

# Acute exercise increases insulin sensitivity in adult sheep: a new preclinical model

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<sup>1</sup>Institute of Sport, Exercise and Active Living, College of Sport and Exercise Science, Victoria University, Melbourne, Victoria, Australia; <sup>2</sup>College of Health and Biomedicine, Victoria University, Melbourne, Victoria, Australia; and <sup>3</sup>Robinson Research Institute and School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia, Australia

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**McConnell GK, Kaur G, Falcão-Tebas F, Hong YH, Gattford KL.** Acute exercise increases insulin sensitivity in adult sheep: a new preclinical model. *Am J Physiol Regul Integr Comp Physiol* 308: R500–R506, 2015. First published January 7, 2015; doi:10.1152/ajpregu.00466.2014.—In healthy humans and rodents, chronic and acute exercise improves subsequent insulin sensitivity of skeletal muscle. A large animal species with similar metabolic responses to exercise would permit longitudinal studies, including repeated biopsies of muscle and other tissues not possible in rodents, and enable study of interactions with insulin-resistant physiological states not feasible in humans. Therefore, we examined whether acute exercise increases insulin sensitivity in adult sheep. Insulin sensitivity was measured by hyperinsulinemic euglycemic clamp (HEC) in mature female sheep ( $n = 7$ ). Sheep were familiarized to treadmill walking and then performed an acute exercise bout (30 min, 8% slope, up to 4.4 km/h). A second HEC was conducted ~18 h after the acute exercise. *Musculus semimembranosus* biopsies were obtained before and after each HEC. Glucose infusion rate during the HEC increased 40% ( $P = 0.003$ ) and insulin sensitivity (glucose infusion rate/plasma insulin concentration) increased 32% ( $P = 0.028$ ) after acute exercise. Activation of proximal insulin signaling in skeletal muscle after the HEC, measured as Ser<sup>473</sup> phosphorylation of Akt, increased approximately five-fold in response to insulin ( $P < 0.001$ ) and was unaltered by acute exercise performed 18 h earlier. PGC<sub>1 $\alpha$</sub>  and GLUT4 protein, glycogen content and citrate synthase activity in skeletal muscle did not change in response to insulin or exercise. In conclusion, improved insulin sensitivity and unchanged proximal insulin signaling on the day after acute exercise in sheep are consistent with responses in humans and rodents, suggesting that the sheep is an appropriate large-animal model in which to study responses to exercise.

exercise; insulin sensitivity; muscle; sheep

REGULAR EXERCISE TRAINING increases insulin-stimulated whole body and skeletal muscle glucose uptake in humans, in healthy individuals as well as in those who have Type 2 diabetes (T2D) or are obese or aged (reviewed by Refs. 14, 31, 45). Increased expression of hexokinase 2, some insulin signaling proteins and GLUT4, and greater insulin-induced vasodilation are implicated as mechanisms for enhanced insulin-stimulated glucose uptake after training (14, 31, 45). Exercise training probably acts mainly at distal sites to increase glucose uptake, with the balance of evidence, suggesting that proximal insulin signaling is not upregulated (14, 38). Importantly, a single bout of exercise also increases the insulin sensitivity of glucose uptake during the following 4–48 h in healthy humans (34, 46,

52, 54, 55). Similar effects have been demonstrated in rodents, where an acute exercise bout increases in vitro insulin-stimulated glucose uptake in mouse muscle 85 min after exercise (18) and in rat muscle collected 3–16 h after a single bout of exercise (8, 15, 19, 43, 44). Available evidence from studies in humans and rodents suggests that acute exercise, like exercise training, increases insulin sensitivity without changes in proximal insulin signaling (reviewed in Ref. 31).

