

# Resistance to Aerobic Exercise Training Causes Metabolic Dysfunction and Reveals Novel Exercise-Regulated Signaling Networks

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Low aerobic exercise capacity is a risk factor for diabetes and a strong predictor of mortality, yet some individuals are “exercise-resistant” and unable to improve exercise capacity through exercise training. To test the hypothesis that resistance to aerobic exercise training underlies metabolic disease risk, we used selective breeding for 15 generations to develop rat models of low and high aerobic response to training. Before exercise training, rats selected as low and high responders had similar exercise capacities. However, after 8 weeks of treadmill training, low responders failed to improve their exercise capacity, whereas high responders improved by 54%. Remarkably, low responders to aerobic training exhibited pronounced metabolic dysfunction characterized by insulin resistance and increased adiposity, demonstrating that the exercise-resistant phenotype segregates with disease risk. Low responders had impaired exercise-induced angiogenesis in muscle; however, mitochondrial capacity was intact and increased normally with exercise training, demonstrating that mitochondria are not limiting for aerobic adaptation or responsible for metabolic dysfunction in low responders. Low responders had increased stress/inflammatory signaling and altered transforming growth factor- $\beta$  signaling, characterized by hyperphosphorylation of a novel exercise-regulated phosphorylation site on SMAD2. Using this powerful biological model system, we have discovered key pathways for low exercise training response that may represent novel targets for the treatment of metabolic disease. *Diabetes* 62:2717–2727, 2013

**C**hronic complex diseases such as the metabolic syndrome and diabetes are tremendous burdens to our society, and regular physical activity (~150 min of aerobic training each week) is a primary recommendation for the prevention and treatment of these conditions (1–3). The potential for exercise to prevent chronic disease is exemplified by large-scale epidemiological studies demonstrating that cardiorespiratory fitness (i.e., aerobic exercise capacity) is one of the

strongest predictors of health and longevity (4–6). For example, individuals with low aerobic exercise capacity have a more than four-fold higher risk of development of the metabolic syndrome and diabetes (7) and have up to five-fold higher all-cause mortality rates (8,9). The striking health risks associated with low aerobic exercise capacity are independent of other metabolic risk factors, including obesity and age (9–11), highlighting the importance of investigating the specific mechanisms that link exercise capacity to diabetes risk.

At present, the only clinically validated treatment for the improvement of exercise capacity is exercise training (12,13). However, significant variation exists in the ability to improve aerobic exercise capacity with exercise training in humans (14–16). In response to a standardized laboratory training protocol, changes in aerobic exercise capacity, as measured by  $\dot{V}O_{2\max}$ , can range from negative or no gain in some individuals (nonresponders) to >100% improvement in others (high responders) (14,17). The fact that some individuals are completely unresponsive to aerobic improvements with exercise training infers the existence of “exercise resistance.”

Considering the strong association between low aerobic exercise capacity and metabolic dysfunction (8), identifying mechanisms that contribute to the disparity in exercise training response may provide new targets for the treatment of chronic metabolic disease. However, the exercise capacity phenotype is determined by complex gene–environment interactions involving intrinsic factors (inborn) and those accrued in response to exercise training, making it challenging to isolate the critical mechanisms (17–20). Furthermore, animal models based on single-gene modifications (i.e., knockouts or transgenics) are inadequate to study the interaction between complex traits such as exercise capacity and metabolic disease. As such, we identified a need to develop genetically heterogeneous (noninbred) animal model systems that more closely embody human phenotypes and disease (19).

