

## Fetuin-A is linked to improved glucose tolerance after short-term exercise training in nonalcoholic fatty liver disease

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**Malin SK, Mulya A, Fealy CE, Haus JM, Pagadala MR, Scelsi AR, Huang H, Flask CA, McCullough AJ, Kirwan JP.** Fetuin-A is linked to improved glucose tolerance after short-term exercise training in nonalcoholic fatty liver disease. *J Appl Physiol* 115: 988–994, 2013. First published August 8, 2013; doi:10.1152/jappphysiol.00237.2013.—Fetuin-A is synthesized in the liver and may be associated with nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes. Lifestyle-induced weight loss reduces fetuin-A, but the effect of exercise alone is unknown. We determined the effect of short-term exercise training on plasma fetuin-A in 13 ( $50.5 \pm 3.4$  yr) obese adults (body mass index,  $33.3 \pm 0.9$  kg/m<sup>2</sup>) with clinically diagnosed NAFLD. Subjects participated in 7 days of supervised exercise training (60 min/day at ~85% maximum heart rate) and were instructed to maintain their normal caloric and macronutrient intake. Insulin resistance was assessed by an oral glucose tolerance test. Hepatic triglyceride content (HTGC) was determined by proton MRI. We used C2C12 skeletal muscle cells to examine the direct effect of fetuin-A on 2-deoxyglucose uptake, insulin signaling [phosphorylation of Akt and AS160 (pAkt and pAS160, respectively)], and glucose transporter-4 (GLUT-4) translocation. Insulin resistance was reduced by 29% ( $P < 0.05$ ), and glucose area under the curve (AUC) was decreased by 13% ( $P < 0.01$ ) after the 7 days of exercise. Furthermore, circulating fetuin-A was decreased by 11% ( $4.2 \pm 0.3$  vs.  $3.6 \pm 0.2$  nM;  $P < 0.02$ ), and this change correlated with reduced insulin resistance ( $r = 0.62$ ;  $P < 0.04$ ) and glucose AUC ( $r = 0.58$ ;  $P < 0.04$ ). Importantly, the exercise program did not change body weight ( $P = 0.12$ ), HTGC ( $P = 0.73$ ), or aerobic capacity ( $P = 0.14$ ). In vitro experiments revealed that fetuin-A decreased skeletal muscle glucose uptake by downregulating pAkt and pAS160 and subsequent GLUT-4 translocation to the plasma membrane. Together, our findings highlight a role for fetuin-A in skeletal muscle insulin resistance and suggest that part of the exercise-induced improvement in glucose tolerance in patients with NAFLD may be due to lowering fetuin-A.

obesity; hyperglycemia; inflammation; insulin sensitivity

APPROXIMATELY 35% OF ADULTS in the United States have nonalcoholic fatty liver disease (NAFLD) and are at high risk for type 2 diabetes (T2D) and cardiovascular disease (15, 25, 37, 42). Although the etiology by which NAFLD increases chronic disease risk remains debatable, insulin resistance appears to be a significant factor (39). Fetuin-A (or  $\alpha$ -2-Heremans-Schmid glycoprotein) is a hepatokine that is synthesized in the liver, where it is associated with excess hepatic triglyceride content (HTGC) and insulin resistance (3, 16, 33, 38). In humans,

fetuin-A is positively associated with nonesterified free fatty acids (NEFA), triglycerides, and low-grade chronic inflammation (e.g., C-reactive protein, TNF- $\alpha$ , and IL-6) (3, 16, 33, 38). Fetuin-A has also been shown to inhibit skeletal muscle insulin receptor tyrosine phosphorylation and reduce Akt activity, which in turn, contributes to decreased peripheral glucose uptake (2, 22, 23). Thus, interventions that reduce blood lipids and the proinflammatory state related to insulin resistance may drive reductions in plasma fetuin-A (32).

Physically active adults have lower plasma fetuin-A compared with sedentary age- and weight-matched counterparts (17). However, the effect of exercise and dietary lifestyle interventions on plasma fetuin-A is somewhat equivocal (27, 33, 34, 38, 44). It appears that weight loss or reductions in hepatic steatosis may be important for reducing fetuin-A after lifestyle interventions (33, 38, 44). However, weight loss may also confound interpretation of the effect of exercise per se on fetuin-A. We have reported previously that short-term exercise improves insulin sensitivity in patients with T2D, and this effect is independent of weight loss (19). To date, there are no studies examining the effect of exercise, independent of weight loss or HTGC, on plasma fetuin-A in adults with NAFLD. Secondly, there are limited studies examining the cellular mechanism by which fetuin-A may affect metabolism in insulin-targeted tissue. Therefore, the purpose of this study was to determine the effects of a 7-day exercise program on circulating fetuin-A in adults with NAFLD. Furthermore, we performed in vitro studies using C2C12 skeletal muscle cells to determine whether fetuin-A could directly reduce muscle insulin sensitivity and whether this effect was mediated by impaired insulin signaling through Akt and/or AS160 and a decrease in glucose transporter-4 (GLUT-4) translocation. We hypothesized that exercise would decrease fetuin-A and correlate with improved glucose tolerance and decreased insulin resistance in vivo. We further hypothesized that fetuin-A would induce insulin resistance directly in skeletal muscle by downregulating insulin signaling and GLUT-4 translocation to the plasma membrane.

