IL-6 is not essential for exercise-induced increases in glucose uptake

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Submitted 2 August 2012; accepted in final form 20 February 2013

O’Neill HM, Palanivel R, Wright DC, MacDonald T, Lally JS, Schertzer JD, Steinberg GR. IL-6 is not essential for exercise-induced increases in glucose uptake. J Appl Physiol 114: 1151–1157, 2013. First published August 28, 2013; doi:10.1152/japplphysiol.00946.2012 — Interleukin-6 (IL-6) increases glucose uptake in resting skeletal muscle. IL-6 is released from skeletal muscle during exercise; however, it is not known whether this IL-6 response is important for exercise-induced increases in skeletal muscle glucose uptake. We report that IL-6 knockout (KO) mice, 4 mo of age, have similar body weight to wild-type (WT), and, under resting conditions, oxygen consumption, food intake, substrate utilization, glucose tolerance, and insulin sensitivity are not different. Maximal exercise capacity is also similar to WT. We investigated substrate utilization and glucose clearance in vivo during steady-state treadmill running at 70% of maximal running speed and found that WT and IL-6 KO mice had similar rates of substrate utilization, muscle glucose clearance, and phosphorylation of AMP-activated protein kinase T172. These data provide evidence that IL-6 does not play a major role in regulating substrate utilization or skeletal muscle glucose uptake during steady-state endurance exercise.

interleukin-6; exercise; insulin sensitivity; AMP-activated protein kinase; glucose uptake; fatty acid oxidation

DURING ENDURANCE EXERCISE, the enhanced energy demand of muscle contractions elicits an increase in skeletal muscle glucose uptake. Understanding the underlying molecular mechanisms regulating contraction-stimulated glucose uptake has been a goal of many laboratories, given that this pathway is largely preserved in individuals resistant to the effects of insulin in promoting skeletal muscle glucose uptake. Therefore, exercise represents an important means for allowing glucose uptake as well as restoring insulin sensitivity in patients with insulin resistance. Muscle contractions result in the production of the myokine interleukin-6 (IL-6), which is released from skeletal muscle in an intensity-dependent manner (for review see Ref. 23). Importantly, short-term exposure of skeletal muscle to high concentrations of IL-6 can acutely increase GLUT4 translocation and glucose uptake (2, 7, 10, 14, 20).

The AMP-activated protein kinase (AMPK) is activated during muscle contractions in an intensity- and time-dependent manner (for review see Ref. 24). In resting skeletal muscle, pharmacological activation of AMPK using 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide results in elevated rates of skeletal muscle glucose uptake that is dependent on the presence of an AMPK α2β2γ3 heterotrimer (3, 12, 25). Using the first genetically modified mice completely lacking skeletal muscle AMPK, we have recently demonstrated that AMPK is essential for regulating exercise capacity and increasing skeletal muscle glucose uptake in response to electrically stimulated muscle contractions and treadmill exercise (21). Analogous to mice lacking skeletal muscle AMPK, IL-6 knockout (KO) mice have reduced resting skeletal muscle AMPK activity (14) and lower exercise capacity (8). In addition, the activation of AMPK during exercise is tightly linked to the secretion of IL-6 from skeletal muscle in both rodents (11) and humans (18).

The above findings connecting IL-6 with AMPK and muscle glucose uptake led us to hypothesize that IL-6 may be a critical regulator of skeletal muscle glucose uptake during exercise. Therefore, in the present study, we used IL-6 KO mice to examine whether endogenous IL-6 is required to sustain normal AMPK activity and skeletal muscle glucose clearance during steady-state endurance exercise.

MATERIALS AND METHODS

Animal procedures. Male C57BL/6J wild-type (stock no. 00664) and IL-6 KO B6.129S2-Ile6 KO/J (stock no. 002650) mice were purchased from Jackson Laboratories at 2 mo of age. The C57BL/6J control mice used in this study were recommended by Jackson Laboratories (Bar Harbor, ME) (JAX), as they provided the C57BL/6J background used to backcross IL-6 KO 129B mice (16) for 11 generations. Importantly, at JAX, the IL-6 KO mice used in this experiment are crossed with pure C57BL/6J line every 10 generations to prevent genetic drift. All experiments were approved by the McMaster University Animal Ethics Committee. Mice were housed in specific pathogen-free microisolators and maintained under controlled environmental conditions (12-h:12-h light/dark cycle with lights on at 07:00 and temperature of 23°C) for 2 mo before experiments. All mice received standard chow food (50% carbohydrate, 19% kcal fat; 31% protein) (Diet 8664, Harlan Teklad, Madison, WI) and water ad libitum. At 4 mo of age (before any exercise testing was performed), metabolic monitoring was performed using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH) as described previously (21). For exercise experiments [exercise tolerance, substrate utilization, glucose clearance, and AMPK and acetyl-CoA carboxylase (ACC) phosphorylation], we utilized a group of six wild-type (WT) and seven KO mice, and for basal experiments (metabolic analyses, insulin sensitivity, and AMPK and ACC phosphorylation), we used a group of eight mice for both WT and KO.

