Alison Ludzki,[1] Sabina Paglialunga,[1] Brennan K. Smith,[1,2] Eric A.F. Herbst,[1]
Mary K. Allison,[1] George J. Heigenhauser,[2] P. Darrell Neufer,[3] and
Graham P. Holloway[1]

Rapid Repression of ADP Transport by Palmitoyl-CoA Is Attenuated by Exercise Training in Humans: A Potential Mechanism to Decrease Oxidative Stress and Improve Skeletal Muscle Insulin Signaling

Diabetes 2015;64:2769–2779 | DOI: 10.2337/db14-1838

Mitochondrial ADP transport may represent a convergence point unifying two prominent working models for the development of insulin resistance, as reactive lipids (specifically palmitoyl-CoA [P-CoA]) can inhibit ADP transport and subsequently increase mitochondrial reactive oxygen species emissions. In the current study, we aimed to determine if exercise training in humans diminished P-CoA attenuation of mitochondrial ADP respiratory sensitivity. Six weeks of exercise training increased whole-body glucose homeostasis and skeletal muscle Akt signaling and reduced markers of oxidative stress without reducing maximal mitochondrial H_2O_2 emissions. To ascertain if enhanced mitochondrial ADP transport contributed to the improvement in the in vivo oxidative state, we determined mitochondrial ADP sensitivity in the presence and absence of P-CoA. In the absence of P-CoA, exercise training reduced mitochondrial ADP sensitivity. In contrast, exercise training increased mitochondrial ADP sensitivity with P-CoA present. We further show that P-CoA noncompetitively inhibits mitochondrial ADP transport and the ability of ADP to attenuate mitochondrial H_2O_2 emission. Altogether, the current data provide a potential mechanism for how P-CoA contributes to insulin resistance and highlight the ability of exercise training to diminish P-CoA attenuation in mitochondrial ADP transport.

Skeletal muscle, by virtue of its mass and high rate of insulin-stimulated glucose transport, represents an important tissue in the development of insulin resistance (1). Understanding the mechanisms that result in peripheral insulin resistance is critical to developing novel therapies. Within skeletal muscle, a large emphasis has been placed on establishing a mechanistic link between reactive lipid accumulation and the induction of insulin resistance. In particular diacylglycerol (DAG) (2) and ceramides (3) have been proposed to antagonize insulin signaling by mediating protein kinase C serine phosphorylation of the insulin receptor substrate and Akt phosphorylation, thereby attenuating insulin-stimulated glucose uptake (4–9). Similar to DAG and ceramides, long-chain fatty acyl-CoA (LCFA-CoA) accumulation is associated with insulin resistance in humans, and consumption of a high-fat diet rapidly accumulates LCFA-CoA moieties in association with insulin resistance (10,11). Collectively, these studies suggest a causal link between intramuscular LCFA-CoA accumulation and insulin resistance; however, a potential mechanism remains to be elucidated.

In addition to regulating reactive lipid accumulation, increased mitochondrial reactive oxygen species (ROS) emission has been implicated in the development of insulin resistance. Although the exact manner by which mitochondrial ROS causes insulin resistance has not been delineated, it has been suggested that activation of the NF-κB/IκB/IKKβ pathway results in serine phosphorylation of the insulin receptor substrate 1 (IRS1), attenuating insulin signaling at a proximal step (12,13). Two lines

---

[1] Department of Human Health & Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

[2] Department of Medicine, McMaster University, Hamilton, Ontario, Canada

[3] East Carolina Diabetes and Obesity Institute, Departments of Physiology and Kinesiology, East Carolina University, Greenville, NC

Corresponding author: Graham P. Holloway, ghollowa@uoguelph.ca.

Received 4 December 2014 and accepted 28 March 2015.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-1838/-/DC1.

© 2015 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

of evidence suggest that mitochondrial ROS has a primary role in the etiology of insulin resistance. First, mitochondrial ROS generation increases in response to fatty acid exposure (14,15) and high-fat feeding (16) in association with the development of insulin resistance. Second, attenuating mitochondrial ROS emission using either a mitochondrial-targeted antioxidant (SS31) or overexpression of mitochondrial antioxidant enzymes (catalase [CAT] and SOD2) prevents diet-induced insulin resistance (16–18). Therefore treatment modalities that target both reactive lipids and mitochondrial ROS may be particularly beneficial at recovering insulin sensitivity. ADP binding to F_1F_0 ATP synthase decreases membrane potential and the overall rate of superoxide production (15,19,20) while simultaneously increasing rates of substrate oxidation. Therefore, attenuations in mitochondrial ADP sensitivity can influence both reactive lipid accumulation and mitochondrial ROS emissions. Supporting this notion, we have recently shown in Zucker diabetic fatty rats that submaximal ADP-stimulated respiration is impaired in association with increased mitochondrial ROS emission in the presence of ADP (21). More recently, it was predicted in humans that adenine nucleotide translocase 1 (ANT1), which is required for ADP/ATP exchange across the inner mitochondrial membrane, is inhibited through acetylation of lysine 23 (22). In addition, ANT1 lysine 23 acetylation was reduced after acute exercise (22), suggesting that chronic exercise interventions may increase mitochondrial ADP sensitivity in association with improving insulin sensitivity. However, this remains to be determined, and the effect of exercise training on submaximal ADP transport kinetics is currently ambiguous. For instance, a cross-sectional analysis in humans with varying training statuses (23), as well as a chronic training program in rats (24), suggests that training decreases the sensitivity to ADP in muscle, as the apparent K_m for ADP increased approximately threefold (23). In contrast to these in vitro assessments of mitochondrial ADP sensitivity, a classic response of exercise training is a decrease in free ADP concentrations during exercise (25,26), indicating an improvement in ADP sensitivity. These conflicting data suggest additional regulation exists on ADP kinetics that is not reflected in the in vitro environment.

One potential regulatory mechanism is palmitoyl-CoA (P-CoA), which has previously been suggested to competitively bind with ADP on ANT (27). Theoretically, the increase in P-CoA content observed in the skeletal muscle of insulin-resistant individuals could impair ADP kinetics, accounting for the strong correlation between P-CoA content and insulin resistance (11). Decreased mitochondrial ADP transport could increase mitochondrial ROS emission and reactive lipid accumulation, and therefore represents a mechanism converging the two prominent working models for the development of insulin resistance. However, although acyl-CoA concentrations are increased with insulin resistance (10,11), exercise training does not reduce acyl-CoA content in association with an improvement in insulin sensitivity (28), challenging the direct relationship between P-CoA content and insulin resistance. Therefore, the purpose of the current study was to determine if exercise training altered the sensitivity of mitochondria to the inhibitory effects of P-CoA on mitochondrial ADP transport, and the potential mechanistic link with mitochondrial ROS emission and redox balance. These data highlight a novel mechanism by which elevated P-CoA can influence skeletal muscle insulin sensitivity in humans.

RESEARCH DESIGN AND METHODS

Human Participants

Middle-aged males were screened using a medical questionnaire and were excluded if they were diagnosed as diabetic, were taking medications to control blood glucose, or had a fasting blood glucose of >7 mmol/L. All participants indicated that they were sedentary prior to commencing training by self-report. No participant changed medication during, or for the 4 months prior to commencing, the experiment. However, two participants were taking antidepressant medication, and two other participants were taking medication for hypertension. Specifically, one was taking an ACE inhibitor, and the other was taking a calcium channel blocker with a diuretic. Participants (n = 14) provided written informed consent prior to experiments, all of which conformed to the Declaration of Helsinki and were approved by the University of Guelph and Hamilton Integrated Research Ethics Boards.