### METHODS

**Subjects.** The study included 13 middle-aged obese men and women (Table 1), who were nonsmoking, weight stable (<2 kg weight loss in the previous 6 mo), and sedentary (exercising <30 min/day and <3 days/wk). Subjects were excluded if they were suffering from chronic disease (i.e., renal or cardiovascular), took known supplements or medications that affected glucose metabolism, or had a history of alcohol use (>20 g/day for men and >10 g/day for

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Table 1. Effects of a 7-day exercise training program on anthropometrics, fitness, and metabolism

	Pre	Post	P
n (M/F)	13 (6/7)		
Age, years	50.5 ± 3.4		
Weight, kg*	99.2 ± 4.4	99.8 ± 4.3	0.12
Body mass index, kg/m <sup>2</sup>	33.3 ± 0.9	33.5 ± 0.9	0.14
Fat mass, kg	42.9 ± 2.3	43.0 ± 2.3	0.92
Fat-free mass, kg	56.3 ± 3.9	56.8 ± 3.8	0.16
Hepatic triglyceride content, %	23.1 ± 4.1	22.5 ± 3.9	0.73
Trunk fat, %	53.8 ± 1.5	54.2 ± 1.6	0.45
VO <sub>2max</sub> , ml · kg <sup>-1</sup> · min <sup>-1</sup>	24.9 ± 1.5	26.3 ± 1.6	0.14
VO <sub>2max</sub> , ml · kg fat-free mass <sup>-1</sup> · min <sup>-1</sup>	44.1 ± 2.2	45.9 ± 1.8	0.20
Fasting plasma glucose, mg/dl*	110.8 ± 4.0	105.5 ± 4.0†	0.02
Fasting plasma insulin, μU/ml	24.8 ± 2.5	19.5 ± 1.6†	0.03
2-h Plasma glucose, mg/dl*	202.3 ± 16.6	181.8 ± 12.0†	0.001
2-h Plasma insulin, μU/ml*	149.3 ± 28.1	101.8 ± 16.9†	0.02
Glucose AUC, mg/dl, 3 h	33,235.5 ± 2,555.4	31,201.2 ± 2,020.9†	0.02
Insulin AUC, μU/ml, 3 h	20,205.4 ± 317.3	15,422.6 ± 2,159.1†	0.001
IR index	94.9 ± 12.5	60.9 ± 6.0†	0.001
HOMA-IR	6.1 ± 0.9	5.1 ± 0.5†	0.03
Adipose-IR	12.2 ± 1.5	12.3 ± 1.4	0.95

Data are mean ± SE. Pre/Post, pre-/postexercise training; VO<sub>2max</sub>, maximal oxygen consumption; AUC, area under the curve. Insulin concentrations and homeostatic model assessment of insulin resistance (HOMA-IR)/IR index (n = 12 of 13). \*Data were log transformed for statistical analysis; †Significant compared with Pre.

women). Subjects were recruited from the Liver Center at the Cleveland Clinic. All men and women had confirmed hepatic steatosis of >5% HTGC using MRI (4). Subjects were verbally briefed about the study and signed informed consent documents, approved by the Cleveland Clinic's Institutional Review Board.

**Exercise training.** Subjects underwent 7 days of supervised exercise, which consisted of aerobic treadmill walking and cycle ergometry, performed at ~85% maximum heart rate (HR<sub>max</sub>) for 60 min. All subjects wore heart-rate monitors to ensure appropriate exercise intensity (Polar Electro, Lake Success, NY). The first and last 5 min of each session consisted of a warm-up and cool-down. Metabolic testing was conducted approximately 16–18 h after the last exercise session.

**Control period.** Subjects were given detailed instructions on maintaining usual dietary intake and habitual physical activity levels during the intervention. On the day before metabolic testing, subjects were instructed to consume a mixed diet, i.e., ~55% carbohydrate, 30% fat, and 15% protein.

