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High Oxidative Capacity Due to Chronic Exercise Training Attenuates Lipid-Induced Insulin Resistance

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Fat accumulation in skeletal muscle combined with low mitochondrial oxidative capacity is associated with insulin resistance (IR). Endurance-trained athletes, characterized by a high oxidative capacity, have elevated intramyocellular lipids, yet are highly insulin sensitive. We tested the hypothesis that a high oxidative capacity could attenuate lipid-induced IR. Nine endurance-trained (age = 23.4 ± 0.9 years; BMI = 21.2 ± 0.6 kg/m²) and 10 untrained subjects (age = 21.9 ± 0.9 years; BMI = 22.8 ± 0.6 kg/m²) were included and underwent a clamp with either infusion of glycerol or intralipid. Muscle biopsies were taken to perform high-resolution respirometry and protein phosphorylation/expression. Trained subjects had ~32% higher mitochondrial capacity and ~22% higher insulin sensitivity ($P < 0.05$ for both). Lipid infusion reduced insulin-stimulated glucose uptake by 63% in untrained subjects ($P < 0.05$), whereas this effect was blunted in trained subjects (29%, $P < 0.05$). In untrained subjects, lipid infusion reduced oxidative and nonoxidative glucose disposal (NOGD), whereas trained subjects were completely protected against lipid-induced reduction in NOGD, supported by dephosphorylation of glycogen synthase. We conclude that chronic exercise training attenuates lipid-induced IR and specifically attenuates the lipid-induced reduction in NOGD. Signaling data support the notion that high glucose uptake in trained subjects is maintained by shuttling glucose toward storage as glycogen. *Diabetes* 61:2472–2478, 2012

Fat accumulation in skeletal muscle strongly associates with the development of muscle insulin resistance (IR), the main risk factor in the development of type 2 diabetes (T2D). Indeed, elevated intramyocellular triglycerides (IMTGs) are associated with obesity and T2D (1–6). However, the question of why triglycerides accumulate in skeletal muscle has not been answered yet. In recent years, mitochondrial dysfunction has received large attention as a putative candidate underlying IMTG accumulation and thereby the development of IR. Several studies showed compromised in vivo and ex vivo mitochondrial function as a contributor to the development of IR and T2D (7–11). Studies have indicated that intrinsic mitochondrial function (i.e., respiratory capacity per

mitochondria) (10–12) as well as mitochondrial content (13,14) is reduced in T2D and in first-degree relatives. However, whether mitochondrial function and content are important in the prevention of lipid-induced muscular fat accumulation and IR has not been firmly established.

An interesting model in this context is the model of lipid infusion, whereby elevation of circulating fatty acids rapidly leads to the accumulation of IMTGs in skeletal muscle and the development of IR within 3–4 h after the onset of lipid infusion (15–20). Thus, this model can be used to investigate whether mitochondrial function and content affect lipid-induced IR by comparing subjects with high and low mitochondrial function. Therefore, the primary aim of our study was to test the hypothesis that a high mitochondrial oxidative capacity could prevent the development of lipid-induced IR. To this end, the current study examined the development of lipid-induced IR in endurance-trained athletes, who were selected based on a high maximal oxygen uptake in a graded exercise test and compared with untrained, but otherwise healthy, subjects.

RESEARCH DESIGN AND METHODS

Subjects. Ten healthy, young, untrained males and nine endurance-trained males were included in this study. Medication use, a family history of diabetes, and unstable dietary habits were the exclusion criteria. Subjects were included as trained when they participated in endurance exercise activities such as running and/or cycling three times per week for at least 2 years. Furthermore, all subjects underwent an incremental aerobic cycling test until exhaustion was reached (21), and subjects were included as trained if $\dot{V}O_{2peak}$ was $>55 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Subjects were included as untrained when they had a sedentary lifestyle and did not participate in any kind of sports or physical exercise for at least 2 years. The institutional medical ethics committee approved the study, and all participants gave their informed written consent. Subjects were recruited through advertisements in the medical university and local sport facilities. All subjects underwent hydrostatic weighing (22) to determine body composition. **Hyperinsulinemic-euglycemic clamp.** Each subject underwent two test days on which insulin sensitivity was measured during a 6-h hyperinsulinemic-euglycemic ($40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) clamp according to DeFronzo et al. (23), with a primed infusion of $[6,6]\text{-}^2\text{H}_2$ glucose ($0.03 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) to calculate rates of glucose appearance (R_a), glucose disposal (R_d), and hepatic glucose production (EGP) (24). Test days with either the infusion of glycerol (1.32 mL/min as control condition) or a heparinized ($0.2 \text{ U} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) infusion of triglycerides (1.35 mL/min; Intralipid, Baxter, Utrecht, the Netherlands) were randomly assigned. Plasma free fatty acid (FFA) concentrations were elevated to physiological levels seen during a high-fat diet or fasting plasma concentrations in insulin-resistant, nondiabetic, obese humans and patients with T2D (25,26). Two days before both test days, subjects refrained from participating in sport activities. On the day of the clamp, subjects reported to the laboratory at 7:30 A.M. after an overnight fast. A fasting blood sample was drawn to measure glucose, insulin, and FFAs followed by the start of the primed $[6,6]\text{-}^2\text{H}_2$ glucose infusion. Baseline blood samples were drawn, and indirect calorimetry measurements were performed to measure carbohydrate and lipid oxidation, and to calculate nonoxidative glucose disposal (NOGD) (27). A muscle biopsy was taken from the m. vastus lateralis under local anesthesia (1% lidocaine) according to the technique of Bergström et al. (28). After the muscle biopsy, the infusion of insulin and 20% variable glucose was started ($t = 0$) combined with simultaneous infusion of 4% glycerol (1.32 mL/min) or 20% intralipid (1.35 mL/min). Indirect calorimetry measurements were repeated during the last 30 min of the clamp ($t = 330$). Blood glucose was

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See accompanying commentary, p. 2397.

maintained as euglycemic, ~ 5.0 mmol/L. A second muscle biopsy was taken immediately after the clamp.