Experimental Design

Participants reported to the University of Guelph after a 12-h overnight fast. Weight and height measures were taken and a catheter was inserted into the antecubital vein by trained phlebotomists. Blood was collected before (t = 0) and 15, 30, 60, 90, and 120 min after the ingestion of a 75-g dextrose drink (Trutol; Thermo Scientific). Post-training oral glucose tolerance tests (OGTTs) were performed ~72 h after the last exercise session. HDL cholesterol, total cholesterol, serum triglycerides, and hs-CRP were measured from serum samples processed by a certified clinical laboratory (LifeLabs, Guelph, Ontario, Canada). Fasting plasma samples were analyzed in-house for nonesterified fatty acids and insulin using commercially available kits (Wako Diagnostics and Millipore, respectively). Plasma glucose was measured at all time points during the OGTT using a standard plate assay. VO_2peak was measured using a MOXUS metabolic cart (AEI Technologies) and an electronically braked cycle ergometer (Lode) using standard protocols before and after 6 weeks of supervised exercise training.

Muscle samples were obtained from the vastus lateralis using a Bergstrom needle at 8:00 A.M. after an overnight fast (29). Two biopsies were taken under basal conditions: a first sample was used for the preparation of permeabilized muscle fibers and a second sample was immediately frozen in liquid nitrogen. A third sample was taken 30 min after participants consumed the same 75-g dextrose drink and was immediately frozen for subsequent Western blotting.

Exercise Training

Subjects began training 1 week after muscle biopsy procedures. Training sessions were supervised, 5 days/week for 6 weeks. Participants completed endurance sessions on Monday, Wednesday, and Friday and high-intensity interval training sessions on Tuesday and Thursday. Sessions were completed on Monark bicycles and included a 5-min warm up and cool down with very low resistance. Only one session was missed by one participant due to a work-related emergency. Full details on the training progression are outlined in Supplementary Fig. 1.

Western Blotting

Western blotting was performed on whole-muscle homogenate as previously described (21) using the following commercially available antibodies: α-tubulin (Ab7291; Abcam), ANT1 (MSA02; MitoSciences), ANT2 (AP1057; Millipore), 4HNE (HNE11-S; Alpha Diagnostic International), OXPHOS (MS604; MitoSciences), COXIV (Invitrogen), CAT (AB1877; Abcam), and SOD2 (AB11889; Abcam), and for protein carbonylation, the OxyBlot Protein Oxidation Detection Kit (S7150; Millipore) was used. Ponceau staining was used to confirm equal loading for antibodies that required the entire membrane (e.g., 4HNE and protein carbonylation). In addition, Western blotting was performed on recovered permeabilized fibers following respiration protocols as previously described (30). All samples for a given protein were detected on the same membrane using chemiluminescence and the FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA).

Mitochondrial Respiration in Permeabilized Fibers

Permeabilized fiber preparation and high-resolution respirometry (Oroboros, Innsbruck, Austria) were performed as described previously (29,30). The following substrate concentrations were used: pyruvate (10 mmol/L), malate (5 mmol/L), ADP (5 mmol/L), glutamate (10 mmol/L), succinate (10 mmol/L), and creatine (20 mmol/L). Titration experiments were stopped at these concentrations. Additionally, ADP respiratory kinetics were measured in the presence of 60 μmol/L P-CoA or palmitate complexed to BSA (31). All titrations were performed with saturating pyruvate and malate concentrations. Cytochrome C was added to all experiments to ensure respiration was stimulated $<10\%$ to confirm mitochondrial membrane integrity.

Mitochondrial H_2O_2 Emission in Permeabilized Fibers

Mitochondrial H_2O_2 emission was measured fluorometrically (Lumina; Thermo Scientific) in a constantly stirring cuvette at 37°C (Peltier controlled) containing a standard reaction buffer (Buffer Z) supplemented with 25 μmol/L blebbistatin, 40 units/mL CuZnSOD, 10 μmol/L Amplex Red (Invitrogen), and 0.5 units/mL horseradish peroxidase in the presence of various substrate combinations (60 μmol/L P-CoA, 100 μmol/L ADP, and 10 mmol/L succinate).

Acute Rodent Experiments

The red gastrocnemius muscle from male C57 mice (8–10 weeks old, 22.3 ± 0.7 g) from our mouse-breeding colony were used, and all protocols were identical to those outlined above, unless specified. ADP-stimulated respiration was induced in the presence of 5 mmol/L malate + 10 mmol/L pyruvate, and the response to lipids (250 μmol/L palmitate, 250 μmol/L palmitate + 1 mmol/L CoA, and 60 μmol/L P-CoA) was determined. In separate experiments, fibers were incubated in Oxygraph chambers with MiR05 and various lipids in the presence or absence of 5 mmol/L ADP + 2 mmol/L L-carnitine for 15 min. Fibers were then washed in a chamber with standard MiR05 for 15 min before determining state III respiration. These experiments were approved by the University of Guelph Animal Care Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Statistical Analysis

All values are presented as means ± SEM. Apparent K_m values were determined using Prism (GraphPad Software, Inc., La Jolla, CA) using Michaelis-Menten kinetics for standard ADP titrations. However, ADP titrations in the presence of P-CoA did not display Michaelis-Menten kinetics, and therefore a one-phase association was used to estimate the concentration of ADP required to reach half-maximal respiration. V_{max} is presented as the highest respiration rate directly measured in titrations. Pre- versus posttraining measures were compared using a paired Student *t* test, with $P < 0.05$ considered statistically significant. A two-way ANOVA with a least significant difference post hoc analysis was also used to compare the effects of P-CoA on ADP sensitivity before and after training.

RESULTS

Exercise Improves Serum Profiles, Glucose Tolerance, and Cardiorespiratory Fitness

We first aimed to characterize the training adaptations in our participants to ensure that improvements in classical responses occurred. Specifically, training reduced BMI and increased VO_2peak and peak power during a VO_2peak test (Table 1). Exercise training also reduced fasting blood glucose, LDL cholesterol, LDL/HDL cholesterol, and hs-CRP (Table 1). There was a strong trend ($P = 0.05$) for an improvement in whole-body insulin sensitivity (HOMA) (Table 1), a finding supported by a reduction in the area under the curve during a 2-h OGTT (Fig. 1*A* and *B*). Exercise training also increased skeletal muscle Akt serine

Table 1—Subject characteristics and blood profile pre- and posttraining

	Pre	Post
Subject characteristics		
Age (years)	51 ± 1.7	51 ± 1.7
Body weight (kg)	110 ± 4	109 ± 5
Height (cm)	181 ± 1	181 ± 1
BMI (kg/m^2)	33.4 ± 1.2	32.8 ± 1.3*
VO$_2$peak (L/min)	3.3 ± 0.1	3.7 ± 0.1*
VO$_2$peak (mL/min/kg)	30.4 ± 1.4	34.9 ± 2.1*
Peak power (watts)	258 ± 12	302 ± 11*
Blood characteristics		
Glucose (mmol/L)	5.8 ± 0.1	5.3 ± 0.2*
Insulin (mmol/L)	63.3 ± 13	53.5 ± 9.3
HOMA	2.4 ± 0.5	1.9 ± 1.3**
FFA (mmol/L)	0.48 ± 0.03	0.45 ± 0.04
TG (mmol/L)	1.7 ± 0.3	1.9 ± 0.3
LDL (mmol/L)	3.3 ± 0.2	2.9 ± 0.2*
HDL (mmol/L)	1.2 ± 0.1	1.2 ± 0.1
LDL/HDL	2.8 ± 0.2	2.5 ± 0.1*
Total cholesterol (mmol/L)	5.2 ± 0.3	5.0 ± 0.3
hs-CRP (mg/L)	3.0 ± 0.5	1.8 ± 0.4*

Data are means ± SEM. n = 12–14. FFA, free fatty acid; TG, triglycerides. *, Significantly ($P < 0.05$) different from Pre. **, $P = 0.05$.