**Cardiovascular fitness.** An incremental treadmill exercise test was used to determine maximal oxygen consumption (VO<sub>2max</sub>) (Jaeger Oxycon Pro; Viasys; CareFusion, San Diego, CA) before and after the intervention, as described previously (28). The HR<sub>max</sub> obtained during this test was used to create the target heart rate during exercise training. Resting metabolic rate and substrate oxidation were determined using indirect calorimetry after ~30 min of rest in the supine position (Vmax Encore; Viasys; CareFusion), and standard equations were applied (11).

**Body composition and HTGC.** Height was measured without shoes using a wall-mounted stadiometer, and body weight was recorded on a digital platform scale with subjects wearing a hospital gown. Dual-energy x-ray absorptiometry (Prodigy; Lunar, Madison, WI) was used to quantify total body fat mass and fat-free mass. HTGC was determined in a Magnetom Verio 3T MRI scanner (Siemens, Munich, Germany), as reported previously (10). In short, a body array MRI coil was centered and aligned with the subject's spine and shoulders for accurate repositioning during post-testing. An 8-cm<sup>3</sup> voxel was positioned within the right posterior lobe of the subject's liver with guidance from the high-resolution localization images. The data were Fourier transformed, filtered, baseline corrected, and phased to obtain the spectra.

**Insulin resistance and glucose tolerance.** After an overnight fast, blood samples were collected from an antecubital vein for fetuin-A,

NEFA, glucose, and insulin. A standard 75-g oral glucose tolerance test (OGTT) was administered to assess glucose and insulin at 30, 60, 90, 120, and 180 min. Glucose and insulin area under the curve (AUC) during the OGTT were calculated using the trapezoidal method. Insulin resistance, which primarily reflects skeletal muscle glucose uptake, was estimated by multiplying insulin and glucose AUC and dividing by 10<sup>6</sup> (9). The homeostatic model assessment of insulin resistance (HOMA-IR), which reflects mostly hepatic insulin resistance (fasting glucose × fasting insulin) and adipose insulin resistance (fasting NEFA × fasting insulin), was also calculated (1, 24).

**Plasma biochemical analysis.** Plasma fetuin-A was analyzed using an ELISA kit (Quantikine; R&D Systems, Minneapolis, MN). Plasma glucose was determined using a glucose oxidase assay (YSI 2300 STAT Plus; YSI Life Sciences, Yellow Springs, OH). Plasma insulin was measured by radioimmunoassay (Millipore, Billerica, MA). Plasma NEFA was measured by colorimetric assay (Wako Chemicals, Richmond, VA).

**Cell culture studies.** C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were maintained at subconfluent conditions in DMEM (4.5 g/l glucose), supplemented with 10% FBS and antibiotics. Myotube differentiation was induced by switching growth media to DMEM, supplemented with 2% horse serum, and maintained for 5–6 days with medium changes every other day.

**2-Deoxyglucose uptake.** 2-Deoxyglucose uptake was performed as described previously (8) with minor modifications. Differentiated myotubes were treated with or without 50 or 100 nM fetuin-A (R&D Systems) in DMEM low-glucose media, supplemented with 1% BSA for 24 h. Cells were then preincubated with Krebs-Ringer HEPES buffer (KRH; in mM: 20 HEPES, pH 7.4, 136 NaCl, 4.7 KCl, 1.25 MgSO<sub>4</sub>, and 1.25 CaCl<sub>2</sub>) ± 100 nM insulin for 1 h. Post-1-h insulin stimulation, the glucose uptake experiment commenced by adding 0.5 μCi/ml 2-deoxy-D-[<sup>3</sup>H]-glucose (PerkinElmer, Waltham, MA) and 100 μM 2-deoxy-D-glucose (Sigma, St. Louis, MO) in KRH buffer. After 5 min, cells were washed four times with ice-cold PBS and lysed with 0.5% SDS. Cell-associated radioactivity was measured, and noncarrier-mediated glucose uptake was assessed by parallel incubations in the presence of 10 μM cytochalasin B (Sigma). The protein content was determined with a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). C2C12 glucose uptake was expressed as specific uptake after subtracting the total uptake with nonspecific background (nmoles mg protein<sup>-1</sup>·min<sup>-1</sup>). All samples were run in duplicate, and each experiment was repeated twice.