Calculations. Calculations for non-steady state R_a and R_d during the clamp were performed as described by Steele (24). EGP was calculated as R_a minus glucose infusion rate. Fat and carbohydrate oxidation were calculated as described by Frayn (27). In addition, NOGD was calculated as R_d minus whole-body carbohydrate oxidation.

Plasma assays. Blood was collected in tubes containing EDTA and immediately centrifuged. Plasma was frozen in liquid nitrogen and stored at -80°C until assayed. Plasma FFAs, glucose, and insulin concentration as well as isotopic enrichment of plasma glucose were determined as previously described (11).

Muscle biopsy handling. About 20 mg of the muscle tissue was directly placed in a conservation medium (BIOPS; Oroboros Instruments, Innsbruck, Austria). On a Petri dish, muscle fibers were separated using small needles, and the muscle membrane was permeabilized with saponin (stock solution, 5 mg/mL BIOPS) as previously described (11). Subsequent to several washing steps with respiration medium (Mir05; Oroboros Instruments, Innsbruck, Austria) to ensure removal of saponin, $\sim 3\text{--}4$ mg wet-weight fiber was transferred into the oxygraph, and respiratory measurements were performed at 37°C . Additional muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C for later analyses.

Mitochondrial density. Mitochondrial DNA (mtDNA) copy number, the ratio of NADH dehydrogenase subunit 1 (ND1) to lipoprotein lipase (LPL) (mtDNA/nuclear DNA), was determined in whole muscle as a measure of mitochondrial density, as described previously (29). As an additional marker for mitochondrial density, we measured protein levels involved in the oxidative phosphorylation (OXPHOS) in whole muscle protein levels in whole muscle by Western blotting (30).

IMTGs. Fresh cryosections of $5\ \mu\text{m}$ were stained for IMTGs by Oil Red O staining combined with fibertyping and immunolabeling of the basal membrane marker laminin to allow quantification, as described previously (31).

Protein phosphorylation. Muscle biopsies were homogenized in Bioplex lysis buffer containing protease and phosphatase inhibitors (Bio-Rad, Munich, Germany) using an Ultraturrax mixer. Homogenates were analyzed for expression and phosphorylation of components of insulin action by Western blotting using antibodies recognizing the insulin receptor and pyruvate dehydrogenase kinase 4 (PDK4) (both from Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated AKT-Ser473, proline-rich AKT substrate of 40 kDa (PRAS40-Thr246), glycogen synthase kinase $\beta 3$ (GSK3 β -Ser9), and glycogen synthase (GS) (Ser641) (all from Cell Signaling Technology, Beverly, MA). Bound antibodies were visualized using enhanced chemiluminescence, quantified using a VersaDoc 4000 MP (Bio-Rad) workstation, and normalized by reprobating the stripped filters with α -tubulin antibody (Calbiochem, Darmstadt, Germany).

High-resolution respirometry. Muscle fibers were permeabilized as previously described (11). Ex vivo mitochondrial respiration was measured using high-resolution respirometry, under hyperoxic conditions in a two-chamber oxygraph (Oroboros, Innsbruck, Austria), and expressed as $\text{pmol} \cdot \text{mg}^{-1}$ muscle fiber wet weight $\cdot \text{s}^{-1}$, as well as per mtDNA copy number. To evaluate oxidative phosphorylation, 2.0 mmol/L malate, 10.0 mmol/L glutamate, 2.0 mmol/L ADP, and 10.0 mmol/L succinate were added, with or without the presence of octanoyl-carnitine (40 $\mu\text{mol/L}$). Finally, 2.0 $\mu\text{g/mL}$ oligomycin or 0.5 $\mu\text{mol/L}$ titrations of the uncoupler fluoro-carbonyl cyanide phenylhydrazine were added to determine leak respiration or maximal respiratory capacity, respectively.

Statistics. Data are reported as mean \pm SE. Statistical analyses were performed using the statistical computer program SPSS 16.0.2 for Mac OS X. Differences between groups and between conditions were analyzed by a two-way ANOVA in which comparisons between groups for repeated measures were computed with a Bonferroni post hoc correction. Pearson correlation coefficients were computed to correlate parameter values. Differences were considered significant when $P < 0.05$.

RESULTS

Subject characteristics. Subject characteristics of the endurance-trained and untrained subjects are reported in Table 1. Endurance-trained subjects had a significantly higher $\text{VO}_{2\text{peak}}$ and lower BMI compared with untrained subjects (Table 1). Fat-free mass was not different between groups (Table 1).

