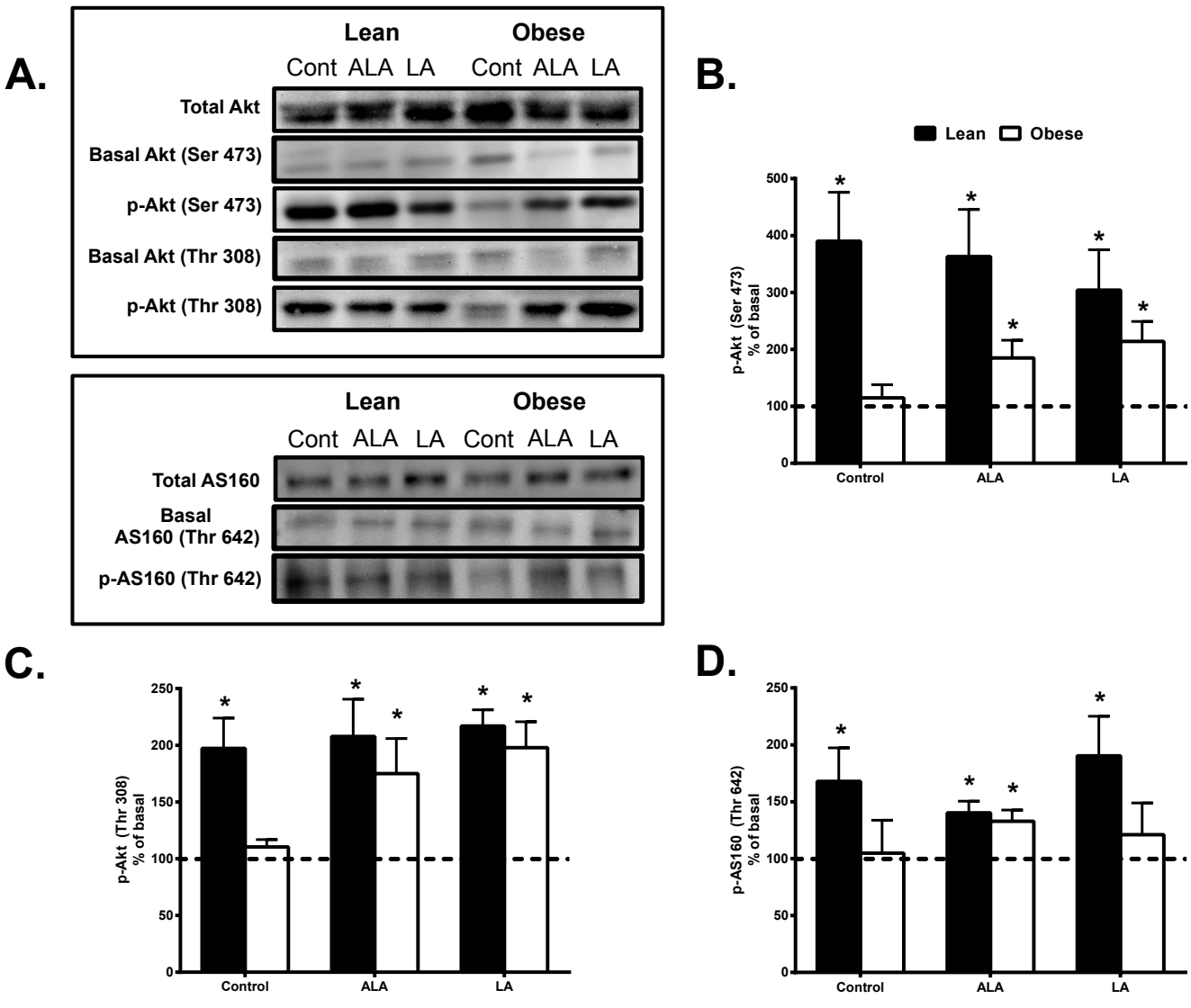

A.
Lean
Obese
Cont ALA LA Cont ALA LA
Total Akt
Basal Akt (Ser 473)
p-Akt (Ser 473)
Basal Akt (Thr 308)
p-Akt (Thr 308)
Lean
Obese
Cont ALA LA Cont ALA LA
Total AS160
Basal AS160 (Thr 642)
p-AS160 (Thr 642)
B.
Lean
Obese
p-Akt (Ser 473) % of basal
500
400
300
200
100
0
Control
ALA
LA
C.
p-Akt (Thr 308) % of basal
250
200
150
100
50
0
Control
ALA
LA
D.
p-AS160 (Thr 642) % of basal
250
200
150
100
50
0
Control
ALA
LA