Whole body insulin sensitivity and glucose tolerance. In 6-h-fasted mice, whole body glucose and insulin tolerance were assessed over 120 min following intraperitoneal injection of either glucose (1 g/kg) or insulin (0.5 U/kg) as previously described (4).

Treadmill running experiments. At 4.5 mo of age, before the exercise experiments, all mice were acclimatized to treadmill running for 3 days with 1 day of rest before the testing day. Acclimatization consisted of running on the treadmill apparatus (Columbus Instruments International Exer4-Oxymax) under the following conditions: day 1: no gradient for 5 min at 10 m/min, day 2: 10 min at 10 m/min, and day 3: 5 min at 10 m/min, 5 min at 12 m/min, and 5 min at 15 m/min. For maximal exercise capacity testing, mice ran at 10 m/min...
for 2 min before intensity (running speed) was increased by 1 m/min every 2 min until mice could not be prompted to continue running by electric shockers at the back of the treadmill. The following week, WT and IL-6 KO mice were run in an Oxymax treadmill (Columbus Instruments) for 40 min at 70% of individual maximal running speed. After 20 min of treadmill running (to ensure steady-state conditions were achieved) oxygen consumption (V\textsubscript{O2}), carbon dioxide production (V\textsubscript{CO2}), and respiratory exchange ratio (RER) were measured. Carbohydrate (CHO) and lipid oxidation rates were calculated by the following equations: \[(4.585 \times V\textsubscript{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{O2}}}}}}}}}}} - (3.226 \times V\textsubscript{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{CO2}}}}}}}}}}})\] and \[(1.695 \times V\textsubscript{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{O2}}}}}}}}}}} - (1.701 \times V\textsubscript{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{CO2}}}}}}}}}}})\], respectively (9).

In vivo exercise protocol for determination of 2-deoxyglucose clearance. On the experimental day, IL-6 KO and WT mice ran at 70% of their individual maximal running capacity for 40 min. After 20 min of treadmill running, when steady-state conditions were achieved, the mice were quickly removed from the treadmill and injected intraperitoneally with a bolus of saline (800 μl/100 g body wt) containing 2-deoxyglucose (2DG) (0.1 mM) and [H\textsubscript{3}]-2DG (60 μCi/ml) corresponding to ~12 μCi/mouse. Blood samples (30 μl) were collected from the tail at −2, 2, 10, 30-, and 40-min time points and transferred to tubes containing EDTA. Blood samples were placed on ice and allowed to clot before centrifugation (3.000 revolution/min) at 4°C for 10 min to collect plasma. Plasma was stored at −80°C until further processed. Plasma radioactivity of tracers was measured by liquid scintillation counting following deproteinization using 0.1 M Ba(OH\textsubscript{2}) and 0.1 M ZnSO\textsubscript{4}. Plasma glucose was determined by a glucometer (Accu-check; Roche Diagnostics, Indianapolis, IN). Plasma IL-6 levels were determined using a mouse IL-6 ELISA (DY406) following manufacturer’s recommendations (R&D Systems, Minneapolis, MN).

After 40 min, mice were euthanized by cervical dislocation. For signaling, 
\textit{gastrocnemius, soleus, quadriceps, tibialis anterior} (TA),
and \textit{extensor digitorum longus} (EDL) from the one leg were quickly removed, immediately frozen in liquid nitrogen, and stored at −80°C until further processing. The same muscles were removed from the other leg and used for determination of glucose clearance as described below and previously (21).

Muscle analyses. Whole muscles were powdered over liquid nitrogen and then homogenized in ice-cold cell lysis buffer containing Tris base (20 mM), leupeptin (4 mM), benzmidine (6 mM), phenylmethylsulfonyl-fluoride (500 μM), sodium fluoride (50 mM), sodium pyrophosphate (5 mM), leupeptin (4 μg/ml), benzimidate (6 mM), phenylmethylsulfonyl-fluoride (50 μM), and soybean trypsin inhibitor (50 μg/ml; pH 7.4) for 30 s using ceramic beads and a Precellys 24 dual homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Lysates were generated by centrifugation at 13,000 revolution/min for 15 min at 4°C. Lysates were either further processed for determination of glucose clearance as described (17, 21) or snap frozen in liquid nitrogen and stored at −80°C for immunoblot analysis. Protein content in lysates was assessed by colorimetric analysis using the bicinchoninic acid method (Pierce, Rockford, IL).