473 and threonine 308 phosphorylation by ~30% after glucose ingestion (Fig. 1*C*), suggesting an improvement in skeletal muscle insulin sensitivity. Altogether, the current training program improved several indices of cardiorespiratory fitness and glucose homeostasis.

Exercise Training Increases OXPHOS and ANT1 Contents, but ANT2 Protein Is Unaltered

Given the improvement in skeletal muscle insulin signaling after exercise training, we next aimed to determine the influence of exercise on several aspects of mitochondrial bioenergetics. We first used a mixed substrate protocol to examine the capacity of the electron transport chain in permeabilized muscle fibers. Mitochondrial oxygen consumption was increased ~60% in all conditions (Fig. 2*A*), suggesting training-induced increases in mitochondrial content. This interpretation is supported by the finding that the content of several subunits of the electron transport chain were increased ~50% in muscle homogenates (Fig. 2*B*), as well as in muscle fiber bundles used for respirometry protocols (Supplementary Fig. 2). The protein content of ANT1 increased similarly to markers of mitochondrial content, whereas in contrast, ANT2 was not altered (Fig. 2*B*). Once again, these patterns were verified in fiber bundles recovered from the respirometer (Supplementary Fig. 2). Altogether, ANT1 increased proportionally to mitochondrial content, whereas ANT2 was unaltered by exercise training.

Training Does Not Alter the Capacity of Mitochondria for H_2O_2 Emission

Genetic ablation of ANT1 decreases basal uncoupled/leak respiration (32), raising the possibility in the current study that the observed lack of change in ANT2 protein may alter basal respiration or ROS emission. Whereas exercise training improved the redox environment of

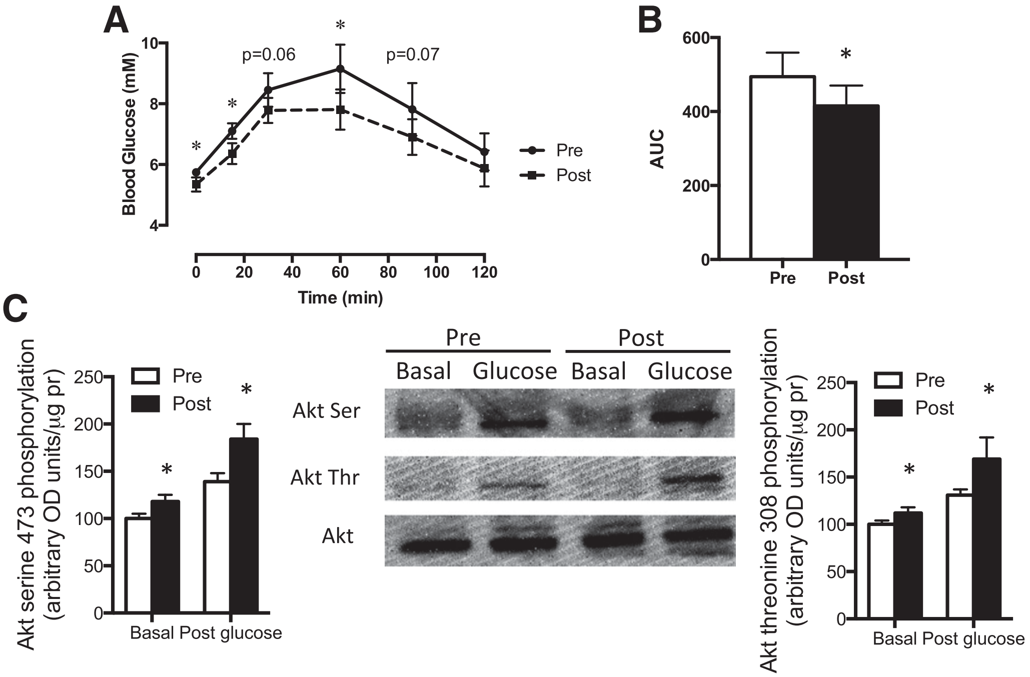


Figure 1—Training improves indices of whole-body glucose tolerance and skeletal muscle insulin sensitivity. Two-hour OGTT response (*A*) and corresponding area under the curve (AUC) (*B*) and skeletal muscle insulin signaling before and after training (*C*). Representative blots are shown in *C* for Akt serine 473 phosphorylation, Akt threonine 308 phosphorylation, and total Akt. Values represent means ± SEM. n = 10. *, Significantly ($P < 0.05$) different from Pre. pr, protein.