Here, we describe novel rat models of low aerobic response to training (LRT) and high aerobic response to training (HRT) that were created by divergent selective breeding to elucidate the mechanistic links between low aerobic adaptation to exercise training and disease risk. These models allowed us to directly test the hypothesis that nonresponders to exercise training have an increased risk for metabolic disease. Furthermore, because many of the health benefits attributed to exercise training stem from activation and remodeling of skeletal muscle, impaired adaptation in this tissue likely underlies the exercise-resistant phenotype. However, the multitude of concurrent

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transcriptional and signaling events that occur in skeletal muscle in response to exercise have made it difficult for previous research to establish which of these are essential for adaptive improvements to exercise capacity and health. Therefore, we used this contrasting animal model system to identify, using an unbiased approach, the molecular and morphological responses in skeletal muscle that are critical for improvements to exercise capacity and, potentially, metabolic health.

## RESEARCH DESIGN AND METHODS

**Artificial selection for LRT and HRT.** To increase genetic heterogeneity, N:NIH out-crossed stock rats ( $n = 152$ ) were used as the founder population (generation 0) rather than inbred strains. At  $\sim 10$  weeks of age, the exercise capacity of each rat was measured using an incremental treadmill running test, which has been described previously (20). Each rat then underwent 24 sessions (3 days/week for 8 weeks) of treadmill running training using a protocol that increased in speed (from 10 to 20 m/min) and duration (from 20 to 30 min) each session. This moderate protocol was designed to ensure that all rats could complete the entire training schedule, regardless of their initial exercise capacity or relative change in capacity across training sessions (21). After completion of exercise training, the exercise capacity of each rat was measured (as described) and the training response was calculated as the change in exercise capacity as follows: posttraining exercise capacity – pretraining exercise capacity. Rats with the highest response to training were chosen for one line of selective breeding (HRT; 10 families/generation), and rats with the lowest response to training were chosen for an independent line (LRT; 10 families/generation). Approximately 100 offspring per line for each generation were assessed for training response and this selection process was repeated for 15 generations ( $N = 3,114$  rats). Rats were fed rodent pellet diet (diet #5001; Purina Mills, Richmond, IN) and given free access to water. All procedures were performed in accordance with the University Committee on Use and Care of Animals at the University of Michigan.

**Acute exercise bout.** Forty female rats ( $n = 20$  LRT and  $n = 20$  HRT) from generation 12 were sent from University of Michigan to the Joslin Diabetes Center. After a 2-week acclimatization period, a subset of rats ( $n = 13$ /group) underwent an acute bout of exercise consisting of 25 min of treadmill running (15% incline) at a moderate speed (15 m/min). A second group of rats did not exercise and acted as sedentary controls ( $n = 7$ /group). Blood and tissue collection for the exercised rats occurred either immediately after the exercise bout ( $n = 7$ /group) or 3 h after the exercise bout ( $n = 6$ /group). All experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Joslin Diabetes Center. The  $\dot{V}O_2$  and  $\dot{V}CO_2$  were measured using the Comprehensive Laboratory Monitoring System (Columbus Instruments).

**Chronic exercise training.** Forty female rats ( $n = 20$  LRT and  $n = 20$  HRT) from generation 15 underwent exercise capacity testing (as described) at 10–12 weeks of age. A subset of rats ( $n = 10$ /group) then underwent an 8-week exercise training protocol (as described). A second group of rats did not undergo exercise training and acted as sedentary controls. After the training period, all rats (exercise-trained and sedentary) underwent a second exercise capacity test for the calculation of exercise response as described. Blood and tissues were collected from exercise-trained rats 48 h after the last exercise bout to wash-out the potentially confounding effects of the last exercise bout.

**Insulin and glucose tolerance tests.** To assess glucose and insulin tolerance, sedentary rats were administered glucose (2 g/kg body weight) or insulin (0.75 units/kg body weight) by intraperitoneal injection, and blood glucose concentration was measured using a OneTouch Ultra (Lifescan) glucose monitoring system at specified time points after injection.