**Subcellular fractionation.** Subcellular fractionation of myotubes was carried out as described previously (26). Briefly, C2C12 myotubes were treated in the absence or presence of 100 nM fetuin-A for 24 h. At the end of treatment, cells were stimulated  $\pm$  100 nM insulin for 1 h and were then harvested. The cell pellet was collected by centrifugation at 700 g for 10 min and resuspended in 1 ml cold (in mM) 20 HEPES, 4 EDTA, 255 sucrose, pH 7.4 (HES buffer), in the presence of protease inhibitors, followed by homogenization using a glass homogenizer. The homogenate was centrifuged at 760 g for 5 min to remove nuclei and unbroken cells. The supernatant was centrifuged further at 31,000 g for 60 min using a TLA-120.2 rotor in an Optima TLX ultracentrifuge (Beckman Coulter, Brea, CA) to pellet the crude plasma membrane (CPM). The light microsomal (LM) fraction was collected from the 31,000 g supernatant by centrifugation at 190,000 g for 60 min. Both CPM and LM pellets were suspended in HES buffer and frozen at  $-20^{\circ}\text{C}$ . Protein concentration was determined by BCA, and GLUT-4 expression in CPM and LM fractions was detected by Western blot analysis. GLUT-4 expression was determined in duplicate from three independent experiments.

**Akt and AS160 phosphorylation.** To investigate insulin stimulation of Akt on serine 473 and AS160 on threonine 642 phosphorylation sites, C2C12 myotubes were treated with or without fetuin-A (R&D Systems) and stimulated with insulin for 5 min and 1 h, respectively, as described above. Cells were collected and lysed with cell extraction buffer (Invitrogen, Carlsbad, CA) in the presence of protease inhibitors (Sigma) and PhosSTOP (Roche Applied Science, Indianapolis, IN). Lysate was collected by centrifugation at 12,000 g for 10 min at  $4^{\circ}\text{C}$ , protein content was measured by BCA, and Akt as well as AS160 phosphorylation (pAkt and pAS160, respectively) was determined by Western blot. Akt and AS160 samples were run in duplicate from three independent experiments.

**Western blotting.** Proteins were separated by Novex Tris glycine SDS-PAGE (Invitrogen), transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA), and blocked with 5% BSA in PBS with 0.1% Tween-20 (PBST) for 1 h. Membranes were incubated overnight with anti-GLUT-4 (Sigma), anti-pAS160 (Thr642; Novus Biologicals, Littleton, CO), anti-AS160 (Rab-GTPase-activating protein; Millipore), or anti- $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed in PBST and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies. Immunoreactive proteins were visualized by an enhanced chemiluminescence reagent (ECL Prime; GE Healthcare Life Sciences, Pittsburgh, PA) and quantified by densitometric analysis using ImageQuant TL software (GE Healthcare Life Sciences).

**Statistical analysis.** Pre- and postgroup mean values were compared using R (version 2.4.0, 2006; The R Foundation, Vienna, Austria). Non-normally distributed data were log transformed for statistical analysis. One subject's insulin levels were  $>2$  SD from the mean, and these data were removed for statistical analysis. Paired *t*-tests were used for human data to determine statistical differences before and after the intervention. A two-way (insulin  $\times$  fetuin-A) ANOVA was used for the cell experiments. Pearson's product moment correlation was used to examine associations, and significance was accepted as  $\alpha \leq 0.05$ . Data are expressed as mean  $\pm$  SE unless noted otherwise.

## RESULTS

**Cardiovascular fitness, body composition, and HTGC.** Exercise adherence was 100%. Seven days of exercise did not statistically increase  $\text{VO}_{2\text{max}}$ , whether scaled to body weight or fat-free mass (Table 1). Exercise did not reduce total body weight, total fat mass, or HTGC (Table 1), but it did increase fasting fat oxidation ( $0.06 \pm 0.01$  vs.  $0.08 \pm 0.01$  g/min;  $P = 0.04$ ).

**Glucose tolerance and insulin resistance.** Relative to baseline, exercise decreased fasting and 2-h plasma glucose concentrations by  $\sim 4\%$  and  $8\%$ , respectively ( $P < 0.05$ ). Exercise also improved glucose tolerance by  $\sim 13\%$  (Table 1). Exercise reduced fasting and postprandial insulin responses, as indicated by lower-insulin AUC ( $P < 0.05$ ; Table 1). Consistent with lower glucose and insulin, exercise decreased HOMA-IR by  $19\%$  ( $P < 0.05$ ) and reduced whole-body insulin resistance by  $\sim 29\%$  ( $P < 0.05$ ; Table 1). Fasting plasma NEFAs were unaltered after the intervention ( $0.54 \pm 0.04$  vs.  $0.59 \pm 0.04$  meq/l;  $P = 0.30$ ), as was adipose insulin resistance (Table 1).

**Plasma fetuin-A.** Exercise reduced fasting plasma fetuin-A by  $\sim 11\%$  (Fig. 1). Reductions in fetuin-A correlated with reductions in insulin resistance ( $r = 0.62$ ;  $P < 0.04$ ; Fig. 2A) and glucose AUC ( $r = 0.58$ ;  $P < 0.05$ ; Fig. 2B). Pretraining fasting plasma glucose correlated with reduced fetuin-A ( $r = -0.65$ ;  $P < 0.05$ ). There was also a trend for pretraining 2-h glucose levels to correlate with reduced fetuin-A ( $r = -0.52$ ;  $P = 0.06$ ). Changes in fetuin-A did not correlate with changes in HTGC after exercise ( $r = 0.18$ ;  $P = 0.55$ ; Fig. 2C).