A large animal model with similar metabolic responses to exercise as occur in humans would facilitate further mechanistic studies of the effects of acute exercise and training on insulin sensitivity. In particular, whether the metabolic benefit of exercise is normal in other physiological states that reduce insulin sensitivity, such as pregnancy (9) or following in utero growth restriction (17), is presently unclear, and requires establishment of a nonrodent large animal model in which to study such interactions. Such a model would ideally allow investigations of effects of physiological perturbations, such as obesity and environmental cues, including evaluation of exercise as an intervention after early-life challenges, prior to translation to human studies, and permit studies of lifetime consequences of early exercise within years, rather than decades as would be required in humans. A large animal model would also enable repeated muscle biopsies to be collected for analysis of progressive training responses over the medium to long term, which is not possible in rodents. To date, the only large animal in which insulin sensitivity responses to exercise has been studied is the horse. Unlike humans and rodents, however, whole body insulin-stimulated glucose uptake measured by hyperinsulinemic euglycemic clamp did not increase in horses during the 24 h following acute exercise (42), suggesting that metabolic responses to exercise may differ in horses from those seen in humans. Cross-bred sheep such as those used in this study reach puberty at around 7–8 mo of age (13) and mature skeletal size by 2 yr of age (7), making life-course studies more feasible than in humans. Acute exercise and exercise training utilizing treadmills have previously been used to study physiological responses to exercise in sheep, which like humans, improve exercise performance in response to exercise training (5, 26, 37). In adult sheep, moderate duration training (3 mo) increased endurance and peak oxygen consumption during sustained moderate-intensity exercise (37), and shorter-duration training (3 wk) reduced the lactate rise during moderate and strenuous exercise (5). Whether exercise increases insulin sensitivity in this species has not been tested. Therefore, in the present study, we evaluated the suitability of sheep as a large animal model in which to study exercise-induced improvements in insulin action. We

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hypothesized that acute exercise would increase whole body insulin sensitivity of adult sheep on the day after an acute exercise bout, without changes in proximal insulin signaling.

## MATERIALS AND METHODS

**Ethical approval.** All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee (approval M-2013-94) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th ed., 2004).

**Animal procedures.** Seven nonpregnant Border Leicester  $\times$  Merino ewes were obtained from a paddock-housed breeding flock of mature animals at  $\sim$ 6 yr of age. Insulin sensitivity measures and muscle biopsies were obtained from six of the seven sheep included in exercise response measures, due to loss of catheter patency in one sheep.

Ewes were held in individual floor pens in an animal holding room on a 12:12-h light-dark cycle and were fed 1.5 kg Lucerne chaff plus 500 g Rumevite pellets each day (Ridley Agriproducts, Melbourne, Australia) with ad libitum access to water throughout the study. After a 1-wk period to adjust to housing and feeding conditions, sheep were acclimatized to stand and walk slowly (2–3 min at 0.8 km/h, 0% slope) on a treadmill (T970, Power First Fitness International, Tai Chung, Taiwan) for 3 days prior to surgery (Fig. 1). To minimize stress, sheep were managed in pairs during treadmill sessions, so that one ewe was on the treadmill, and the second ewe of the pair was held in a pen immediately in front of the treadmill during each treadmill acclimatization and test period. Sheep were then fasted overnight prior to surgery. Polyvinyl chloride catheters (ID 1.5 mm, OD 2.7 mm) were inserted into the left jugular vein and left carotid artery of each ewe, under general anesthesia and asepsis. Anesthesia was induced by intravenous injection of thiopentone sodium (1.5 g/kg body wt in sterile water; Troy Laboratories, Glendenning, NSW, Australia), sheep were intubated, and anesthesia was then maintained by 1.5–2.5% isoflurane in oxygen. Antibiotics (Cephalazolin; 1 g in 3 ml sterile water im; Hospira Australia, Mulgrave, VIC, Australia) were administered after induction of anesthesia, and analgesia was given at extubation and again on the day after surgery (300 mg im ketoprofen; Troy Laboratories). Catheters were flushed daily for the remainder of the experiment with heparinized saline (500 U/ml) to maintain patency. After a day of recovery, sheep again walked slowly on a treadmill as above on the next 2 days following surgery, and insulin sensitivity was measured on the following day, as described below (Fig. 1). After the preexercise hyperinsulinemic euglycemic clamp, sheep were further acclimatized to walking at increasing speed (peak at 1.6 km/h on *day 1*, 4.8 km/h on *day 10*) and slope (0% on *day 1*, 8% from *day 4* onward) on the treadmill over 10 days, with an acute exercise test on the 11th day, as described below (Fig. 1). A postexercise measure of insulin sensitivity was obtained  $\sim$ 18 h after the acute exercise test.

**Acute exercise test.** The acute exercise test was carried out at a slope of 8%. Treadmill speed was increased from 0.8 to 4.4 km/h over a 3-min period, and sheep were then exercised at 4.4 km/h for 15 min,

walked at 1.6 km/h for 3 min, and then at 4.0 km/h for 15 min. Sheep were returned to pens at the end of the exercise period and had access to feed during recovery and for the following hour, before the overnight fast was commenced. Arterial blood (3 ml) was collected into heparinized syringes and placed on ice, before the acute exercise test, after 15 min of acute exercise, at the end of the acute exercise, and then during recovery at 15-min intervals for 60 min after acute exercise. Blood samples were centrifuged, and plasma was frozen for later measurement of plasma glucose and lactate.