Comparison of trained and untrained subjects

Plasma metabolites. Fasting glucose and insulin levels were comparable between trained and untrained subjects; however, fasting FFA levels were significantly lower in trained versus untrained subjects (Table 1).

Mitochondrial metabolism. As predicted, endurance-trained subjects showed a significantly higher mitochondrial

TABLE 1
Subject characteristics

	Untrained (n = 10)	Trained (n = 9)	Significance
Age (years)	21.9 \pm 0.9	23.4 \pm 0.9	NS
Body weight (kg)	75.8 \pm 3.0	67.8 \pm 2.8	$P = 0.07$
BMI (kg/m^2)	22.8 \pm 0.6	21.2 \pm 0.6	$P = 0.05$
Fat (%)	19.1 \pm 1.4	11.7 \pm 1.1	$P < 0.01$
Fat-free mass (kg)	61.2 \pm 2.1	60.0 \pm 2.5	NS
$\text{VO}_{2\text{peak}}$ ($\text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)	43.0 \pm 1.1	61.5 \pm 1.2	$P < 0.01$
W_{max} (W/kg)	3.7 \pm 0.2	5.3 \pm 0.2	$P < 0.01$
Fasting glucose (mmol/L)	5.1 \pm 0.1	5.0 \pm 0.1	NS
Fasting insulin (mU/mL)	10.3 \pm 1.2	10.5 \pm 1.3	NS
Fasting FFAs ($\mu\text{mol/L}$)	469 \pm 34	342 \pm 32	$P < 0.05$

Data are mean \pm SE. W_{max} , maximal workload.

respiration rate in permeabilized muscle fibers compared with the untrained subjects for both multisubstrate protocols, including malate, glutamate, and succinate with and without the addition of octanoyl-carnitine (Fig. 1A). In addition, mitochondrial mass, measured as mtDNA copy number (ND1/LPL), was higher in trained versus untrained subjects ($P < 0.05$). It is noteworthy that when respiratory values were normalized to mitochondrial content, the difference in ex vivo mitochondrial respiration between trained and untrained subjects disappeared (Fig. 1B), indicating similar intrinsic mitochondrial capacities. Similar results were obtained if respiration rates were corrected for OXPHOS protein content, another marker of mitochondrial density, which was also higher in trained subjects (99 ± 15 vs. 61 ± 13 arbitrary units, respectively; $P < 0.05$).

Insulin sensitivity. Insulin sensitivity, as defined by insulin-stimulated glucose uptake (ΔR_d , clamp minus basal), was significantly higher in trained compared with untrained subjects (Fig. 2A). The difference in insulin sensitivity was entirely due to a higher insulin-stimulated increase in oxidative glucose disposal in trained subjects (Fig. 2B). Insulin-stimulated increase in NOGD was similar in trained and untrained subjects (Fig. 2C). In line, insulin-induced suppression of lipid oxidation was also more pronounced in trained compared with untrained subjects (Table 2). Another way to express these findings is to define metabolic flexibility, which reflects the increase in respiratory exchange ratio (RER) from baseline to insulin-stimulated conditions. This metabolic flexibility was significantly higher in trained compared with untrained subjects (Table 2). No differences in insulin-induced suppression of hepatic glucose output were observed between trained and untrained subjects (Table 2).

Effect of oxidative capacity on lipid-induced IR. Upon the infusion of intralipid, FFA concentrations rose to a similar extent in trained and untrained subjects (from 401 ± 45 to $1,016 \pm 118\ \mu\text{mol/L}$ in trained subjects and from 512 ± 52 to $1,211 \pm 100\ \mu\text{mol/L}$ in untrained subjects, $P < 0.05$ for both groups). Lipid infusion markedly lowered insulin-stimulated glucose uptake by $\sim 63\%$ in untrained subjects (from 35.7 ± 2.7 to $12.0 \pm 3.1\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Fig. 2A). Lipid-induced IR was significantly alleviated in trained subjects, in whom insulin-stimulated glucose uptake only decreased by $\sim 29\%$ (from 45.9 ± 3.6 to $31.6 \pm 3.0\ \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Fig. 2A). The difference in lipid-induced IR between trained and untrained subjects was entirely due to a difference in lipid-induced lowering of NOGD; insulin-stimulated NOGD decreased by 52% in untrained subjects upon lipid infusion (from 29.1 ± 2.7 to