**Fetuin-A and skeletal muscle insulin resistance.** In a dose-dependent manner, fetuin-A induced a marked reduction in insulin-stimulated glucose uptake in C2C12 myotubes compared with untreated control cells (Fig. 3A; fetuin-A main effect:  $P < 0.02$ ), thus supporting the view that fetuin-A impairs skeletal muscle insulin sensitivity. To test whether a decrease in insulin-stimulated glucose uptake was associated with a decrease in GLUT-4 translocation to the plasma membrane or microsomal GLUT-4 protein content, we performed subcellular fractionation on the C2C12 myotubes. Fetuin-A treatment lowered insulin-stimulated GLUT-4 translocation from the microsomal fraction to the plasma membrane, although this did not reach statistical significance (Fig. 3B; fetuin-A main effect:  $P = 0.16$ ). To investigate the mechanism by which fetuin-A decreases glucose uptake in relation to GLUT-4 translocation, we tested the insulin signaling pathway via activation of Akt and AS160. Fetuin-A treatment significantly lowered insulin-stimulated pAkt ( $P < 0.02$ ) and pAS160 (fetuin-A main effect:  $P < 0.02$ ) in the myotubes compared with controls (Fig. 3, C and D).

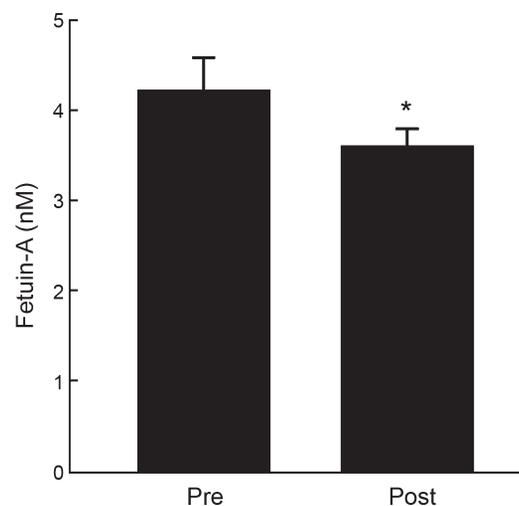


Fig. 1. Effects of a 7-day exercise training program on plasma fetuin-A concentrations. Data are mean  $\pm$  SE. \*Pre- vs. postexercise training (Pre vs. Post, respectively),  $P < 0.02$ .