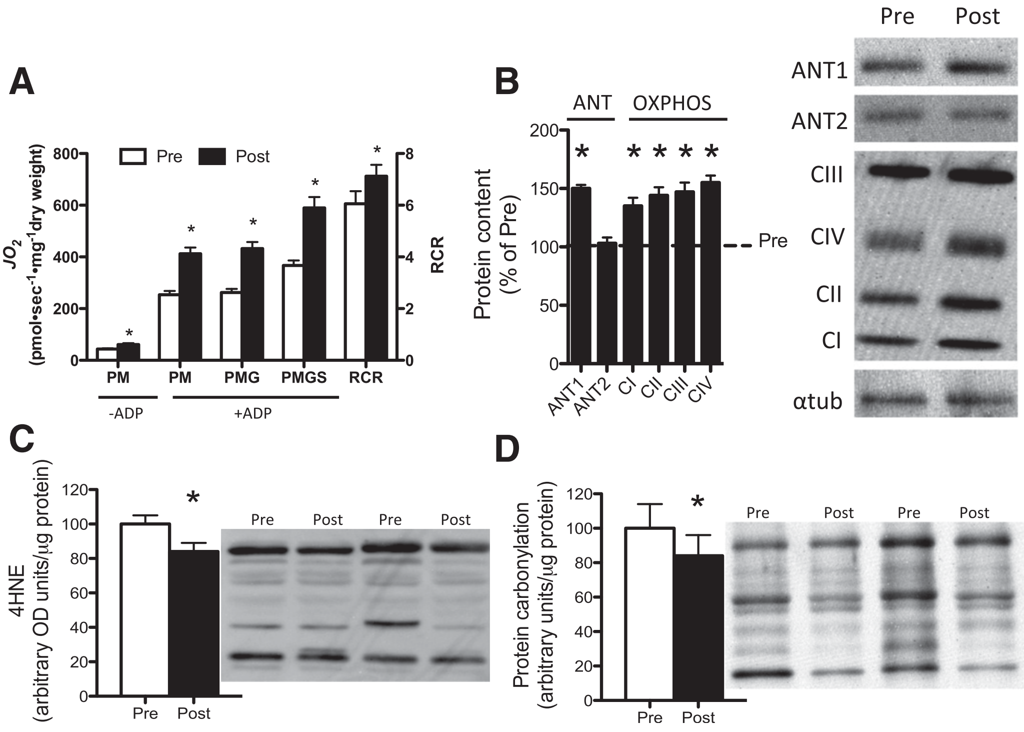


Figure 2—Training improves mitochondrial capacity and skeletal muscle oxidative state. *A*: Leak (−ADP) and ADP-stimulated mitochondrial respiration in the presence of various substrates (P, 10 mmol/L pyruvate; M, 5 mmol/L malate; G, 10 mmol/L glutamate; S, 10 mmol/L succinate). *B*: The content of subunits from the electron transport chain protein complexes I–IV (V is absent because the gel was cut for α-tubulin [αtub], which is at the same molecular weight), ANT1, and ANT2, with corresponding representative blots in whole-muscle homogenates. 4HNE (*C*) and protein carbonylation contents (*D*). *E*: Mitochondrial H_2O_2 emission capacity. *F*: Content of antioxidant proteins CAT and SOD2, with representative blots. *G*: Leak respiration in the presence of 60 μmol/L P-CoA. *H*: Mitochondrial H_2O_2 emission in the presence of P-CoA. Data are means ± SEM. *n* = 10–12. *, Significantly ($P < 0.05$) different from Pre. RCR, respiratory control ratio. pr, protein.

skeletal muscle, as assessed by a reduction in 4HNE (Fig. 2*C*) and protein carbonylation contents (Fig. 2*D*), mitochondrial H_2O_2 emission was not altered (Fig. 2*E*). Whereas CAT did not change, the mitochondrial antioxidant enzyme SOD2 increased after training (Fig. 2*F*), potentially explaining this discrepancy. However, given the ability of lipids to interact with ANT and induce uncoupling (33,34), we also examined the potential for training to increase the ability of P-CoA to induce mitochondrial uncoupling and decrease ROS emission rates. However, regardless of the training status, P-CoA did not alter state IV respiration (Fig. 2*G*) or mitochondrial H_2O_2 emission rates (Fig. 2*H*). Combined, these data suggest that in the absence of ADP, P-CoA does not affect mitochondrial bioenergetics.

Exercise Training Decreases the Apparent Sensitivity of Mitochondria to ADP

We next aimed to determine the respiratory sensitivity to ADP in permeabilized fibers as a potential alternative functional consequence to the observed alterations in ANT1/ANT2 ratios. Regardless of the presence of creatine, titrating ADP in the presence of saturating pyruvate and malate resulted in a typical Michaelis-Menton kinetic curve (Fig. 3*A* and *D*), which could be used to estimate maximal respiration and ADP sensitivity. Similar to the mixed substrate protocol, exercise training increased maximal respiration (V_{max}) in a creatine-independent manner (Fig. 3*B* and *E*). In addition, exercise training increased the apparent ADP K_m ~40% in the presence of creatine (Fig. 3*C*), and there was a strong trend for an increase in the apparent ADP K_m in the absence of creatine (Fig. 3*F*). These data suggest that exercise training decreased mitochondrial ADP sensitivity in association with improvements in insulin sensitivity, contrary to our working model. We therefore next aimed to determine external regulation on ANT that could be influenced by exercise training.

Exercise Training Decreases the Sensitivity to P-CoA Inhibition of ADP Respiration

Intramuscular P-CoA concentrations are elevated in insulin-resistant skeletal muscle and are known to inhibit ANT function (11,27). We therefore next aimed to directly determine if training altered the ability of P-CoA to attenuate ADP sensitivity. Before training, the presence of 60 μmol/L P-CoA almost completely prevented the ability of ADP to stimulate respiration (Fig. 4*A*). After training, the V_{max} was increased ~75% (Fig. 4*B*) and the ability of ADP to stimulate respiration was approximately fourfold greater (Fig. 4*A*), suggesting decreased sensitivity to P-CoA inhibition. In support of this, the ADP

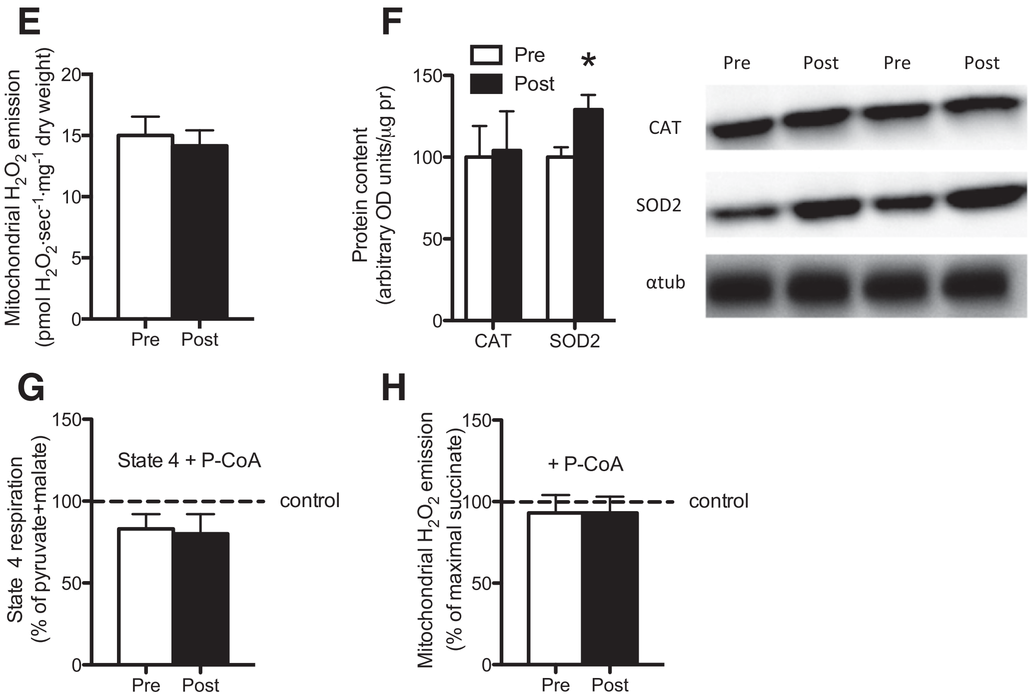


Figure 2—*Continued*.

concentration required to reach half-maximal respiration was reduced by ~50% after training when P-CoA was present (Fig. 4*C*). In addition, respiration in the presence of 100 µmol/L ADP, which represents a submaximal free ADP concentration within muscle, was increased after training in the presence of P-CoA (depicted in Fig. 4*A*). Altogether, whereas P-CoA impaired ADP sensitivity, training improved the sensitivity to ADP when P-CoA was present, a response that is in contrast to typical assessments of ADP sensitivity in permeabilized fibers (Fig. 4*D*).