**Hyperinsulinemic euglycemic clamp with collection of muscle biopsies.** Whole body insulin sensitivity was measured in the unexercised state and again  $\sim$ 18 h after acute exercise in conscious relaxed animals, by hyperinsulinemic euglycemic clamp (120 min, 2 mU insulin $\cdot$ min $^{-1}\cdot$ kg $^{-1}$ ), as described previously (16), with blood glucose clamped to unstressed fasting concentrations measured prior to the muscle biopsy of each clamp. Sheep were fasted overnight prior to the clamp, weighed to enable calculation of insulin dose, rested in their floor pens for an hour, and then a sample of arterial blood (2 ml) was collected. Under local anesthesia (1% Lignocaine without adrenaline) and using a percutaneous skeletal muscle biopsy needle (5.0-mm diameter) with aspiration, biopsies were collected from the left *Musculus semimembranosus* in the non-insulin-stimulated state (prior to the clamp) and from the right *M. semimembranosus* in the insulin-stimulated state (immediately following the hyperinsulinemic euglycemic clamp). For the postclamp sample, insulin and glucose infusions were continued at the final rate used during the hyperinsulinemic euglycemic clamp until after collection of the biopsy, to maintain elevated circulating insulin. Biopsy samples were immediately snap-frozen in liquid nitrogen and stored frozen at  $-80^{\circ}\text{C}$  for later analysis. Analgesic was administered following the second biopsy (300 mg ketoprofen im; Troy Laboratories). The *M. semimembranosus* was selected because it is one of the larger load-bearing muscles in the sheep hindlimb, consisting predominantly of type II fast fibers in postnatal animals, and it is active during the type of exercise used in the present study, evidenced by increases in the proportion of type I slow fibers after chronic treadmill exercise (1, 32). Insulin responsiveness appears to increase similarly after exercise in both types of muscle fiber, with increased insulin-stimulated glucose uptake 3 h after exercise in both predominantly type I fiber muscles and predominantly type II fiber muscles in rats (56).

**Plasma insulin, glucose, and lactate analyses.** Plasma insulin was measured in duplicate in samples collected before the biopsy, at 10, 5, and 0 min before infusion of insulin (fasting concentrations) and at 15-min intervals during the last hour of the clamp (steady-state concentrations), by a double-antibody, solid-phase radioimmunoassay (Human insulin-specific RIA, HI-14K, Millipore, St. Charles, MO), which has been validated for use in ovine samples (16). All samples from a single sheep were analyzed in the same assay. The intra-assay and inter-assay coefficients of variation (CV) for the insulin assay were 8.6% and 7.6%, respectively, for a quality control sample containing 46.4 mU/l insulin ( $n = 2$  assays). Plasma lactate and glucose concentrations from the acute exercise test were measured

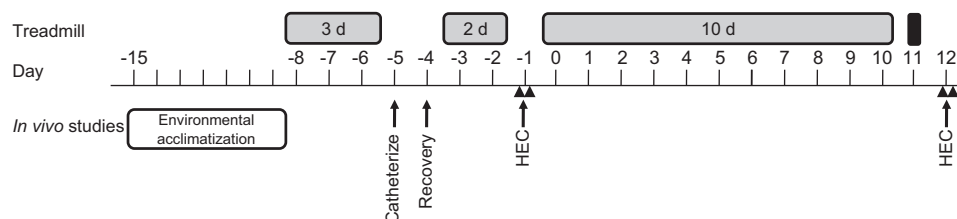


Fig. 1. Study timeline. Gray bars indicate days of acclimatization to standing and walking on treadmill; solid bar shows day of acute exercise test; arrows indicate timing of surgery and in vivo tests; black triangles indicate timing of muscle biopsies, taken 1 h prior to and immediately following each hyperinsulinemic euglycemic clamp (HEC).