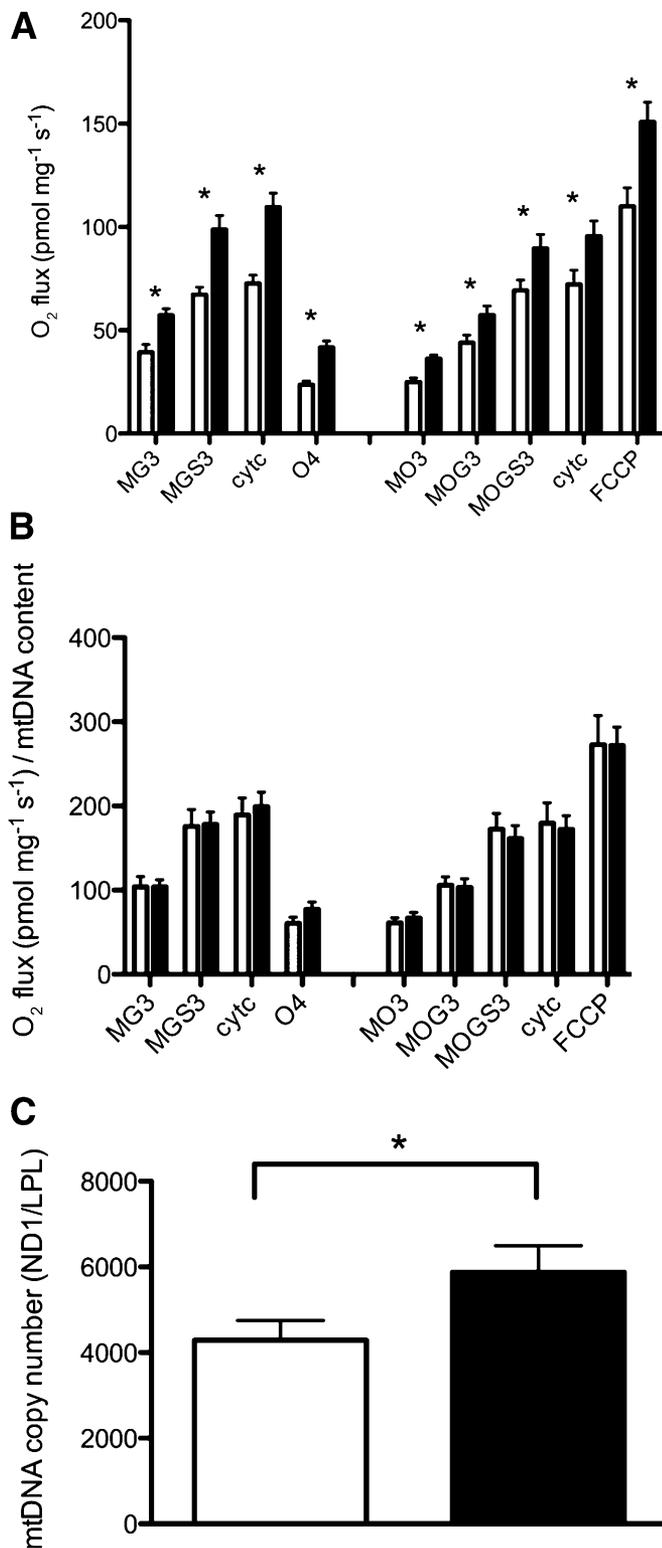


FIG. 1. Mitochondrial oxygen flux ($\text{pmol} \cdot \text{mg}^{-1} \cdot \text{s}^{-1}$) measured in permeabilized muscle fibers in trained (black bars) and untrained (white bars) subjects (A) and normalized to mitochondrial content (B). * $P < 0.05$, trained vs. untrained subjects. mtDNA copy number calculated as the ratio ND1/LPL in arbitrary units (C). All data are expressed as mean \pm SE. cytc, cytochrome c; FCCP, fluoro-carbonyl cyanide phenylhydrazone; M, malate; MG3, malate glutamate and ADP; MGS3, MG3 and succinate; MO, malate octanoyl-carnitine; MO3, MO and ADP; MOG3, MO3 and glutamate; MOGS3, MOG3 and succinate; O4, oligomycin.