P-CoA Attenuation of ADP Transport Influences Mitochondrial ROS Emission

To further examine a potential mechanism of action for how P-CoA alterations in mitochondrial bioenergetics could influence insulin sensitivity, we conducted several experiments in wild-type mice. During state III respiration, the real-time addition of 60 µmol/L P-CoA rapidly depressed state III respiration by ~50% within 15 min (Fig. 5*A* and *B*). The subsequent addition of dinitrophenol recovered respiration rates up to ~90% of maximal ADP-stimulated respiration (Fig. 5*A* and *B*), indicating that P-CoA was not affecting electron flux through the electron transport chain, implicating a primary inhibition of ADP transport. The presence of palmitate similarly inhibited ADP-supported respiration (Fig. 5*C*). Furthermore, these effects are not complex I specific, as other substrate protocols (provision of succinate and G3P) yield the same inhibition pattern (Supplementary Fig. 3). Together, these data suggest that lipids in general attenuate ADP transport, supporting previous findings that lipids competitively bind to the ADP binding site on ANT (27).

Given that we observed an exercise-training interaction with P-CoA–mediated attenuations in ADP sensitivity, we reasoned that this process was not simply regulated by competitive inhibition as classically suggested (27). Therefore, we next performed experiments whereby mitochondria were exposed to various lipids for 15 min, washed for 15 min in a lipid-free environment, and then stimulated to respire in the presence of saturating ADP and pyruvate + malate. Using this approach, ADP-stimulated respiration was not affected by a 15-min preincubation with palmitate (Fig. 5*D*). However, when CoA was included with palmitate in the preincubation media, subsequent state III respiration was reduced similarly to the effects of P-CoA (Fig. 5*D*). These data suggest that, unlike palmitate, P-CoA inhibition of ADP transport is not simply through competitive inhibition, and suggests a more rigid interaction with ANT. Intriguingly, when L-carnitine was included in the preincubation media, the negative influence of P-CoA was prevented (Fig. 5*C*), suggesting that the availability of P-CoA in the intermembrane space, and not the matrix, is required for the observed inhibition of ADP-stimulated respiration.

To investigate the potential link between reactive lipid inhibition of ADP transport and ROS production, we examined the impact of P-CoA on the propensity to generate mitochondrial ROS in the presence of ADP. Exposure of mitochondria to P-CoA did not alter maximal succinate-supported H_2O_2 emission (Fig. 2*G* [humans] and Fig. 6 [mice]). Whereas 100 µmol/L ADP consistently decreased mitochondrial H_2O_2 emission rates in rodents, exposure to P-CoA attenuated this response (Fig. 6). These data suggest that P-CoA inhibition of ADP transport can influence mitochondrial ROS emissions.

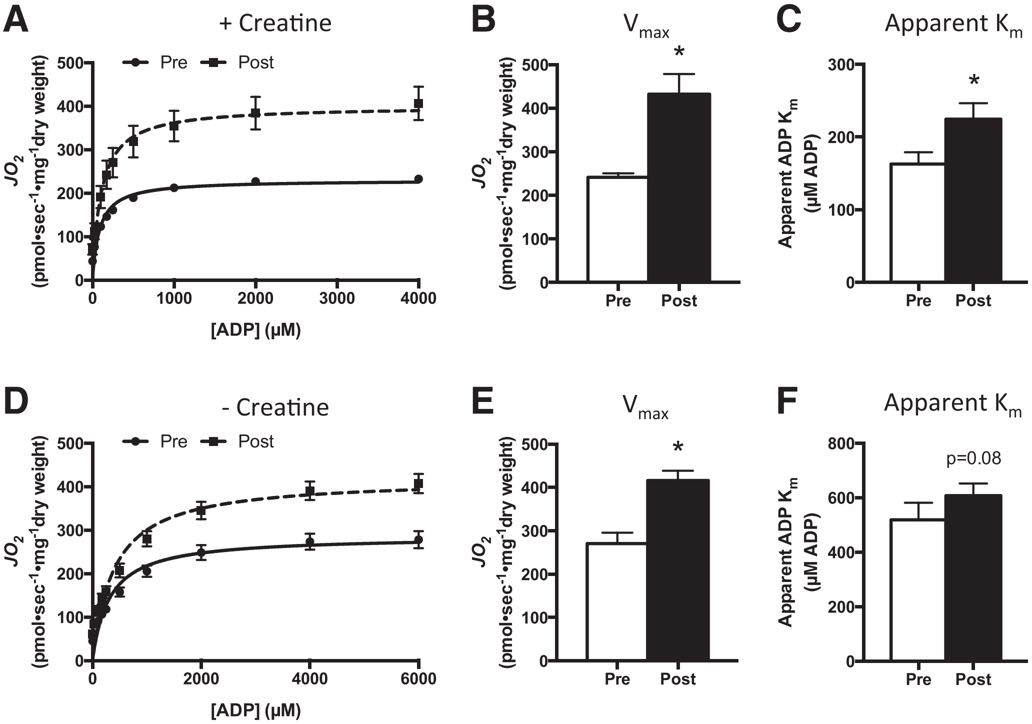


Figure 3—Apparent mitochondrial ADP sensitivity is reduced after exercise training. ADP titrations in the presence (*A*–*C*) and absence (*D*–*F*) of creatine are shown with 10 mmol/L pyruvate and 5 mmol/L malate present in the respiration media. Corresponding V_{max} (*B* and *E*) and apparent ADP K_m (*C* and *F*) were calculated. Values represent means ± SEM. n = 11–13. *, Significantly ($P < 0.05$) different from Pre.

Combined, these data provide a plausible mechanism for the observed reduction in oxidative stress, improved insulin sensitivity, and the association between skeletal muscle Akt phosphorylation and ADP-stimulated respiration in the presence of P-CoA that was apparent after aerobic training in the current study.

DISCUSSION

In the current study, we show that the ability of P-CoA to inhibit mitochondrial ADP-stimulated respiration is dramatically attenuated by exercise training. This response is strongly associated with skeletal muscle Akt phosphorylation, whole-body insulin sensitivity, and an improvement in the oxidative state of the muscle. Altogether, the present data provide a plausible mechanism for how P-CoA can influence insulin sensitivity and highlight mitochondrial ADP transport as a potential nexus point between reactive lipids and mitochondrial ROS during the development of insulin resistance.