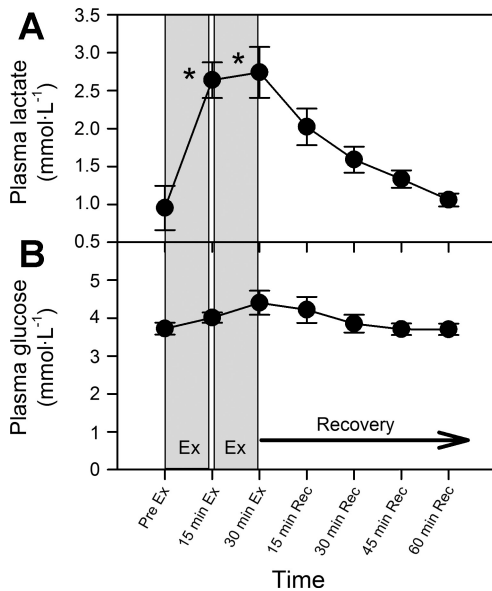


Fig. 2. Plasma lactate (A) and glucose (B) concentrations during and after acute exercise. Concentrations were compared with preexercise values;  $n = 7$  sheep. \* $P < 0.05$ .

using the YSI 2300 STAT Plus glucose and lactate analyzer (John Morris Scientific, Chatswood, NSW, Australia).

**Western blot analyses.** The abundance of GAPDH, PGC<sub>1 $\alpha$</sub> , and GLUT4, and total and phosphorylated Akt proteins in the muscle biopsies were determined using Western blot analysis of whole muscle homogenate, as described elsewhere (35). Frozen muscle biopsies were cryosectioned and homogenized with solubilizing buffer [ $\sim 100 \mu\text{l}/\text{mg}$  tissue: 0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT, and 0.001% bromophenol blue]. Protein content of homogenate was determined using the RED 660 protein assay kit (G-Biosciences, St. Louis, MO). Samples for analysis of total and phosphorylated Akt were heated at 95°C for 5 min prior to SDS-PAGE. Equal amounts (6  $\mu\text{g}$ ) of protein for each sample were subjected to SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membrane, blocked for 1 h at room temperature and then incubated overnight at 4°C with primary antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA; cat no. sc-25778), total Akt or Ser<sup>473</sup> phospho-Akt (Cell Signaling Technology, Danvers, MA; cat no. 9272 and 4058, respectively), PGC<sub>1 $\alpha$</sub>  (Calbiochem, San Diego, CA; cat no. ST1202), or GLUT4 (Thermo Scientific, Rockford, IL; cat no: PAI-1065). Membranes were washed in Tris-buffered saline with 1% Tween-20 (TBST) four times for 5 min each at room temperature, and bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and Super-signal West Femto chemiluminescence reagent (Thermo Scientific). Total Akt protein was measured on the same membrane as Ser<sup>473</sup> phospho-Akt, following stripping and washing to remove antibody to Ser<sup>473</sup> phospho-Akt. Membranes were stripped in buffer (10% SDS; 0.7%  $\beta$ -mercaptoethanol, 0.5 M Tris, pH 6.8) at 56°C for 30 min, followed by four washes each of 5 min in TBST at room temperature. PGC<sub>1 $\alpha$</sub>  and GLUT4 were able to be measured concurrently due to differing protein sizes, and the membrane was then washed and incubated with primary antibody to GAPDH as a loading control. The blots were imaged using the Versadoc MP4000 imaging system (Bio-Rad Laboratories, Hercules, CA), and density of bands was quantified using Quantity One software (Bio-Rad Laboratories). The ratio of phosphorylated to total Akt was calculated as an index of activation of the proximal insulin signaling pathways. The abundance of PGC<sub>1 $\alpha$</sub>  and GLUT4 was normalized against the abundance of GAPDH.

**Muscle glycogen content.** Three to six milligrams of frozen muscle was added to 2 M HCl and incubated at 95–100°C for 120 min.

Samples were neutralized with 0.66 M NaOH and analyzed in triplicate for glucose units using an enzymatic, fluorometric method (39), modified for use in 96-well plates.

**Muscle citrate synthase activity.** Frozen samples (2–4 mg) of each muscle were homogenized (final dilution ratio of 1:400) in potassium phosphate buffer (100 mM KH<sub>2</sub>PO<sub>4</sub> and 100 mM K<sub>2</sub>HPO<sub>4</sub>, at pH 7.5). Samples were then frozen and thawed in liquid nitrogen three times, and the supernatant was collected after centrifugation at 900  $g$  for 10 min at 4°C. Citrate synthase (tricarboxylic acid cycle enzyme) activity of supernatant was measured in triplicate by examining the increase of 5,5-dithiobis-2-nitrobenzoate (DTNB) at a wavelength of 412 nm (48).