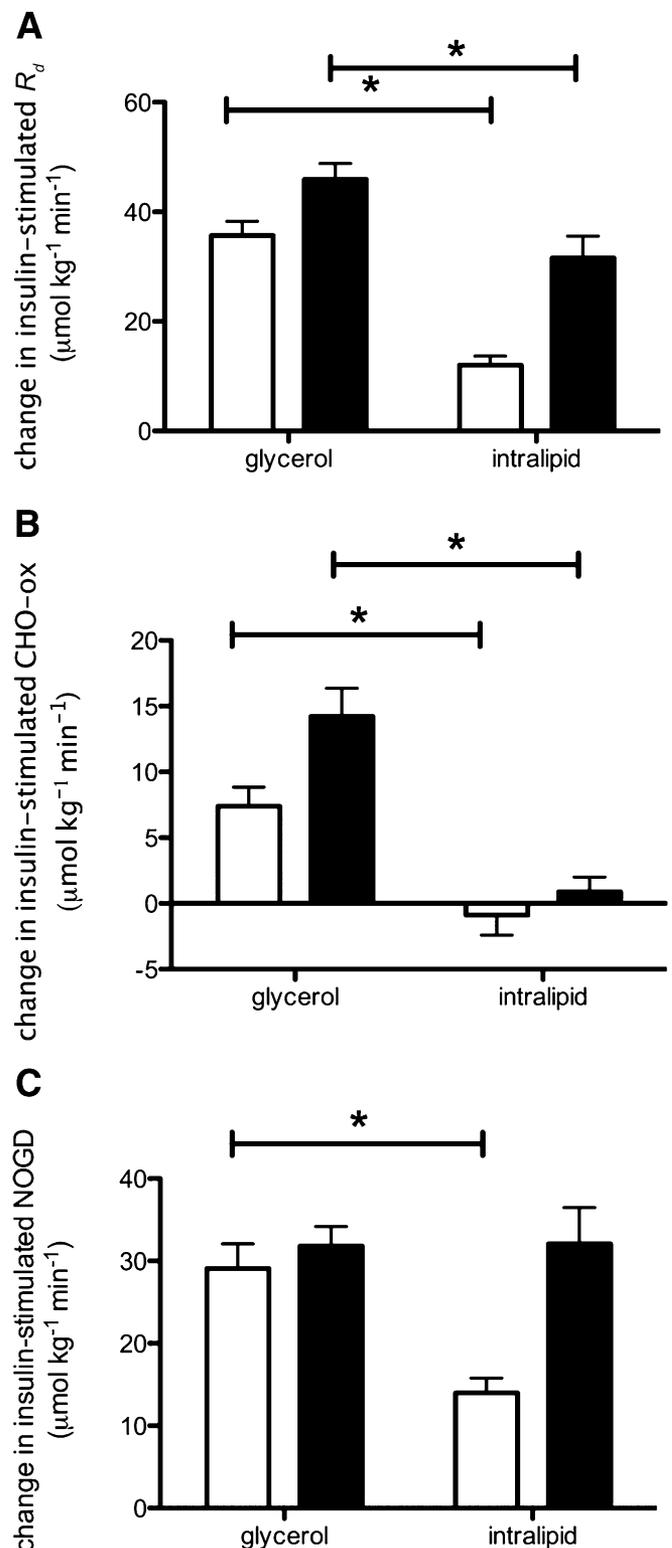


FIG. 2. Insulin sensitivity expressed as insulin-stimulated change of ΔR_d (A), carbohydrate oxidation (B), and NOGD (C) (all parameters expressed as $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in trained (black bars) and untrained (white bars) subjects during a hyperinsulinemic-euglycemic clamp with simultaneous infusion of glycerol or lipid. Data expressed as mean \pm SE. * $P < 0.05$.

$14.0 \pm 3.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Fig. 2C), whereas NOGD was completely unaffected in trained subjects (from 31.8 ± 2.8 to $32.1 \pm 3.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P = \text{NS}$) (Fig. 2C).

As a consequence, the 29% decrease in insulin sensitivity upon lipid infusion that we observed in trained subjects was

TABLE 2
Substrate kinetics

	Glycerol		Lipid	
	Untrained	Trained	Untrained	Trained
EGP ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)				
Basal	15.6 \pm 0.6	14.0 \pm 0.8	18.1 \pm 2.3	13.5 \pm 0.6
Clamp	-0.5 \pm 1.0#	-1.5 \pm 1.3#	4.8 \pm 1.1#	2.4 \pm 1.5#
$\Delta\text{EGP}_{\text{clamp}} - \text{EGP}_{\text{basal}}$	-16.1 \pm 1.2	-15.1 \pm 1.3	-13.3 \pm 2.0	-11.2 \pm 2.0
Lipid oxidation ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)				
Basal	1.2 \pm 0.1*	1.5 \pm 0.1*	1.2 \pm 0.1*	1.7 \pm 0.1*
Clamp	0.6 \pm 0.1*	0.4 \pm 0.1*	1.5 \pm 0.1#	1.8 \pm 0.1*#
Δ Lipid oxidation _{clamp} - lipid oxidation _{basal}	-0.6 \pm 0.1*	-1.1 \pm 0.1*	0.3 \pm 0.1#	0.2 \pm 0.1#
RER				
Basal	0.82 \pm 0.01*	0.80 \pm 0.02*	0.82 \pm 0.02*	0.78 \pm 0.01*
Clamp	0.91 \pm 0.01*	0.95 \pm 0.01*	0.80 \pm 0.01*	0.78 \pm 0.01*
$\Delta\text{RER}_{\text{clamp}} - \text{RER}_{\text{basal}}$	0.09 \pm 0.02*	0.14 \pm 0.02*	-0.02 \pm 0.02#	-0.001 \pm 0.01#

Data are mean \pm SE. * $P < 0.05$ between trained vs. untrained subjects. # $P < 0.05$ between lipid vs. glycerol trial.

completely accounted for by a reduction of insulin-stimulated glucose oxidation (from $+14.2 \pm 1.9$ to $+0.9 \pm 1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Fig. 2B). In untrained subjects, the insulin-stimulated glucose oxidation was also completely reduced (from $+7.4 \pm 1.7$ to $-0.9 \pm 1.3 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Fig. 2B) as well as NOGD, as previously mentioned. In line, the suppression of lipid oxidation, which is normally observed under insulin-sensitive conditions, was completely abolished upon lipid infusion in both trained (from -1.1 ± 0.1 to $+0.2 \pm 0.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Table 2) and untrained subjects (from -0.6 ± 0.1 to $+0.3 \pm 0.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$) (Table 2). Again, this is reflected in altered metabolic flexibility upon lipid infusion, which was similar ($P > 0.05$) in both groups (from 0.14 ± 0.02 to -0.001 ± 0.01 in trained subjects and from 0.09 ± 0.02 to -0.02 ± 0.02 in untrained subjects) (Table 2).

Intramyocellular lipid accumulation. In type I fibers, the mean IMTG area fraction was increased in untrained subjects ($n = 7$) but not in trained subjects ($n = 10$) upon intralipid infusion (clamp minus baseline, %), leading to a significant difference in muscle fat accumulation upon intralipid infusion ($+0.2 \pm 1.2$ vs. $+4.1 \pm 1.1\%$, $P < 0.05$). In type II muscle fibers, the mean intramyocellular IMTG area fraction did not change upon intralipid infusion (clamp minus baseline, %) in trained and untrained subjects (-0.2 ± 0.5 vs. $+0.5 \pm 0.9\%$).