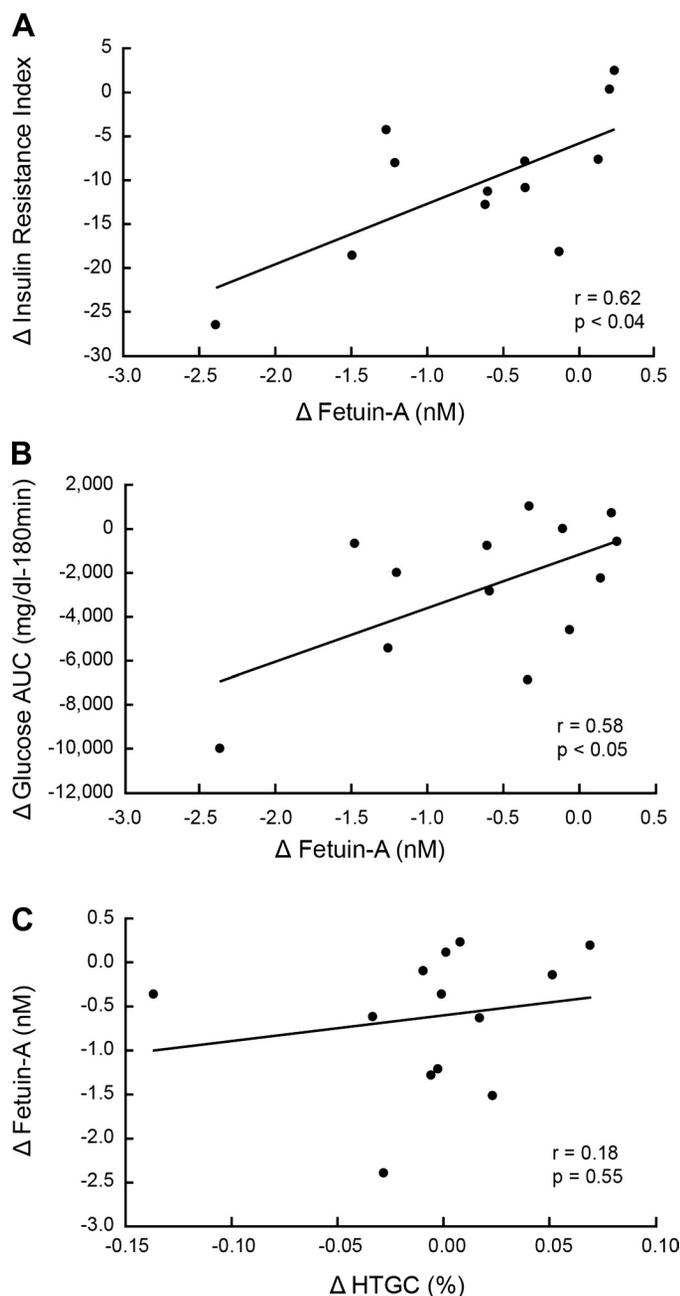


Fig. 2. Correlation between the change ( $\Delta$ ) in fetuin-A concentrations and the  $\Delta$  in insulin resistance (A),  $\Delta$  in glucose tolerance (B), and  $\Delta$  in hepatic triglyceride content (HTGC; C). Insulin resistance index ( $n = 12$  of 13). AUC, area under the curve.

## DISCUSSION

Fetuin-A is known as an inflammatory mediator and is thought to play a role in systemic insulin resistance (29–31, 43). In this study, the major, new finding was that short-term exercise training, which produced no change in body weight or aerobic fitness, led to a decrease in plasma fetuin-A levels (Fig. 1), and this decrease in fetuin-A correlated with a reduction in insulin resistance (Fig. 2A) and improvement in glucose tolerance (Fig. 2B). We also report for the first time that fetuin-A impairs insulin-mediated glucose uptake in C2C12 myotubes by down-regulating GLUT-4 translocation to the plasma mem-

brane, and this effect may be mediated through decreased pAkt and pAS160 (Fig. 3). Taken together, these data support a role for fetuin-A in exercise-induced improvements in insulin action.

Although there is currently no effective therapy for NAFLD, lifestyle-induced weight loss is widely prescribed with the intent of reducing HTGC (20, 35, 41). Lifestyle intervention is also effective in ameliorating insulin resistance and reducing cytokines that are known to induce glucose intolerance (20). Interestingly, lifestyle interventions that induce weight loss and reduce HTGC have led to lower plasma fetuin-A levels and raise the possibility that fetuin-A may play a role in regulating peripheral insulin sensitivity (17, 33, 38). However, weight loss confounds the ability to assess the effect of exercise independently on fetuin-A. Recently, three studies reported that 6–12 wk of aerobic or resistance exercise without weight loss had no effect on plasma fetuin-A in both nondiabetic and T2D individuals (27, 34, 44). This suggests that weight loss rather than exercise itself is the primary factor that regulates fetuin-A and insulin resistance. In contrast, there are also data to show that fetuin-A is higher in physically inactive men and women compared with those who are highly active (17). Furthermore, there was an inverse correlation between  $VO_{2max}$  and fetuin-A, suggesting that physical fitness may regulate fetuin-A concentrations. The reason for these inconsistencies is not readily apparent. However, it appears that disease status (i.e., NAFLD vs. T2D vs. obese), medication use, and higher exercise intensity (e.g.,  $\sim 85\%$   $HR_{max}$ ) are important factors in determining circulating fetuin-A (27, 34, 44). Indeed, one of the strengths of our study is that subjects with NAFLD were not on any diabetic medications and did not lose weight or reduce hepatic HTGC, thereby allowing us to isolate the independent effects of a short-term, high-intensity exercise program on fetuin-A and glucose regulation (27, 34, 44). Future work is required, nevertheless, to test the effects of medication use with exercise at various exercise intensities across different metabolic cohorts to gain additional insight into the regulation of fetuin-A. The time-course effect of exercise on fetuin-A concentrations postexercise also awaits further investigation. Taken together, our work extends previous work in adults with NAFLD (33, 38) and suggests that 7 days of exercise positively affects hepatic cytokine release before weight/fat loss or lower HTGC.