**Statistical analyses.** Plasma lactate and glucose concentrations obtained on each animal before, during, and after the intensive exercise test were analyzed by repeated-measures ANOVA for effects of time, with specific contrasts to values obtained before the test. Measures of body weight and plasma glucose on each animal in the unexercised state and  $\sim 18$  h after acute exercise were analyzed by repeated-measures ANOVA. Insulin concentrations and derived variables (insulin:glucose ratios and insulin sensitivity) failed normality tests, and preexercise and postexercise values were compared using the Wilcoxon signed rank test. Concentrations of specific proteins in muscle biopsies taken at multiple time points on each animal were analyzed for effects of insulin, exercise, and interactions using repeated-measures ANOVA. Data are expressed as means  $\pm$  SE. Significance was accepted at  $P < 0.05$ .

## RESULTS

**Metabolic responses during acute exercise.** Plasma lactate concentrations were increased after 15 and 30 min of acute exercise, compared with preexercise concentrations, and tended ( $P < 0.09$ ) to remain elevated at 15 min during the recovery period (Fig. 2A). Although plasma glucose concentration changed with time ( $P = 0.039$ ), plasma glucose concentrations at each time point during acute exercise or recovery did not differ from preexercise concentrations (Fig. 2B).

**Insulin sensitivity and insulin signaling.** In the 12 days from the unexercised to the post-acute exercise clamp, sheep gained an average of 3.9 kg body wt ( $P = 0.001$ , Table 1). Fasting plasma glucose concentrations, fasting plasma insulin concentrations, and fasting glucose-insulin concentration ratios 18 h after acute exercise were similar to concentrations in preexercise samples (Table 1). Steady-state plasma insulin concentra-

Table 1. Body weights, fasting blood glucose and plasma insulin concentrations, steady-state insulin concentrations, and steady-state glucose infusion rate during HEC

	$n$	Unexercised	18 h After Exercise	Significance
Body weight, kg	7	78.6 $\pm$ 1.4	82.5 $\pm$ 1.3	0.001
Fasting glucose, mmol/l	7	3.60 $\pm$ 0.09	3.49 $\pm$ 0.11	0.280
Fasting insulin, mU/l	7	7.36 $\pm$ 1.39	9.49 $\pm$ 3.42	0.753
Fasting glucose:insulin, mU/mmol	7	0.57 $\pm$ 0.08	0.53 $\pm$ 0.09	0.753
HEC glucose infusion rate, mg·kg <sup>-1</sup> ·min <sup>-1</sup>	6	1.95 $\pm$ 0.13	2.74 $\pm$ 0.21	0.003
HEC Steady state insulin, mU/l	6	168 $\pm$ 11	177 $\pm$ 6	0.173

Data are presented as means  $\pm$  SE. HEC was performed in sheep in the unexercised state and in the same animals  $\sim 18$  h after acute exercise. Body weights and glucose concentrations were analyzed by repeated-measures ANOVA, and insulin concentrations and ratios (nonnormal) were analyzed by Wilcoxon signed rank test. HEC, hyperinsulinemic euglycemic clamps.

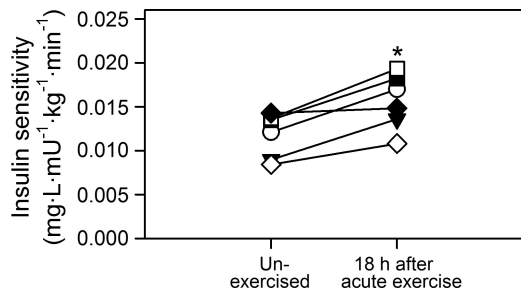


Fig. 3. Exercise increases insulin sensitivity in sheep. Insulin sensitivity in unexercised sheep and in the same animals ~18 h after acute exercise. Insulin sensitivity was measured by hyperinsulinemic euglycemic clamp and paired data ( $n = 6$  sheep) were compared by Wilcoxon signed rank test,  $*P < 0.05$ ; symbols indicate individual animals.

tion during the hyperinsulinemic euglycemic clamp was similar in the unexercised state and 18 h after acute exercise (Table 1). The glucose infusion rate required to maintain euglycemia was 40% higher (Table 1), and insulin sensitivity (glucose infusion rate/plasma insulin concentration) was 32% higher 18 h after acute exercise than in the unexercised condition ( $P = 0.028$ , Fig. 3). Akt phosphorylation at Ser<sup>473</sup> relative to total Akt protein in muscle increased ~5-fold in response to insulin ( $P < 0.001$ ) and was similar in the unexercised condition and 18 h after acute exercise ( $P > 0.6$ ; Fig. 4).