Protein phosphorylation of insulin signaling. No differences were found in the expression level of insulin receptor before versus after glycerol or lipid infusion for both untrained and trained subjects (Fig. 3A). The phosphorylation of AKT (pAKT) significantly increased upon insulin stimulation with glycerol in untrained subjects and tended to increase in trained subjects ($P = 0.09$). In addition, pAKT increased nonsignificantly with lipid infusion in both groups upon lipid infusion (data not shown). Phosphorylation of PRAS40, a downstream target of AKT phosphorylation, was significantly increased in trained subjects after glycerol ($P < 0.05$) and lipid infusion ($P < 0.05$), in contrast to the untrained subjects (data not shown). Phosphorylation of GSK3 β (pGSK3 β), inhibiting GSK3 β activity, was increased in trained subjects upon lipid infusion. This change was not observed in untrained subjects (Fig. 3B). Phosphorylation of GSK3 β causes dephosphorylation of GS, resulting in a blunted suppression of glycogen synthesis. In trained subjects, insulin dephosphorylates GS upon both glycerol and lipid infusion, which was not observed in

untrained subjects (Fig. 3C). The phosphorylation of fork-head box class O1 (pFOXO1) significantly increased upon glycerol infusion in both untrained and trained subjects. Upon hyperinsulinemia, during lipid infusion, pFOXO1 remained responsive in both groups (Fig. 3D). The expression of PDK4, a target gene of FOXO1, tended to increase upon glycerol infusion in untrained subjects and significantly increased in trained subjects. Upon the infusion of lipid, PDK4 significantly increased in trained subjects (Fig. 3E).

Correlations. Baseline respiratory values ($\text{pmol} \cdot \text{mg}^{-1} \cdot \text{s}^{-1}$) correlated positively with insulin sensitivity, defined as insulin-stimulated glucose uptake (clamp minus basal ΔR_{G} ; $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). Insulin sensitivity correlated with state 3 respiration on glutamate and succinate ($R^2 = 0.34$, $P < 0.01$) (Supplementary Fig. 1A) and state 3 respiration on glutamate, succinate, and octanoyl-carnitine ($R^2 = 0.34$, $P < 0.01$) (Supplementary Fig. 1B). All other respiratory states correlated with insulin sensitivity as well (data not shown).

Furthermore, IMTG accumulation upon lipid infusion (clamp minus baseline, %) tended to correlate negatively with insulin sensitivity (clamp minus basal ΔR_{G} ; $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $R^2 = 0.21$, $P = 0.07$) (Supplementary Fig. 1C).

DISCUSSION

A high oxidative capacity has been suggested to be important in the prevention of IR and ultimately T2D. Here we show that a high oxidative capacity due to chronic exercise training is indeed able to attenuate acute lipid-induced IR. Thus, endurance-trained athletes with a higher oxidative capacity, reflected by a higher mitochondrial content but not in enhanced intrinsic mitochondrial function, reduced insulin sensitivity only by 29% upon lipid infusion versus 63% in untrained subjects. This difference was entirely due to the lack of an effect of lipid infusion on NOGD in the trained subjects, whereas insulin-stimulated glucose oxidation reduced similarly in trained and untrained subjects upon lipid infusion.

Interestingly, in this study, we show that trained subjects have a higher mitochondrial capacity in skeletal muscle, but that intrinsic mitochondrial function was similar between trained and untrained subjects. This is in line with training-induced increases in mitochondrial function in obese control and T2D subjects observed in the 12-week training program, which was almost entirely accounted for by an