Figure 3 – Revised blots

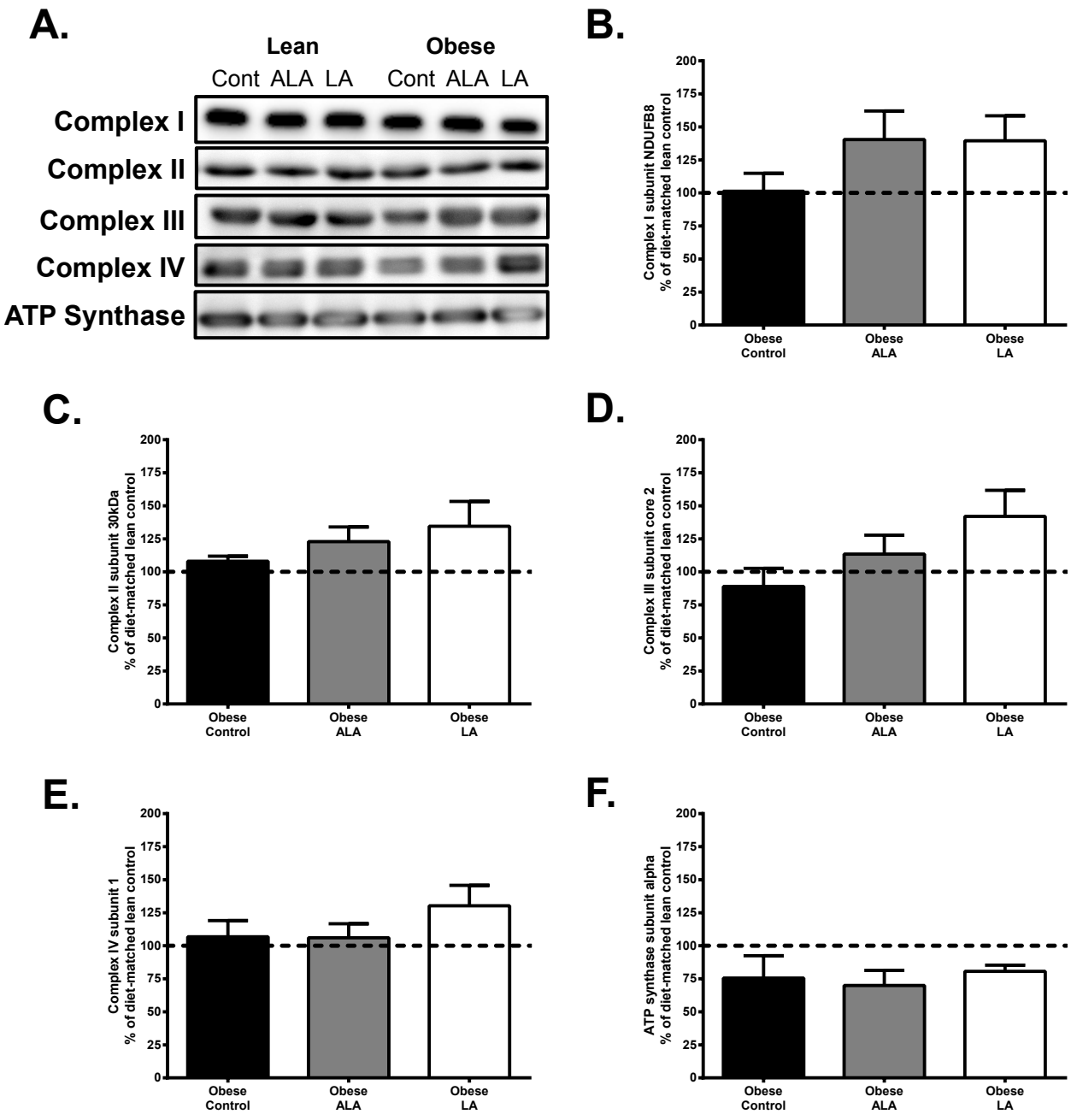


Figure 4

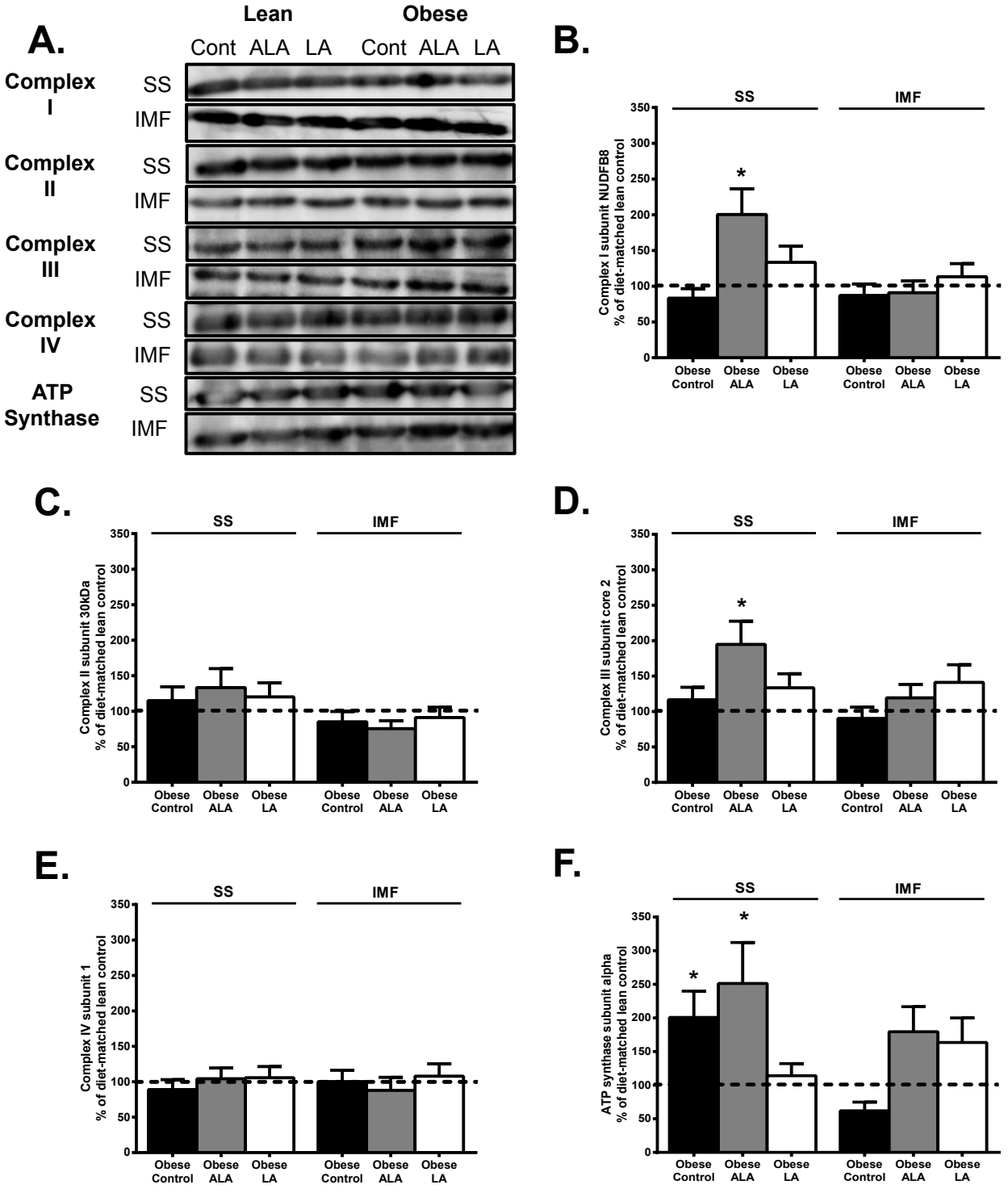

A.
Lean
Obese
Cont ALA LA Cont ALA LA
Complex I
Complex II
Complex III
Complex IV
ATP Synthase
SS
IMF
B.
SS
IMF
Complex I subunit NUDFB8
% of diet-matched lean control
C.
Complex II subunit 30kDa
% of diet-matched lean control
D.
Complex III subunit core 2
% of diet-matched lean control
E.
Complex IV subunit 1
% of diet-matched lean control
F.
ATP synthase subunit alpha
% of diet-matched lean control
Obese Control
Obese ALA
Obese LA