**Muscle indicators of exercise training status.** We evaluated skeletal muscle parameters that increase in response to exercise training, to confirm that the present study represented an acute exercise protocol with no effect of treadmill walking during familiarization. PGC<sub>1 $\alpha$</sub>  and GLUT4 protein expression did not change in response to insulin and were each similar in the unexercised condition and 18 h after acute exercise (Fig. 5). GAPDH protein expression was not changed by insulin and was similar in the unexercised condition and 18 h after acute exercise (Fig. 5). Similarly, muscle glycogen content (unexercised, non-insulin-stimulated:  $70.3 \pm 10.2$ ; unexercised, insulin-stimulated:  $91.2 \pm 14.5$ ; postexercise, non-insulin-stimu-

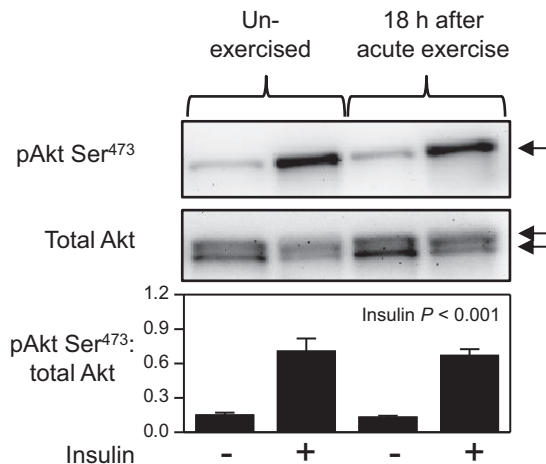


Fig. 4. Basal and insulin-induced expression and phosphorylation of Akt in *M. semimembranosus* sampled from unexercised sheep and in the same animals ~18 h after acute exercise ( $n = 6$  sheep). Muscle protein expression in biopsies taken at multiple time points on each animal were analyzed for effects of insulin, exercise, and interactions using repeated-measures ANOVA. Arrows indicate bands used for densitometry.

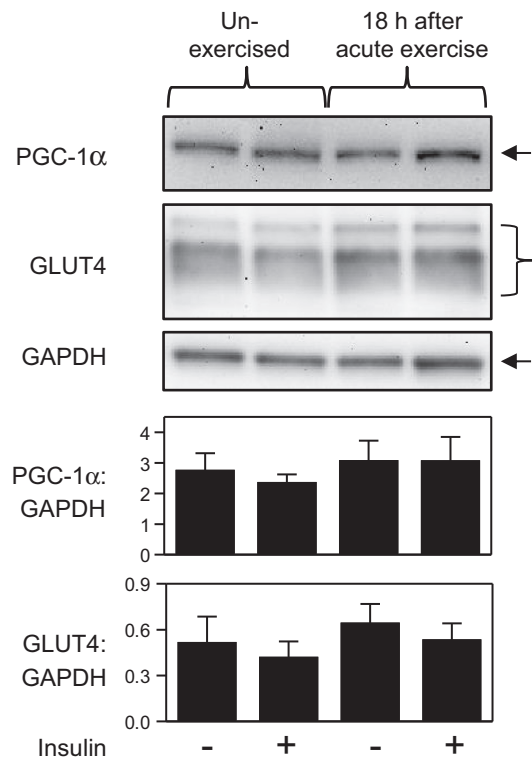


Fig. 5. Basal and insulin-induced expression of PGC<sub>1 $\alpha$</sub>  and GLUT4 in *M. semimembranosus* sampled from unexercised sheep and in the same animals ~18 h after acute exercise ( $n = 6$  sheep). Muscle protein expression in biopsies taken at multiple time points on each animal were analyzed for effects of insulin, exercise and interactions using repeated-measures ANOVA. Arrows and brackets indicate bands/regions used for densitometry.

lated:  $77.3 \pm 5.9$ ; postexercise, insulin-stimulated:  $77.9 \pm 8.3$  mmol glycosyl units/kg wet wt) and citrate synthase activity (unexercised, non-insulin-stimulated:  $12.4 \pm 1.1$ ; unexercised, insulin-stimulated:  $11.3 \pm 1.0$ ; postexercise, non-insulin-stimulated:  $15.1 \pm 3.5$ ; postexercise, insulin-stimulated:  $9.8 \pm 2.1$   $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g protein}^{-1}$ ) did not change in response to insulin and was similar in the unexercised condition and 18 h after acute exercise.