Immunoblotting. Protein phosphorylation and expression levels were determined by SDS-PAGE followed by immunoblotting using muscle lysates that were adjusted to equal protein concentration (2 μg/μl) and boiled for 5 min at 95°C in 4X sample buffer (Tris-HCl (50 mM, pH 6.8), SDS (2%), glycerol (10%), DTT (1%), EDTA (1%), and bromophenol blue (0.02%)). A sample (~30 μg) was loaded per well, and proteins were separated on 7.5% Tris-HCl gels and transferred to polyvinylidene difluoride membrane by wet blotting (40 mA overnight). Membranes were blocked in skim milk/PBST (5%) for 1 h at room temperature before incubation with primary antibodies (1: 1,000) overnight at 4°C. Antibodies for determination of AMPK α2, AMPK phospho-εT172, ACC phospho- S79/212, and horseradish peroxidase (HRP)-conjugated streptavidin (total ACC) (1:2,000) were purchased from (Cell Signaling Technology, Beverly, MA). Membranes were then washed for 3 × 10 min in PBST and incubated with HRP-conjugated secondary antibody (1:10,000) for 1 h at room temperature. Protein bands were visualized using Fusion Image Dock Station (Vilber Lourmat, Germany) and enhanced chemiluminescence (ECL\textsuperscript{*}). Bands were quantified using ImageJ software and were expressed as a ratio of the phosphorylated to total protein. Membranes used for detection of phosphorylated ACC and AMPK were stripped with a buffer containing 2-mercaptoethanol (100 mM), SDS (2%), and Tris-HCl (62.5 mM; pH 7.8). Membranes were reprobed with the corresponding total antibody.

Muscle glycogen. Muscle glycogen content was determined as glycosyl units after acid hydrolysis as previously described (22). In short, 10–15 mg wt/wt muscle was hydrolyzed in HCl (1 M) at 98°C for 1 h and analyzed using an automatic analyzer (Hitachi automatic analyzer 912; Boehringer Mannheim, Ingelheim, Germany).

Statistics. All data was expressed as means ± SE. Results were analyzed using paired t-test using GraphPad Prism software. The insulin and glucose tolerance tests were analyzed using a two-way ANOVA for repeated measures and Bonferroni as a post hoc. Significance was accepted at P < 0.05.

RESULTS

IL-6 KO mice have normal body mass and resting energy metabolism. The body mass of mice at 4 mo of age was ~29 g, and there was no detectable difference between WT and IL-6 KO mice (Fig. 1A). Over a 24-h light/dark cycle, IL-6 KO mice had similar RER, oxygen consumption, spontaneous activity levels, and food intake compared with WT littermates (Fig. 1, B–E). Whole body glucose and insulin tolerance in IL-6 KO mice was not different compared with WT as illustrated by a similar area under the curve (Fig. 1, F, G, inset).

IL-6 KO mice have normal carbohydrate oxidation during submaximal treadmill exercise. In resting skeletal muscle, IL-6 can increase glucose uptake, but it is not known whether physiological levels of IL-6 released during exercise are important for regulating substrate utilization. Therefore, we examined maximal exercise tolerance and substrate utilization during an acute bout of submaximal treadmill running. We found that maximal exercise tolerance (WT: 25 m/min and IL-6 KO: 26 m/min) was not different between WT and IL-6 KO mice (Fig. 2A). We then ran mice at 70% of individual maximal running speed (~15 m/min) (Fig. 2A), which significantly elevated plasma IL-6 levels in WT (~39 pg/ml) but not IL-6 KO mice (not detected) (Fig. 2B). As anticipated, given the similar workload between WT and IL-6 KO mice, VO\textsubscript{2} was not different between genotypes (Fig. 2, C and D). In addition, under steady-state conditions, RER was not different between WT and IL-6 KO mice (Fig. 2, E and F), indicating that substrate utilization (Fig. 2, G and H) during endurance exercise is not regulated by IL-6. These data indicate that IL-6 is not essential for controlling maximal exercise capacity or substrate utilization during steady-state endurance exercise.