Figure 5

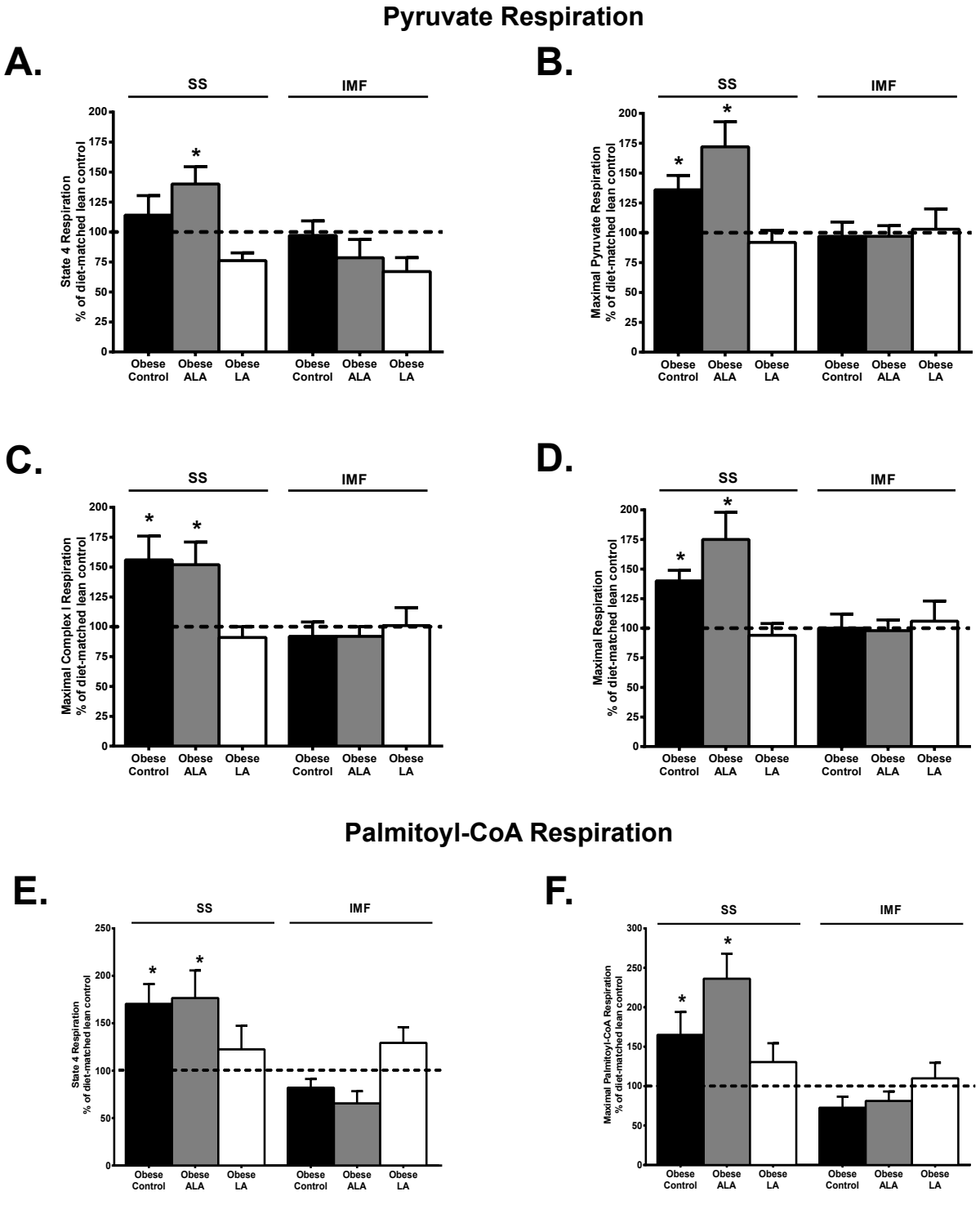

Pyruvate Respiration
A.
B.
C.
D.
SS
IMF
State 4 Respiration
% of diet-matched lean control
Maximal Pyruvate Respiration
% of diet-matched lean control
Maximal Complex I Respiration
% of diet-matched lean control
Maximal Respiration
% of diet-matched lean control
Obese Control
Obese ALA
Obese LA
Palmitoyl-CoA Respiration
E.
F.
Maximal Palmitoyl-CoA Respiration
% of diet-matched lean control