Skeletal muscle glucose uptake is dependent on phosphorylation and activation of the canonical insulin signaling cascade, leading to translocation of GLUT-4 to the plasma membrane (6). Furthermore, exercise training upregulates total and plasma membrane GLUT-4 expression in animal and human studies (5, 6, 12, 14, 36). Exercise training also increases insulin signaling (18), although the downstream insulin effects on glucose uptake have been questioned (13). There is evidence that fetuin-A acts on the insulin-signaling pathway, and data show that it inhibits insulin receptor tyrosine phosphorylation and tyrosine kinase activity in skeletal muscle, adipose, and the liver of cell and animal models (2, 7, 22, 23). Furthermore, Mathews et al. (22, 23) have shown that fetuin-A null mice have improved postprandial glucose disposal and signaling through MAPK and Akt in liver and skeletal muscle. Fetuin-A has also been linked to impaired skeletal muscle glycogen synthesis in rodents, suggesting that it regulates multiple sites of insulin-mediated metabolism (23). Herein, we

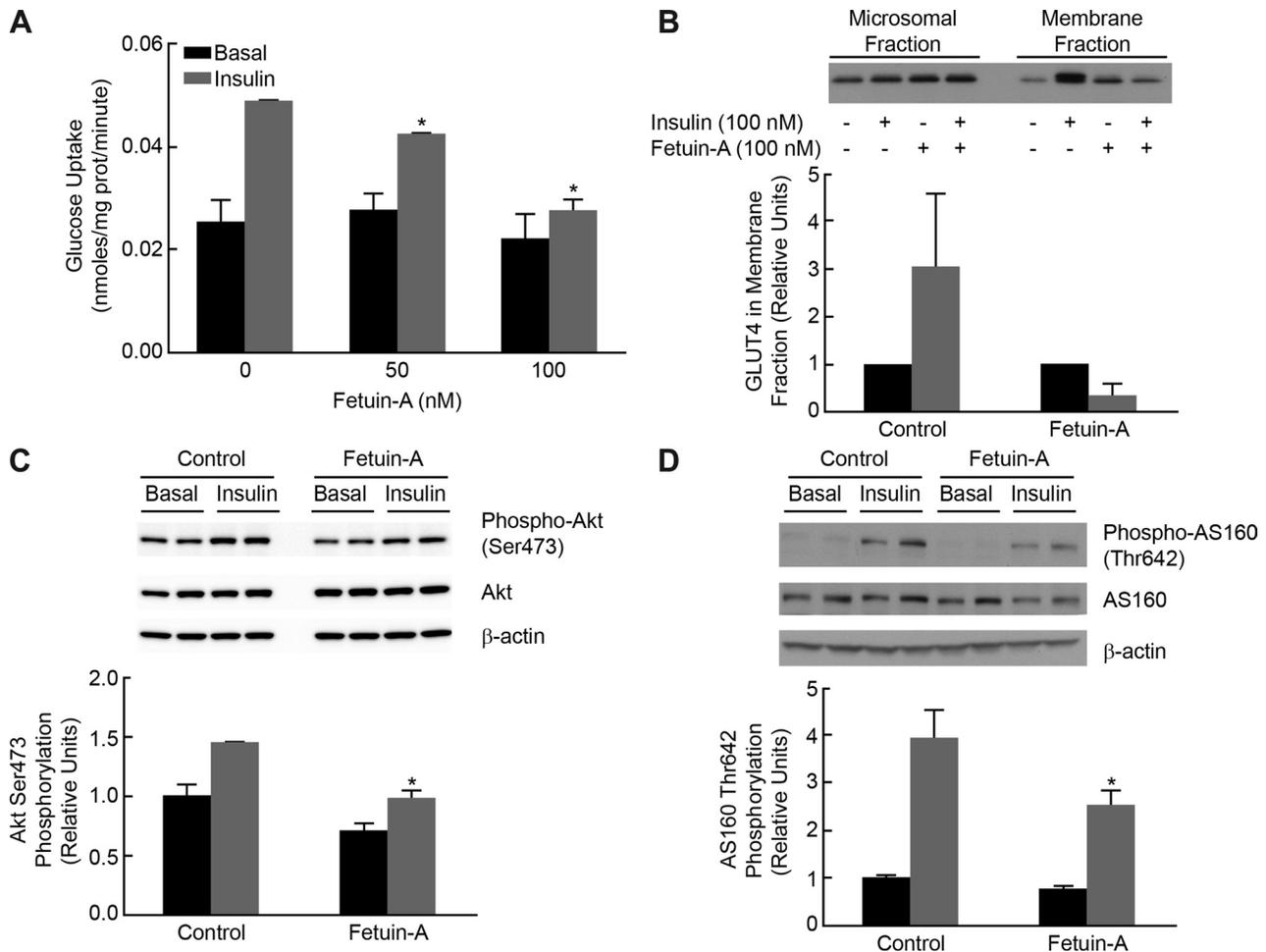


Fig. 3. Effect of fetuin-A on insulin-mediated glucose uptake in skeletal muscle (A), glucose transporter-4 (GLUT-4) translocation (B), Akt phosphorylation (C), and AS160 phosphorylation (D). C2C12 cells were plated and differentiated as described in the experimental protocol. Differentiated C2C12 myotubes were treated with or without 50 and 100 nM fetuin-A for 24 h and then stimulated with 100 nM insulin. Results represent the mean  $\pm$  SE from 3 independent experiments. All experiments were run in duplicate. \*Compared with control (fetuin-A main effect,  $P < 0.02$ ).