Exercise Training and Mitochondrial ADP Sensitivity

The role of mitochondrial ADP transport as a regulator of mitochondrial bioenergetics has remained largely unexplored, although recent literature has elucidated biologically relevant ADP K_m values (35), solidified the regulation of mitochondrial ADP transport by mitochondrial creatine kinase (36,37), and uncovered the functional consequence of acute exercise (29). In the current study, we advance our understanding of the regulation of ADP transport by providing evidence that chronic exercise training in obese individuals attenuates the apparent sensitivity of mitochondria to ADP, supporting a previous cross-sectional analysis in healthy humans (23) and treadmill training in rodents (24). These data are in contrast to the well-established decrease in free ADP that occurs after training (25). However, although the reduction in ADP respiratory sensitivity after training appears perplexing, in the current study, respiration at a given submaximal ADP concentration was higher after training, an index that may have greater relevance to whole-body physiology. Nevertheless, the increase in the apparent ADP K_m that occurred with training suggests that an alteration occurred within this system that attenuated the sensitivity to ADP. This likely occurred on ANT, and not mitochondrial creatine kinase, given the apparent creatine-independent response. Therefore, potential mechanisms for the observed impairment in ADP sensitivity include an increase in the acetylation of lysine 23 (22), a decrease in ANT1 tyrosine 194 phosphorylation (38), and a decrease in glutathionylation/carbonylation of ANT (39,40). Alternatively, an alteration in the expression of ANT isoforms may mediate this response, as in the current study, exercise training did not alter the content of ANT2. The functional effects of ANT isoforms are not currently known; however, in rodent skeletal muscle,

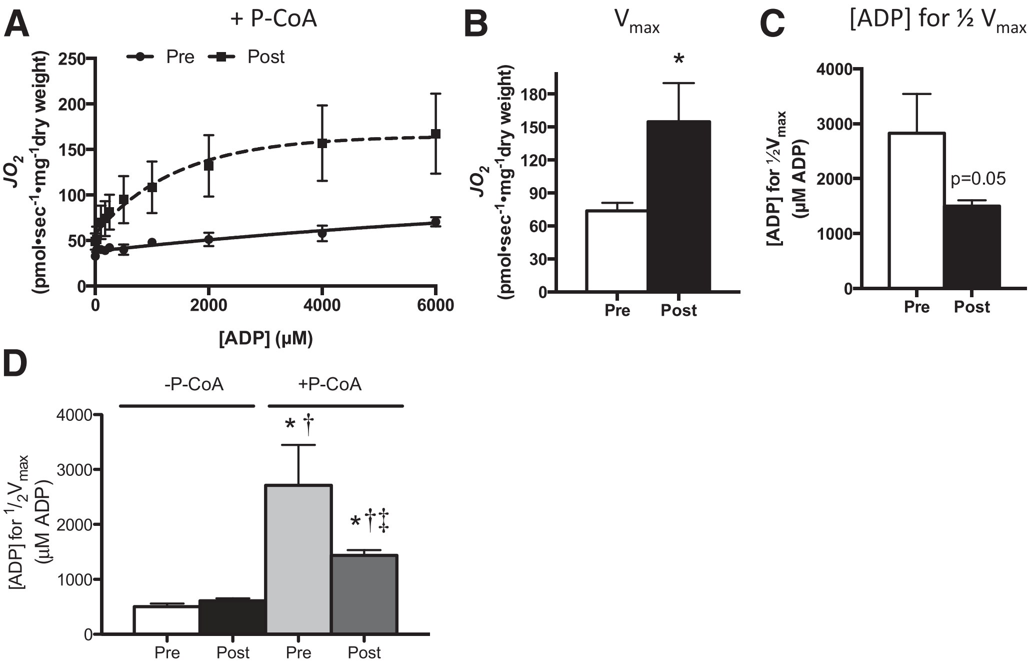
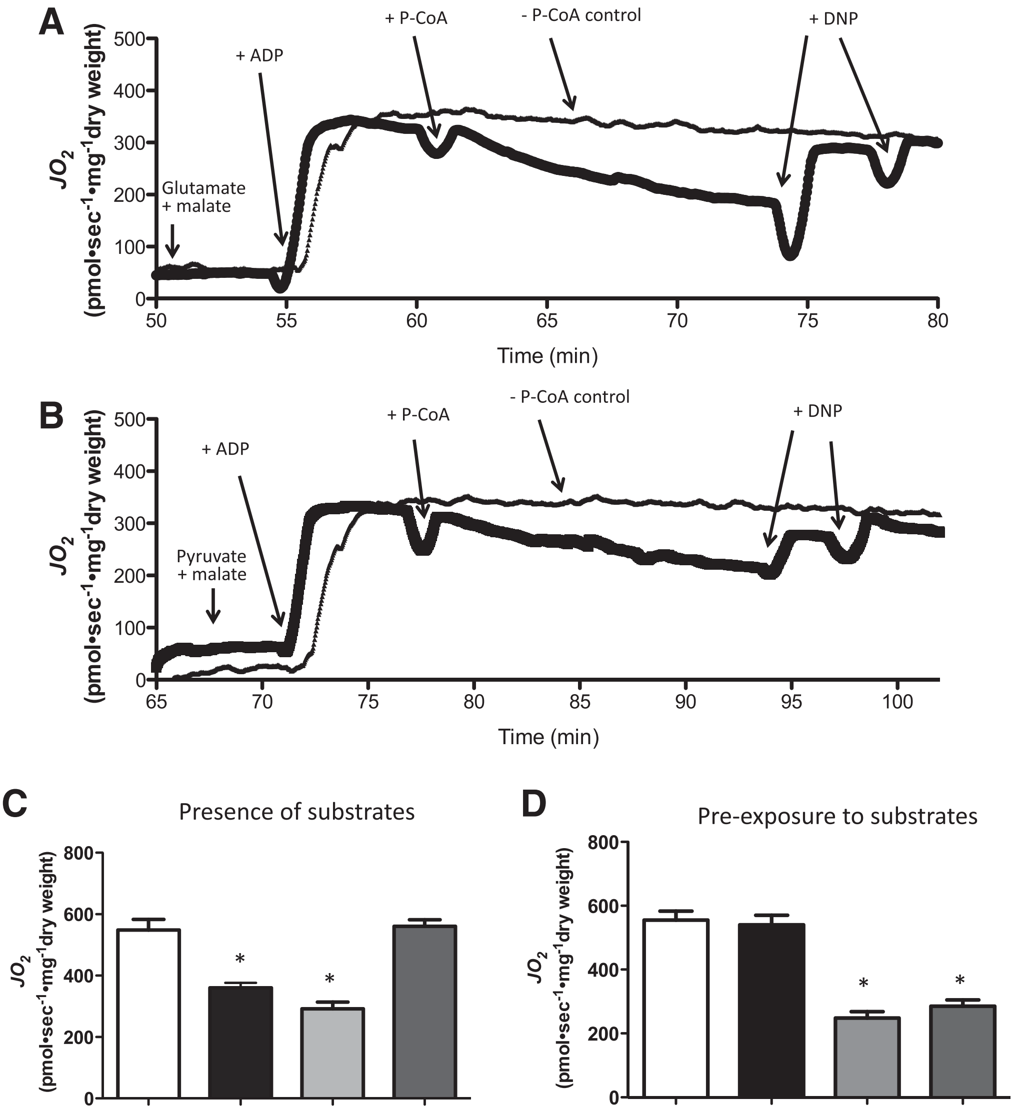


Figure 4—Impairment of mitochondrial ADP sensitivity by P-CoA is attenuated by exercise training. *A*: ADP titrations in the presence of 60 μmol/L P-CoA, 10 mmol/L pyruvate, and 5 mmol/L malate. Calculated V_{max} (*B*) and [ADP] (*C*) required to reach half-maximal respiration, as estimated using a one-phase association fit. *D*: [ADP] required to reach half-maximal respiration is reduced by training only with P-CoA present in the media. Values represent means ± SEM. *n* = 8–11. *, Significantly ($P < 0.05$) different from Pre; †, different from Post in the absence of P-CoA; ‡, different from Pre with P-CoA present.

a reduction in ANT2 protein is associated with impaired submaximal, but not maximal, ADP-stimulated respiration (21). It is therefore tempting to suggest that ANT2 displays a greater ADP sensitivity, and the relative abundance of ANT2/ANT1 observed in the current study influenced the overall ADP respiratory sensitivity. Regardless of the potential mechanism, exercise training consistently displays a reduction in mitochondrial ADP sensitivity when determined in the absence of lipid moieties (current study and Zoll et al. [23], Zoll et al. [24], and Guerrero et al. [41]).