Figure 6

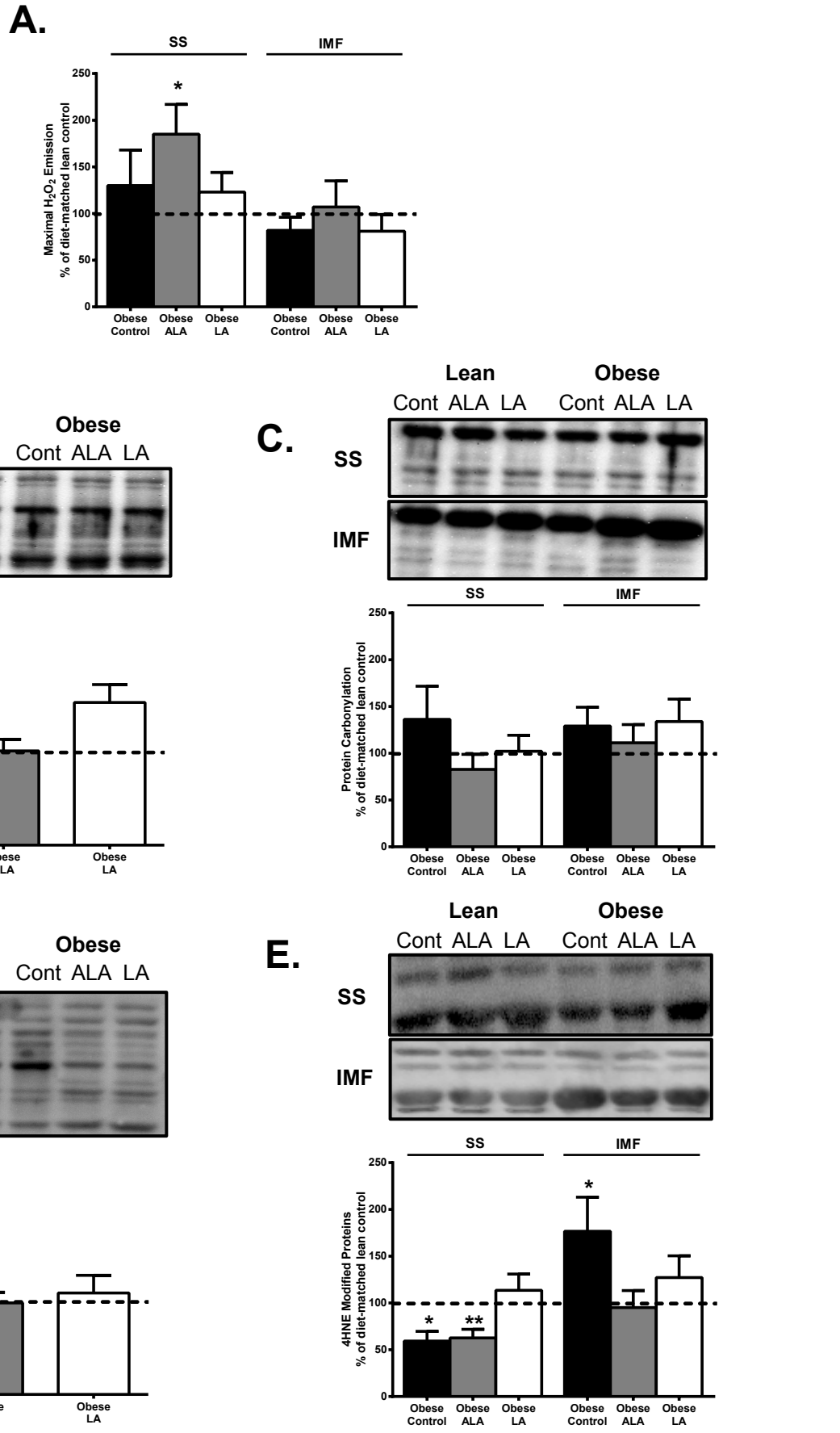
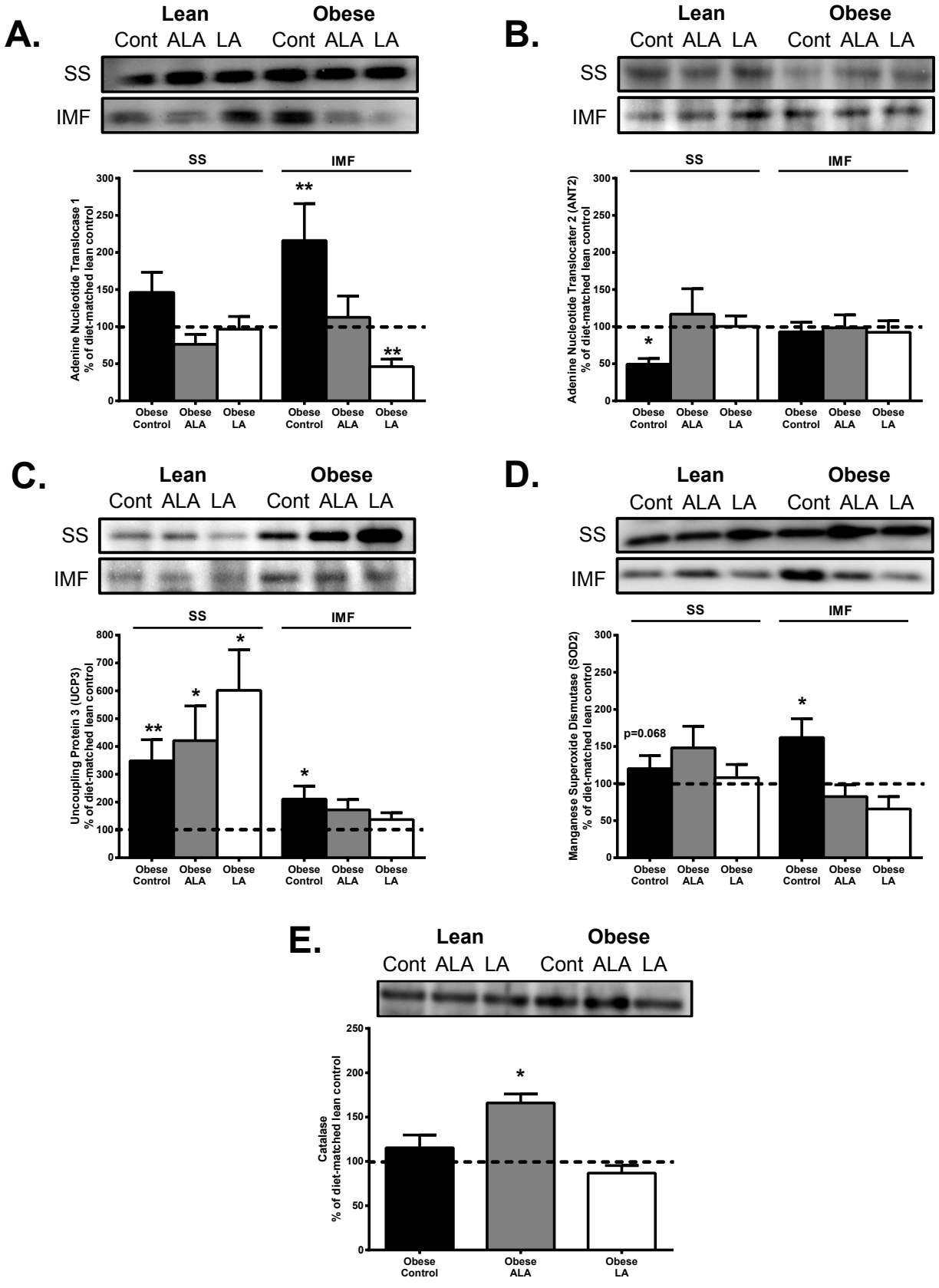

A.
SS
IMF
Maximal H_2O_2 Emission
% of diet-matched lean control
Obese Control
Obese ALA
Obese LA
B.
Lean
Obese
Cont ALA LA Cont ALA LA
Protein Carbonylation
% of diet-matched lean control
C.
SS
IMF
D.
4HNE Modified Proteins
% of diet-matched lean control
E.

Figure 7


A.
Lean
Obese
Cont ALA LA Cont ALA LA
SS
IMF
SS
IMF
Adenine Nucleotide Translocase 1
% of diet-matched lean control
Obese Control
Obese ALA
Obese LA
B.
Adenine Nucleotide Translocater 2 (ANT2)
% of diet-matched lean control
C.
Uncoupling Protein 3 (UCP3)
% of diet-matched lean control
D.
Manganese Superoxide Dismutase (SOD2)
% of diet-matched lean control
p=0.068
E.
Catalase
% of diet-matched lean control