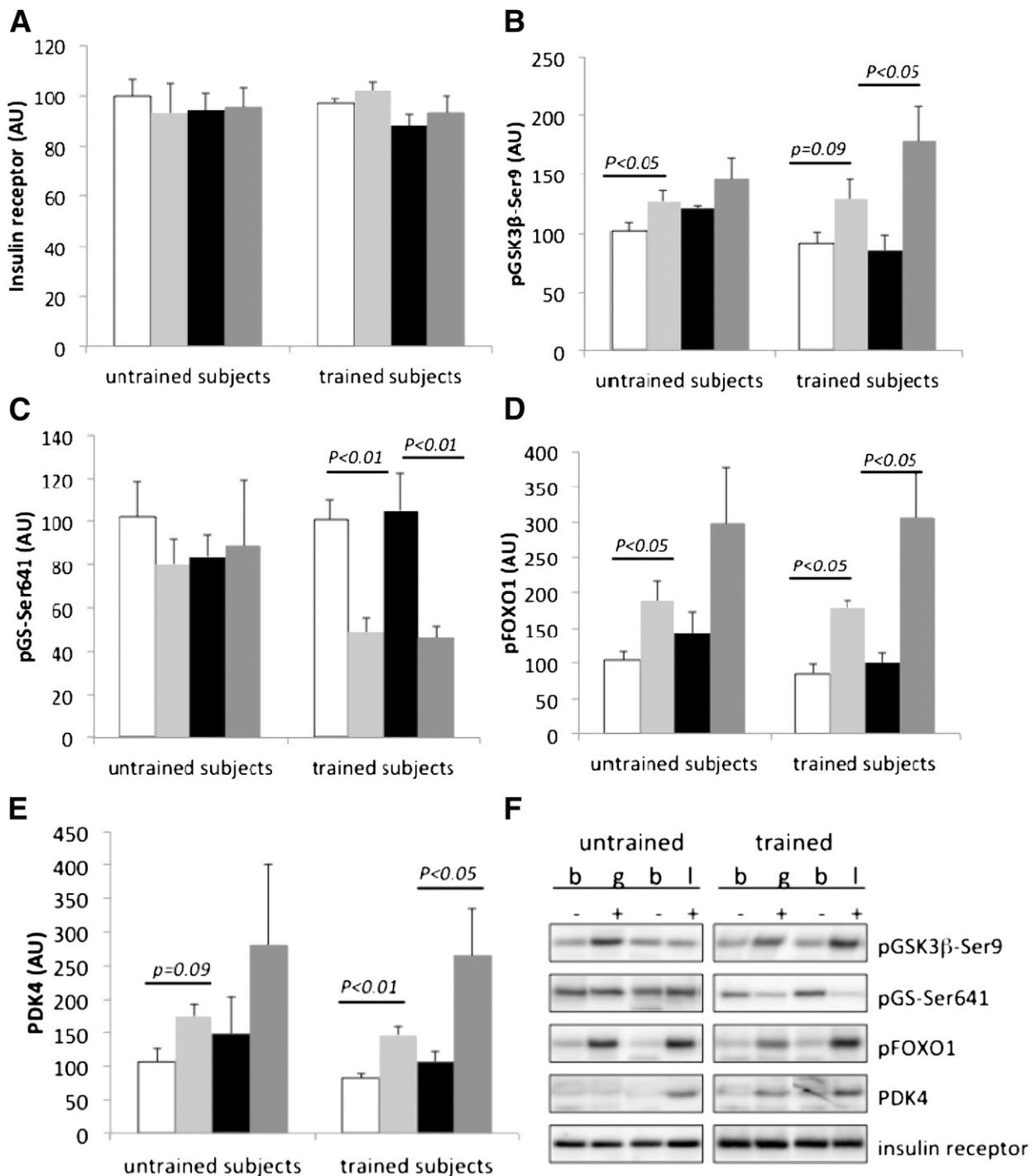


FIG. 3. The expression level of insulin receptor (A), pGSK3 β (B), GS (C), phosphorylation of FOXO1 (pFOXO1) (D), and phosphorylation of PDK4 (E) in trained and untrained subjects before and after glycerol (white and light gray bars, respectively) as well as before and after lipid infusion (black and dark gray bars, respectively). Representative Western blots under baseline conditions (b), after glycerol (g), and after lipid (l) infusion, with (+) and without (-) insulin stimulation (F). Data expressed as mean \pm SE. AU, arbitrary units.

increased mitochondrial mass rather than intrinsic mitochondrial function (32).

It is well known that endurance training improves whole-body and muscular insulin sensitivity in young, healthy, lean individuals, as well as in obese and T2D subjects (33–35). Also in the current study, baseline insulin sensitivity was higher in trained subjects compared with the age-matched untrained individuals. Interestingly, this difference in insulin sensitivity could be entirely attributed to a difference in

insulin-stimulated glucose oxidation, also referred to as metabolic flexibility, and was not due to differences in NOGD. In other words, trained subjects have higher insulin sensitivity because of an increased capability to switch between fat and glucose oxidation upon insulin stimulation. This finding is in accordance with our recent report showing that a 12-week endurance and strength training program was able to increase insulin sensitivity in T2D subjects, solely explained by an increase in insulin-stimulated glucose

oxidation and without any change in insulin-stimulated NOGD (34). In these subjects, mitochondrial function and content improved after the 12-week training program (32). Together these data indicate that mitochondrial oxidative capacity (mitochondrial content and/or intrinsic mitochondrial function) is indeed an important determinant of the oxidative part of muscular insulin sensitivity. In the current study, this finding is substantiated by the positive correlations between respiratory mitochondrial fluxes and insulin sensitivity. In addition, the observation that NOGD rates are similar between untrained and trained subjects, and that NOGD rates did not improve upon 12 weeks of exercise training in T2D patients despite improvements in insulin sensitivity (34), suggests that insulin-stimulated glucose oxidation and storage are two independently regulated mechanisms. Both, however, require functional signaling for myocellular glucose uptake.

We were interested in examining next whether a high oxidative capacity not only associates with a higher insulin sensitivity but also attenuates lipid-induced IR. Thus, a reduced mitochondrial function or lower oxidative capacity has been suggested to underlie the development of IR through increased accumulation of fat and/or lipid intermediates in skeletal muscle. Here we indeed showed that the development of IR upon experimental elevation of circulating fatty acids was attenuated in trained subjects, but the development of IR could not completely be prevented. More specifically, insulin stimulation of glucose oxidation and inhibition of fat oxidation, i.e., metabolic flexibility, were affected to a similar extent in trained and untrained subjects. In fact, the reduction in insulin-stimulated glucose oxidation completely accounted for the reduced insulin sensitivity upon lipid infusion in the trained subjects. In untrained subjects, however, both insulin-stimulated oxidative capacity as well as insulin-stimulated NOGD were markedly reduced upon lipid infusion, in line with recently reported data (36). In contrast, trained athletes were completely protected against a lipid-induced reduction in NOGD.