report that fetuin-A impairs insulin-mediated glucose uptake in skeletal muscle (Fig. 3) in C2C12 myotubes. These *in vitro* data are consistent with our *in vivo* correlation data (Fig. 3, A and B) and suggest that fetuin-A is involved in regulating glucose uptake in insulin-resistant subjects. Furthermore, fetuin-A treatment also reduced insulin signaling through Akt and AS160—two required steps for GLUT-4 translocation. Our findings suggest that fetuin-A, through Akt/AS160, directly or indirectly, led to a downregulation of GLUT-4 translocation that contributed to impaired glucose uptake. It is worth noting, however, that the fetuin-A concentration used in our cell model was higher than that seen *in vivo* before or after exercise, and further work is required to gain mechanistic insight using human tissue. In addition, C2C12 models are of mouse origin, and we cannot rule out the possibility that experiments conducted in other cell models or human tissue would respond the same way. Nevertheless, when taken together, our data suggest that fetuin-A regulates skeletal muscle insulin signaling and influences the capacity of skeletal muscle to facilitate glycemic control.

The current study was not designed to establish the exact mechanism responsible for exercise-reducing fetuin-A in humans, but our data indicate that exercise lowers fetuin-A,

independent of lower hepatic fat content, as suggested previously (33, 38, 44). Thus these data serve to highlight that exercise induces novel effects on plasma fetuin-A in adults with NAFLD. An alternative explanation to exercise-induced changes in fetuin-A may relate to changes in blood lipids. Recent work suggests that high fat diets and saturated fatty acids, such as palmitate, stimulate NF- $\kappa$ B binding to the fetuin-A promoter and increase its expression (7, 21, 29). Moreover, recent work by Pal et al. (31) shows that fetuin-A is an endogenous ligand for Toll-like receptor 4 and thus may promote fatty acid-induced insulin resistance *in vitro* and *in vivo*. If exercise lowered NEFA levels in our study, then this may attenuate actions of a key nutrient stimulus on influencing plasma fetuin-A concentrations. However, plasma NEFAs did not change after the intervention, suggesting that reductions in fetuin-A concentrations are due to different exercise-related mechanisms. Hyperglycemia has also been shown to enhance fetuin-A expression via activation of ERK1/2 in a human hepatoma cell line (HepG2) (40). We report that the effects of exercise on fetuin-A were related to baseline glucose concentrations as well as the improvement in glucose AUC after short-term exercise training. We acknowledge that these associations do not demonstrate causality but speculate that fe-

tuin-A expression, after the exercise intervention, may be due to reduced glucotoxicity. As such, improved glycemia may act as a feedback signal between skeletal muscle and liver that reflects decreased insulin resistance (29).

In conclusion, 7 days of exercise reduced plasma fetuin-A, and our data suggest that exercise regulates this hepatokine before any significant change in hepatic steatosis. The reduction in fetuin-A is clinically relevant, as it was associated with lower blood glucose responses and less insulin resistance. Further work using glucose isotopes and human skeletal muscle biopsies is needed to gain mechanistic insight into the role that exercise has on the interaction of fetuin-A and hepatic vs. peripheral insulin sensitivity in adults at risk for T2D. Collectively, these data suggest that fetuin-A is a novel hepatokine capable of attenuating glucose intolerance via changes in skeletal muscle insulin signaling.

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#### DISCLOSURES

The authors report no conflict of interest.

#### AUTHOR CONTRIBUTIONS

Author contributions: S.K.M., J.M.H., and J.P.K. conception and design of research; S.K.M., A.M., C.E.F., J.M.H., A.R.S., C.A.F., and J.P.K. performed experiments; S.K.M., A.M., C.E.F., J.M.H., M.R.P., H.H., C.A.F., and J.P.K. analyzed data; S.K.M., A.M., C.E.F., J.M.H., M.R.P., A.R.S., H.H., A.J.M., and J.P.K. interpreted results of experiments; S.K.M. and A.M. prepared figures; S.K.M. drafted manuscript; S.K.M., A.M., C.E.F., J.M.H., M.R.P., A.R.S., C.A.F., A.J.M., and J.P.K. edited and revised manuscript; S.K.M., A.M., C.E.F., J.M.H., M.R.P., A.R.S., H.H., C.A.F., A.J.M., and J.P.K. approved final version of manuscript.

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