## DISCUSSION

Whole body insulin sensitivity was increased the day after acute exercise in sheep. This is consistent with previous reports of increased whole body or skeletal muscle insulin sensitivity persisting for 4–48 h after a single bout of exercise in humans (34, 46, 54) and 4–48 h after acute exercise in rodents (8, 18, 44). This is the first report of exercise-induced increases in insulin sensitivity in a nonhuman large animal. Proximal insulin signaling in skeletal muscle, assessed as Akt phosphorylation, was increased by insulin and unchanged by acute exercise, also consistent with effects of exercise in humans and mice (18, 54). The present study develops the sheep as a large animal model in which to study metabolic benefits of exercise and shows that signaling pathways induced by insulin and exercise in skeletal muscle can be studied serially in this species.

Intriguingly, studies in the horse, which is the only other large nonhuman animal in which exercise-induced changes in

insulin sensitivity have been characterized, have shown no increase in whole body insulin sensitivity measured by hyperinsulinemic euglycemic clamp after a single bout of exercise (42). In contrast, exercise training increased insulin-stimulated glucose uptake in some (49, 50), but not all (11), studies in the horse, which is more consistent with the increase in insulin sensitivity induced by training in humans and rodents (45). Pratt et al. (42) hypothesized that the lack of response to acute exercise reflected the relatively small increase in glycogen synthase activity and slow glycogen replenishment in muscle after exercise in horses compared with humans and rodents. Thus, the sheep appears to be more similar to humans and rodents than to horses in its responses to acute exercise, since acute exercise increased insulin-stimulated glucose uptake under euglycemic conditions in the present study.

Our study protocol was designed to provide an acute exercise challenge, with a minimal chronic training effect. We familiarized the sheep to standing and then to walking on the treadmill in the 10 days prior to the acute exercise test, at lower intensities and shorter durations of exercise than used in the acute test. Consistent with our model inducing acute effects of exercise, rather than exercise training effects, we did not see any increases in skeletal muscle  $PGC_{1\alpha}$  or GLUT4 protein expression between biopsies obtained in the unexercised condition compared with 18 h after the acute exercise challenge. Exercise training consistently increases GLUT4 gene and protein expression in humans and rodents, but acute exercise usually causes only transient increases in GLUT4 mRNA expression and protein (reviewed by Ref. 45). The similar muscle glycogen content measured in these animals in the unexercised state and 18 h after the exercise bout in ovine skeletal muscle in the present study is also consistent with a lack of training effect, since exercise training in sheep and in humans increases glycogen contents of resting skeletal muscle (33, 36, 40). It is possible, however, that the lack of change in glycogen content in skeletal muscle sampled 18 h after acute exercise in the present study might partially reflect incomplete restoration of glycogen depleted during exercise (54). Sheep had access to feed for  $\sim 2$  h after acute exercise and then were fasted overnight prior to hyperinsulinemic euglycemic clamp. On the basis of *M. semimembranosus* glycogen content responses to exercise and postexercise repletion rates in mature Merino ewes (20), the 2-h refeeding period used here should have allowed restoration of the majority of glycogen depleted from muscle during the exercise protocol. Activity of the mitochondrial enzyme citrate synthase, which is increased following exercise training in rats and humans (28, 53), but unchanged 3 h after a single exercise bout in humans (28), was also similar in muscle samples collected from sheep in the nonexercised state and 18 h after exercise. These results are, therefore, consistent with the increased insulin sensitivity in the sheep in the present study being induced by acute exercise, rather than an exercise-training effect.