In general, lipid-induced IR has been explained by an inhibitory effect of muscular fatty acid intermediates on insulin signaling, resulting in reduced GLUT4 translocation and hence impeded myocellular glucose uptake (37,38). As a result of this reduced glucose uptake, both oxidative capacity and NOGD are reduced. The results obtained in our untrained subjects upon lipid infusion are consistent with this model, as lipid infusion resulted in lipid accumulation in muscle and both reduced oxidative capacity and NOGD. In the trained subjects where lipid infusion did not affect NOGD, however, an alternative mechanism is emerging. Thus, it has been postulated by Randle et al. (39) that lipid-induced IR is due to competition between glucose and fat for oxidation; high fatty acid availability would prevail fat oxidation over glucose oxidation. The fact that lipid infusion leads to a preferential oxidation of fatty acids over glucose in both trained and untrained subjects fits with the concept of Randle et al. (39) and could be considered a normal adaptation to increased FFA availability. However, according to Randle et al. (39), an increase in fat oxidation would lead to the accumulation of metabolites in the glycolytic pathway, such as glucose-6-phosphate and intracellular glucose, but would not reduce the conversion of glucose or glucose-6-phosphate to glycogen. In other words, in Randle's model, NOGD would not be affected by high FFA availability. Therefore, the finding that in trained subjects, NOGD was not affected upon lipid infusion would fit with the hypothesis put forward by Randle et al. (39). To further

examine this possibility, we examined muscle biopsies taken before and after the clamp. The expression of the insulin receptor was not affected by lipid infusion, and the effect of lipid infusion on protein phosphorylation of AKT was not different between untrained and trained subjects. Insulin-stimulated phosphorylation of PRAS40, a downstream target of AKT phosphorylation, was significantly higher in the trained subjects under insulin-stimulated conditions during the control clamp and lipid infusion, compared with untrained subjects. We next determined phosphorylation of GS, also a downstream target of AKT phosphorylation. It is known that a reduction in GS phosphorylation releases the suppression on glycogen synthesis. We noted that GS phosphorylation was reduced upon insulin stimulation in trained subjects only, even in the lipid infusion condition. These data strongly suggest that trained subjects maintain glucose uptake directed toward glycogen storage upon the infusion of lipids, in contrast to untrained subjects. The dephosphorylation of GS is regulated by the phosphorylation status of GSK3 β . In support of the data above, phosphorylation of GSK3 β markedly increased after lipid infusion in trained subjects, which was not seen in untrained subjects. Overall, phosphorylation data indicate that signaling at the IRS and AKT level is still intact in both trained and untrained subjects, but that signaling upon lipid infusion downstream from AKT is only intact in trained subjects and affected in the untrained subjects. This supports the notion that trained subjects are protected against lipid-induced impairment of insulin signaling and glucose uptake. Instead, glucose is directed into glycogen storage, i.e., NOGD.

Other phosphorylation results showed that pFOXO1 was increased upon intralipid infusion. FOXO1 is suggested to be a transcription factor involved in metabolic adaptation through PDK4 (40). The increased phosphorylation of PDK4 upon lipid infusion of trained subjects may be responsible for the decreased carbohydrate oxidation seen in this group, as phosphorylation of PDK4 results in lower glucose oxidation through inhibition of pyruvate dehydrogenase.

An alternative explanation of why subjects with a high fat oxidative capacity are protected against lipid-induced IR would be the efficient shuttling of fatty acids to inert triglycerides, thereby preventing the accumulation of lipid intermediates that interfere with insulin-stimulated GLUT4 translocation. In that respect, it was recently shown that one bout of acute exercise was able to prevent lipid-induced IR by the efficient shuttling of fatty acids in the muscle into inert triglyceride stores, through activation of diacylglycerol acyltransferase (DGAT) (41). However, our data suggest that in trained subjects, elevation of fatty acids by intralipid infusion did not lead to shuttling of these fatty acids to triglyceride stores, as IMTG content was not increased upon intralipid infusion in this group. It should be noted that conditions between these studies were different; in the current study, we did not investigate the development of lipid-induced IR upon acute exercise, but rather after chronic exercise training. Moreover, in the case of a high availability of FFAs, chronic exercise training seems to prevent the storage of triglycerides in muscle. Interestingly, despite an absent accumulation of IMTGs, insulin-stimulated glucose disposal was still reduced by 29% upon intralipid infusion in trained subjects, which was most likely due to substrate competition, as suggested by Randle et al. (39).

Overall, from the current study, we conclude that chronic exercise training attenuates lipid-induced IR by preventing a reduction in NOGD. Our data may also indicate the importance of considering oxidative glucose disposal separate

from NOGD when investigating the etiology of lipid-induced IR. In the future, more mechanistic studies are needed to fully understand the mechanism by which endurance training affects lipid-induced NOGD.

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E.P. researched data, contributed to discussion, and wrote the manuscript. R.M. researched data, contributed to discussion, and reviewed and edited the manuscript. D.M.O. and L.S. researched data and reviewed and edited the manuscript. J.H., M.K.C.H., and P.S. contributed to discussion and reviewed and edited the manuscript. G.S. and E.M.-K. researched data. P.S. 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.

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