IL-6 is not required for skeletal muscle glucose clearance during treadmill exercise. Plasma glucose levels during exercise (20, 30, and 40 min) were comparable between WT and IL-6 KO mice (Fig. 3A). Skeletal muscle glycogen at the end of treadmill exercise was comparable between genotypes (Fig. 3B). Consistent with RER data during steady-state treadmill running, glucose clearance into oxidative (soleus) and glycolytic (EDL, TA, \textit{gastrocnemius, quadriceps}) muscles were not different between WT and IL-6 KO mice although there was a trend for a 25% reduction in EDL muscle (P = 0.1) (Fig. 3C). These data demonstrate that IL-6 is not required for promoting skeletal muscle glucose clearance during endurance treadmill exercise.
AMPK and ACC phosphorylation are not altered in skeletal muscle of IL-6 KO mice at rest or following treadmill exercise. Under resting conditions, AMPK T172 (Fig. 4A) or ACC S79/212 (Fig. 4B) phosphorylation in soleus and EDL were not different between WT and IL-6 KO mice. Consistent with similar glucose clearance, we found that phosphorylation of AMPK T172 and ACC S79/212 was comparable between WT and IL-6 KO mice at the completion of treadmill exercise (Fig. 4, C and D). There were
Fig. 2. IL-6 KO mice have normal substrate utilization during steady-state treadmill exercise. A: maximal exercise tolerance and 70% of maximal running speed (~15 m/min). B: plasma IL-6 levels in mice at rest (preexercise) and following an acute bout of submaximal treadmill running (70% maximal running speed; 40 min) (postexercise). Normal oxygen consumption (V̇\text{O}_2) over time (C) and average (D) is shown. Respiratory exchange ratio (RER) over time (E) and average (F), and fatty acid (FA) and carbohydrate (CHO) oxidation rates over time (G) and average (H) in IL-6 KO and WT mice during 20 min steady-state treadmill exercise at the same relative workload (70% each individual's mouse maximal running speed) are shown. Data are means ± SE, n = 7 WT and n = 8 KO. \#P < 0.05 compared with preexercise, same genotype. *P < 0.05 compared with WT, same treatment.
also no differences in AMPK or ACC phosphorylation in TA, gastrocnemius, or quadriceps (data not shown). These data indicate that IL-6 does not regulate the phosphorylation of AMPK or ACC at rest or following treadmill exercise.

**DISCUSSION**

The primary focus of our study was to investigate the physiological importance of endogenous IL-6 in regulating substrate utilization during endurance exercise. We found that IL-6 KO mice have normal exercise tolerance, and surprisingly IL-6 produced during treadmill exercise is not essential for regulating substrate utilization or promoting skeletal muscle glucose clearance. Taken together, these studies indicate that physiological levels of IL-6 do not play a major role in regulating in vivo energy metabolism during acute exercise. Alternatively, it may indicate that there are compensatory mechanisms that can account for the absence of IL-6 under these conditions.

Whole body deficiency in IL-6 promotes the development of aging and high-fat-diet-induced obesity and insulin resistance (19, 26). As our study was focused on examining the effects of IL-6 on energy metabolism during endurance exercise, we wanted to avoid these confounding variables. Therefore, we chose to study relatively young adult male mice fed a standard chow diet high in carbohydrates rather than fat. We found that our IL-6 KO mice had comparable body mass, energy intake and expenditure, substrate utilization, and spontaneous activity levels compared with WT controls (Fig. 1, A–E). Glucose and insulin tolerances were also not different between WT and IL-6 KO mice (Fig. 1, F and G). Thus these data demonstrating similar body mass and insulin sensitivity between WT and IL-6 KO mice support the idea that this model was appropriate for studying the effects of IL-6 deficiency on energy metabolism during endurance exercise.

We found that IL-6 KO mice did not have impaired exercise tolerance. These findings are different of those by Faldt et al. (8), who showed that IL-6 KO mice were exercise intolerant compared with WT controls. These differences are likely to be accounted for by variations in exercise protocols. Specifically Faldt et al. (8) assessed endurance exercise capacity at 20° incline, starting at 10 m/min followed by 14 m/min after 10 min, and then 18 m/min after 5 min until exhaustion. In our study, we assessed maximal exercise capacity and ran mice at 0° incline, starting at 10 m/min, and increasing speed by 1 m/min every 2 min until exhaustion. Recently, Benrick et al. (5) showed that both male and female IL-6 KO mice 8 wk of age had normal endurance treadmill running and voluntary wheel activity compared with WT, respectively. This is consistent with our data indicating that IL-6 does not play a major role in maintaining maximal exercise capacity.