P-CoA and Mitochondrial ADP Sensitivity

The shift in the ADP sensitivity after training may suggest external regulation on ANT. To examine one potential mechanism, we determined ADP respiratory sensitivity in the presence of P-CoA. We used this substrate as palmitate represents one of the most abundant dietary lipids, changes in intramuscular P-CoA content are most closely associated with muscle insulin sensitivity (42), and ANT has previously been shown to have the highest sensitivity to inhibition by P-CoA (27). Similar to previous reports, P-CoA dramatically, and almost instantaneously, decreased ADP-stimulated respiration (43). However, whereas previous reports suggest this inhibition occurred as a result of competitive inhibition of ADP binding (27,43), the current study suggests another regulatory mechanism is at play, as the removal of P-CoA from the respiration media did not recover ADP-stimulated respiration. Given that training dramatically attenuated the ability of P-CoA to prevent ADP-stimulated respiration, this system appears to be sensitive to exercise, and corresponding posttranslational modifications to ADP binding proteins (such as ANT) represent one candidate for this regulation. Alternatively, since ANT2 protein did not increase with exercise training, it is tempting to speculate that ANT2 is less susceptible to P-CoA inhibition, and future work should examine this possibility. Regardless, the present data provide novel basic understanding on the regulation of ADP sensitivity and the response to exercise training. To investigate the potential link between P-CoA inhibition of ADP transport and oxidative stress, we examined the impact of P-CoA on the propensity to generate mitochondrial ROS in the presence of ADP. Using this approach, we provide evidence that the ability of ADP to lower mitochondrial ROS production was significantly diminished after mitochondria were exposed to P-CoA. These data are the first to link P-CoA inhibition of ADP transport to increased ROS production and establish a novel mechanism detailing the association between intramuscular P-CoA content, ROS production, and potentially insulin resistance. Although these responses likely contribute to an improvement in insulin sensitivity after training, the increase in antioxidants (SOD2 in current study and reviewed in Powers and Lennon [44]) and known reductions in DAGs and ceramides (45,46) all contribute to the beneficial effects of exercise.



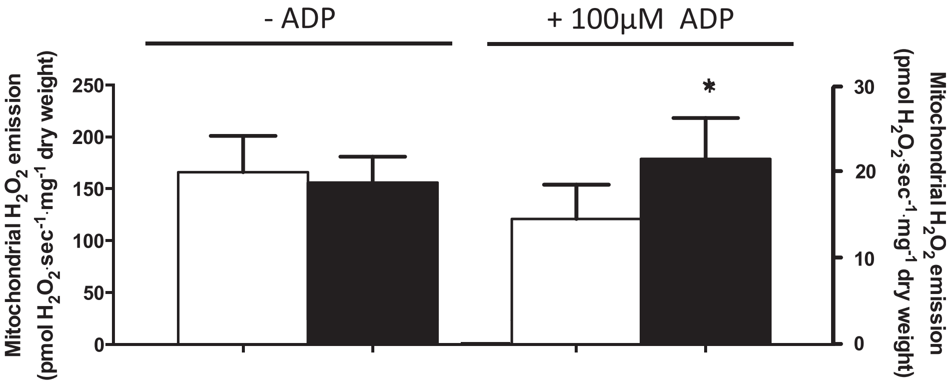
Pyruvate Malate + ADP	+	+	+	+
Palmitate	-	+	-	-
P-CoA	-	+	+	+
LCarn	-	-	-	+

Pyruvate Malate + ADP	+	+	+	+
Palmitate	-	+	+	-
CoA	-	-	+	-
P-CoA	-	-	-	+

Figure 5—Inhibition of ADP transport by P-CoA and palmitate. Representative Oxygraph traces showing real-time inhibition in glutamate (*A*) and pyruvate (*B*) state III respiration (5 mmol/L ADP) in the presence and absence (control) of P-CoA (60 μmol/L). *C*: Respiration in the presence of various lipids. *D*: Respiration after pre-exposure to various lipids. Values represent means ± SEM. *n* = 3–6. *, Significantly ($P < 0.05$) different from maximal pyruvate + malate respiration. DNP, dinitrophenol; LCarn, L-carnitine.

Perspectives and Conclusions

Mitochondrial ROS production is intricately linked to an imbalance between energy supply and energy demand by the interrelated rise in mitochondrial membrane potential ($\Delta\psi_m$). In this context, the current data strongly suggest that an accumulation of cytosolic P-CoA can influence redox balance by attenuating mitochondrial ADP transport. In addition, previous literature shows that elevations in cytosolic P-CoA can promote an increase in carnitine palmitoyl-transferase I (CPT-I) activity by reducing the inhibitory effectiveness of malonyl-CoA (31). Therefore, an increase in cytosolic P-CoA would be expected to inhibit mitochondrial ADP transport while simultaneously increasing matrix P-CoA concentrations and membrane potential, dramatically affecting redox balance. It is likely for these reasons in the current study that the attenuation in P-CoA inhibition of ADP-stimulated respiration was associated with decreased 4HNE and protein carbonylation content



Succinate	+	+	+	+
15 minute P-CoA pre-incubation	-	+	-	+
100 μM ADP	-	-	+	+

Figure 6—P-CoA inhibits ADP attenuation of mitochondrial ROS emission. Values represent means ± SEM. $n = 6$. *, Significantly ($P < 0.05$) different from P-CoA absence.

and insulin signaling. Altogether, the current data strongly implicate P-CoA as regulator of mitochondrial bioenergetics, positioning P-CoA inhibition of ADP transport as a convergence point between reactive lipid accumulation, mitochondrial ROS emission, and insulin resistance—interactions that are attenuated with exercise training.