Interestingly, the postexercise increase in insulin sensitivity that we observed in these sheep was induced by a relatively low intensity and short exercise bout. Previous studies in humans and rodents have reported increased insulin sensitivity after either higher-intensity or longer-duration acute exercise protocols than used in the present study (3, 8, 15, 19, 23, 34, 43, 44, 54). The exercise intensity achieved in the present study by sheep in the intensive exercise test (30 min, 8% slope,

4–4.4 km/h) is probably  $\sim 50$ – $60\%$  of  $\dot{V}O_{2\max}$ , based on studies in pregnant sheep (26).  $\dot{V}O_{2\max}$  has not been reported in nonpregnant sheep, but it is probably similar to that of pregnant sheep at similar body weights, since  $\dot{V}O_{2\max}$  remains similar before and throughout pregnancy in women (21, 30). Our results suggest that even half an hour of acute exercise at moderate exercise intensity in the sheep is sufficient to increase insulin-stimulated whole body glucose uptake for at least 18 h after acute exercise. We suggest that further research should directly characterize the intensity of exercise at which increases in insulin sensitivity are observed in this species. Nevertheless, these findings in sheep suggest that acute exercise may be able to induce short-term increases in the whole body insulin sensitivity of glucose uptake at lower intensities or durations than those reported in humans to date and suggest that studies of acute exercise in humans should be extended to these lower intensities and durations.

Over the experimental period, the mature female sheep in the present study gained  $\sim 4$  kg body wt. This increase in body weight probably largely reflects gain of fat tissue, because these sheep were mature adult ewes, that were transferred from paddock housing and pasture feeding into relatively sedentary conditions of pen housing with abundant nutrition  $\sim 2$  wk prior to the first clamp, conditions shown to increase weight and fat deposition, even in young growing animals (47). The sheep in the present study were relatively insulin-resistant, with preexercise insulin sensitivity approximately three-fold and six-fold lower than we have observed previously in young adult sheep and in 1 mo-old lambs, respectively (16). Importantly, exercise increased insulin sensitivity despite the ewes in the present study gaining significant body weight between preexercise and postexercise tests. This is consistent with the fact that exercise training increases insulin sensitivity before weight is lost in humans (6).

Our findings confirm that whole body insulin sensitivity is increased on the day after acute exercise in sheep, without increased activation of proximal insulin signaling, which is consistent with effects of acute exercise in humans and rodents (reviewed in Ref. 31). Studies of biopsies from mixed-fiber-type muscles in humans (vastus lateralis and quadriceps femoris; Refs. 51 and 54) and in isolated muscles comprising predominantly type I fibers (soleus) or type II fibers (epitrochlearis) in mice and rats (2, 12, 18) have similarly reported a lack of effect of acute exercise on insulin-stimulated Akt serine phosphorylation. Together with the present results obtained in ovine *M. semimembranosus*, which comprises predominantly type II muscle fibers (1, 32), this suggests that the lack of exercise effect on insulin-stimulated Akt serine phosphorylation is common to different muscle fiber types. Repeated muscle biopsies, as conducted in this study in sheep, have similarly been used in human studies to investigate the mechanisms underlying exercise responses in this tissue, including in diabetes (e.g., 27, 33, 46, 55). In addition to muscle, it is possible to repeatedly biopsy fat (29) and liver (24) in sheep, which would permit longitudinal studies of exercise responses in multiple tissues in future studies in this species, as well as to investigate responses in other tissues, such as pancreas and heart at post mortem. The availability of a large animal model also provides the capacity to investigate interactions between many physiological states and exercise effects on insulin action, which may not be feasible or ethically appropriate in

humans. These include several conditions that impair insulin action and sensitivity in sheep and humans, including pregnancy (9, 41), IUGR induced by restricted placental growth and function (10, 25), and obesity (4, 22).

In conclusion, this is the first demonstration of increases in insulin sensitivity after acute exercise in a non-human large animal. This suggests that the sheep will be a useful species in which to investigate underlying mechanisms, longitudinal responses to varying exercise protocols, and the efficacy of exercise in increasing insulin sensitivity in conditions that perturb insulin sensitivity, as well as interactions with other interventions prior to translation to human studies.

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#### DISCLOSURES

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

#### AUTHOR CONTRIBUTIONS

Author contributions: G.K.M. and K.L.G. conception and design of research; G.K.M., G.K., F.F.-T., Y.H.H., and K.L.G. performed experiments; G.K.M., G.K., and K.L.G. analyzed data; G.K.M., G.K., and K.L.G. interpreted results of experiments; G.K.M., G.K., and K.L.G. prepared figures; G.K.M. and K.L.G. drafted manuscript; G.K.M., G.K., F.F.-T., Y.H.H., and K.L.G. edited and revised manuscript; G.K.M., G.K., F.F.-T., Y.H.H., and K.L.G. approved final version of manuscript.

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