Findings that IL-6 treatment enhances skeletal muscle glucose uptake and fatty acid oxidation (7) led us to hypothesize that this myokine may be important for regulating substrate utilization during exercise. Subsequent experiments assessing substrate utilization and glucose clearance were completed at 70% of maximal running speed (i.e., the same relative workload). In our study, IL-6 KO mice had similar steady-state rates of substrate utilization and glucose clearance. These results are different from those by Faldt et al. (8), who reported that fatty acid oxidation was reduced in IL-6 KO mice when run at the same absolute speed (10 m/min). As relative exercise intensity is the most important predictor of substrate utilization with higher exercise intensities associated with lower rates of fatty acid oxidation (6) and given the lower maximal exercise capacity of the IL-6 KO mice used by Faldt et al. (8), it is not surprising that they found that IL-6 KO mice utilized fewer fatty acids than WT controls at the same absolute relative
workload. Our data in which IL-6 KO mice were run at the same relative workload to WT controls suggest that IL-6 deficiency does not result in impaired rates of fatty acid oxidation during exercise.

We hypothesized that, given the robust effects of exercise to increase skeletal muscle glucose uptake, IL-6 may be important for contributing to this effect. We found that muscle glucose clearance into a variety of glycolytic and oxidative muscles was not different between WT and IL-6 KO mice during 20 min of steady-state endurance treadmill exercise although there was a trend for a 25% reduction (*P* < 0.1) in EDL muscle (Fig. 3A). While our manuscript was in preparation, Benrick et al. (5) reported similar findings with the exception that skeletal muscle glucose clearance was significantly reduced in EDL muscle of IL-6 KO mice. Taken together, these data suggest that, with the exception of the EDL muscle, IL-6 does not play an essential role in regulating skeletal muscle glucose uptake during exercise between muscle types are ongoing.

IL-6 increases AMPK phosphorylation in resting myotubes (7, 10) and isolated muscles (20). However, studies in IL-6 KO mice have reported either normal (1) or reduced (14) AMPK phosphorylation in resting and exercised muscle. We found that, in both oxidative and glycolytic muscle types, AMPK phosphorylation was normal under basal resting as well as exercised conditions, indicating that IL-6 does not play a major role in regulating AMPK activity under these conditions.

Why aren’t the acute effects of IL-6 treatment on AMPK activity and glucose uptake in resting muscle translated to endurance exercise? Kelly et al. (13) have shown that the primary mechanism by which IL-6 activates AMPK in resting muscle is through the induction of cAMP and the subsequent disruption of the AMP/ADP/ATP ratio. Importantly, Kelly et al. demonstrate that this is an essential mechanism for IL-6-induced activation of AMPK, as the effects are eliminated by treatment with the β-adrenergic blocker propranolol or the
adenyl cyclase inhibitor 2′-5′ di deoxyadenosine (13). It is known that treadmill exercise in rodents dramatically increases epinephrine and skeletal muscle cAMP (for review see Ref. 15), and, although our mice were acclimatized to the treadmill running protocol, it is likely that there was still a marked stress response as a result of the endurance exercise protocol. Therefore, it seems very likely that the modest effects of IL-6 to increase muscle cAMP levels are masked by the much larger increases in sympathetic innervation that occur due to the treadmill exercise. In summary, our data suggest that, while IL-6 has important effects on substrate utilization and skeletal muscle glucose uptake in resting muscle, it plays a relatively minor role in regulating skeletal muscle AMPK activity and glucose uptake during acute treadmill exercise in rodents.

GRANTS

These studies were supported by grants from the Canadian Institutes of Health Research and Natural Science and Engineering Research Council of Canada (G. Steinberg, D. Wright). J. Schertzer is a Canadian Diabetes Association Scholar. G. Steinberg is a Canada Research Chair in Metabolism and Obesity. D. Wright is a Canada Research Chair in Lipids, Metabolism and Health. H. O’Neill is supported by an Australian Postgraduate Award (The University of Melbourne) and St Vincent’s Institute of Medical Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.M.O. conception and design of research; H.M.O., R.P., D.C.W., T.M., and J.D.S. performed experiments; H.M.O. and G.R.S. interpreted results of experiments; H.M.O. prepared figures; H.M.O. and G.R.S. drafted manuscript; H.M.O., R.P., D.C.W., T.M., J.S.L., J.D.S., and G.R.S. edited and revised manuscript; H.M.O., R.P., D.C.W., T.M., and J.D.S. approved final version of manuscript.

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J Appl Physiol • doi:10.1152/japplphysiol.00946.2012 • www.jappl.org

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