Funding. This work was funded by the Natural Sciences and Engineering Research Council of Canada (G.P.H.) and infrastructure was purchased with the assistance of the Canadian Foundation for Innovation (G.P.H.) as well as the Ontario Research Fund (G.P.H.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.L. and G.P.H. designed the study, performed experiments, and wrote the manuscript. S.P., B.K.S., E.A.F.H., M.K.A., and G.J.H. performed experiments, interpreted data, and contributed to writing the manuscript. P.D.N. designed experiments and edited the manuscript. G.P.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Caro JF, Dohm LG, Pories WJ, Sinha MK. Cellular alterations in liver, skeletal muscle, and adipose tissue responsible for insulin resistance in obesity and type II diabetes. Diabetes Metab Rev 1989;5:665–689
2. Timmers S, Schrauwen P, de Vogel J. Muscular diacylglycerol metabolism and insulin resistance. Physiol Behav 2008;94:242–251
3. Summers SA. Ceramides in insulin resistance and lipotoxicity. Prog Lipid Res 2006;45:42–72
4. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. Diabetes 2002;51:2005–2011
5. Wright LE, Brandon AE, Hoy AJ, et al. Amelioration of lipid-induced insulin resistance in rat skeletal muscle by overexpression of Pgc-1β involves reductions in long-chain acyl-CoA levels and oxidative stress. Diabetologia 2011;54:1417–1426
6. Adams JM 2nd, Pratipanawatr T, Berria R, et al. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes 2004;53:25–31
7. Chibalin AV, Leng Y, Vieira E, et al. Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. Cell 2008;132:375–386
8. Bruce CR, Risis S, Babb JR, et al. Overexpression of sphingosine kinase 1 prevents ceramide accumulation and ameliorates muscle insulin resistance in high-fat diet-fed mice. Diabetes 2012;61:3148–3155
9. Holland WL, Brozinick JT, Wang LP, et al. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. Cell Metab 2007;5:167–179
10. Consitt LA, Bell JA, Houmard JA. Intramuscular lipid metabolism, insulin action, and obesity. IUBMB Life 2009;61:47–55
11. Ellis BA, Poynten A, Lowy AJ, et al. Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. Am J Physiol Endocrinol Metab 2000;279:E554–E560
12. Sinha S, Perdomo G, Brown NF, O'Doherty RM. Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B. J Biol Chem 2004;279:41294–41301
13. Yuan M, Konstantopoulos N, Lee J, et al. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001;293:1673–1677
14. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 2002;277:44784–44790
15. Anderson EJ, Yamazaki H, Neufer PD. Induction of endogenous uncoupling protein 3 suppresses mitochondrial oxidant emission during fatty acid-supported respiration. J Biol Chem 2007;282:31257–31266
16. Anderson EJ, Lustig ME, Boyle KE, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J Clin Invest 2009;119:573–581
17. Lee HY, Choi CS, Birkenfeld AL, et al. Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. Cell Metab 2010;12:668–674
18. Boden MJ, Brandon AE, Tid-Ang JD, et al. Overexpression of manganese superoxide dismutase ameliorates high-fat diet-induced insulin resistance in rat skeletal muscle. Am J Physiol Endocrinol Metab 2012;303:E798–E805

19. Anderson EJ, Neufer PD. Type II skeletal myofibers possess unique properties that potentiate mitochondrial H(2)O(2) generation. Am J Physiol Cell Physiol 2006;290:C844–C851

20. Picard M, Ritchie D, Wright KJ, et al. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell 2010;9:1032–1046

21. Smith BK, Perry CG, Herbst EA, et al. Submaximal ADP-stimulated respiration is impaired in ZDF rats and recovered by resveratrol. J Physiol 2013;591: 6089–6101

22. Mielke C, Lefort N, McLean CG, et al. Adenine nucleotide translocase is acetylated in vivo in human muscle: modeling predicts a decreased ADP affinity and altered control of oxidative phosphorylation. Biochemistry 2014;53:3817–3829

23. Zoll J, Sanchez H, N'Guessan B, et al. Physical activity changes the regulation of mitochondrial respiration in human skeletal muscle. J Physiol 2002;543:191–200

24. Zoll J, Koulmann N, Bahi L, Ventura-Clapier R, Bigard AX. Quantitative and qualitative adaptation of skeletal muscle mitochondria to increased physical activity. J Cell Physiol 2003;194:186–193

25. Dudley GA, Tullson PC, Terjung RL. Influence of mitochondrial content on the sensitivity of respiratory control. J Biol Chem 1987;262:9109–9114

26. Phillips SM, Green HJ, Tarnopolsky MA, Heigenhauser GJ, Grant SM. Progressive effect of endurance training on metabolic adaptations in working skeletal muscle. Am J Physiol 1996;270:E265–E272

27. Ho CH, Pande SV. On the specificity of the inhibition of adenine nucleotide translocase by long chain acyl-coenzyme A esters. Biochim Biophys Acta 1974; 369:86–94

28. Bruce CR, Kriketos AD, Cooney GJ, Hawley JA. Disassociation of muscle triglyceride content and insulin sensitivity after exercise training in patients with type 2 diabetes. Diabetologia 2004;47:23–30

29. Perry CG, Kane DA, Herbst EA, et al. Mitochondrial creatine kinase activity and phosphate shuttling are acutely regulated by exercise in human skeletal muscle. J Physiol 2012;590:5475–5486

30. Herbst EA, Paglialunga S, Gerling C, et al. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. J Physiol 2014;592:1341–1352

31. Smith BK, Perry CG, Koves TR, et al. Identification of a novel malonyl-CoA IC (50) for CPT-I: implications for predicting in vivo fatty acid oxidation rates. Biochem J 2012;448:13–20

32. Brand MD, Pakay JL, Ocloo A, et al. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. Biochem J 2005;392:353–362

33. Andreyev AYu, Bondareva TO, Dedukhova VI, et al. The ATP/ADP-antiporter is involved in the uncoupling effect of fatty acids on mitochondria. Eur J Biochem 1989;182:585–592

34. Skulachev VP. Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation. FEBS Lett 1991;294:158–162

35. Perry CG, Kane DA, Lin CT, et al. Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. Biochem J 2011;437:215–222

36. Saks VA, Khuchua ZA, Vasilyeva EV, Belikova OYu, Kuznetsov AV. Metabolic compartmentation and substrate channelling in muscle cells. Role of coupled creatine kinases in in vivo regulation of cellular respiration—a synthesis. Mol Cell Biochem 1994;133–134:155–192

37. Walsh B, Tonkonogi M, Söderlund K, Hultman E, Saks V, Sahlin K. The role of phosphorylcreatine and creatine in the regulation of mitochondrial respiration in human skeletal muscle. J Physiol 2001;537:971–978

38. Feng J, Zhu M, Schaub MC, et al. Phosphoproteome analysis of isoflurane-protected heart mitochondria: phosphorylation of adenine nucleotide translocator-1 on Tyr194 regulates mitochondrial function. Cardiovasc Res 2008;80:20–29

39. Yan LJ, Sohal RS. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. Proc Natl Acad Sci U S A 1998;95:12896–12901

40. Queiroga CS, Almeida AS, Martel C, Brenner C, Alves PM, Vieira HL. Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis. J Biol Chem 2010;285:17077–17088

41. Guerrero K, Wuyam B, Mezin P, et al. Functional coupling of adenine nucleotide translocase and mitochondrial creatine kinase is enhanced after exercise training in lung transplant skeletal muscle. Am J Physiol Regul Integr Comp Physiol 2005;289:R1144–R1154

42. Houmard JA, Tanner CJ, Yu C, et al. Effect of weight loss on insulin sensitivity and intramuscular long-chain fatty acyl-CoAs in morbidly obese subjects. Diabetes 2002;51:2959–2963

43. Pande SV, Blanchaer MC. Reversible inhibition of mitochondrial adenosine diphosphate phosphorylation by long chain acyl coenzyme A esters. J Biol Chem 1971;246:402–411

44. Powers SK, Lennon SL. Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. Proc Nutr Soc 1999;58:1025–1033

45. Dubé JJ, Amati F, Stefanovic-Racic M, Toledo FG, Sauers SE, Goodpaster BH. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. Am J Physiol Endocrinol Metab 2008;294:E882–E888

46. Holloway GP, Han XX, Jain SS, Bonen A, Chabowski A. Chronic muscle stimulation improves insulin sensitivity while increasing subcellular lipid droplets and reducing selected diacylglycerol and ceramide species in obese Zucker rats. Diabetologia 2014;57:832–840