**Blood metabolite and cytokine measurements.** Plasma free fatty acid concentration was determined using an enzymatic colorimetric method (NEFA C; Wako Chemicals). Plasma interleukin-6, interleukin-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , and leptin concentrations were measured using a multiplex ELISA assay (Milliplex; Millipore). Plasma transforming growth factor (TGF)- $\beta$ 1 concentration was measured using an ELISA assay (MB100B; R&D Systems). Plasma triglycerides were measured using a colorimetric assay (Stanbio Laboratory 2200–430). Plasma glucose was measured using hexokinase reagent (Eagle Diagnostics #2821).

**Citrate synthase activity.** Whole-cell lysates from soleus and plantaris muscles were analyzed for citrate synthase activity as previously described (22).

**Mitochondrial maximal ATP production rate.** Twenty-four sedentary male rats ( $n = 12$  LRT and  $n = 12$  HRT) from generation 13 and 24 trained male rats ( $n = 12$  LRT and  $n = 12$  HRT) from generation 14 were sent from University of

Michigan to University of Nottingham. Mitochondria were isolated from freshly isolated soleus muscles  $\sim 1$  week after completion of training and the rates of maximal ATP production in the presence of various substrates were determined using a luminescence technique as described previously (23). The substrate solutions tested were (final concentrations) as follows: glutamate 16.4 mmol/L and succinate 15 mmol/L; palmitoyl-L-carnitine and 5  $\mu$ mol/L and malate 1.5 mmol/L (with human serum albumin 0.14 mg/mL); pyruvate 50 mmol/L and malate 22 mmol/L; succinate 2.5 mmol/L; and glutamate 32.75 mmol/L and malate 22 mmol/L.

**Muscle glycogen content.** An aliquot ( $\sim 20$  mg) of pulverized gastrocnemius muscle was hydrolyzed with HCl and neutralized with NaOH. The glucose concentration of the resulting lysate was analyzed using hexokinase reagent (Eagle Diagnostics #2821).

**Liver triglyceride content.** Triacylglycerol was extracted and saponified from an aliquot of liver ( $\sim 25$  mg) in ethanol/KOH at 55°C. Glycerol content was determined using a colorimetric assay (Sigma).

**Western blotting.** Gastrocnemius muscle lysates were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated with antibodies specific for the following: pERK 1/2 T202/Y204 (CST #4370); pSMAD4 T277 (Abgent AP7753); pSMAD3 S208 (Abgent AP9995); pSMAD2 S245/250/255 (CST #3104); pSMAD2 S465/467 (CST #3104); SMAD2 (CST #5339); pSMAD3 S423/425 (CST #9520); SMAD4 (CST #9515); active c-Jun N-terminal kinase (JNK; Promega V7931); JNK (CST #9252); pP38 mitogen-activated protein kinase (MAPK; CST #9211); P38 MAPK (CST #9212); pAkt (CST #9271); Akt (CST #9272); pAMPK (CST #2531); pACC (Millipore 07–303); ACC (Upstate); pTAK1 S412 (CST #9339); TGF- $\beta$ -activated kinase (CST #4505); pCaMKII T286 (CST #3361); calcium/calmodulin-dependent protein kinase II (CaMKII; BD611292); CaMKK (BD); and AS160 (Upstate). The immunoreactive proteins were detected with enhanced chemiluminescence and quantified by densitometry.

**Immunohistochemistry.** Plantaris muscles from sedentary and exercise-trained rats were frozen in  $N_2$ -cooled isopentane and cut into 6- $\mu$ m cross-sections. Sections were stained with antibodies against laminin (Sigma-Aldrich) and either myosin heavy chain I (4.951; DHSB) or myosin heavy chain IIA (SC-71; DHSB), or the endothelial marker CD31 (Serotec MCA1334EL), and visualized using fluorescent secondary antibodies under 100 $\times$  magnification. Analysis of muscle capillary density (capillaries/mm<sup>2</sup>) based on CD31 immunofluorescence was performed by OracleBio Limited using a customized vessel detection algorithm within Definiens Tissue Studio 3.5 image analysis software. Quantification of all images was performed in a blinded manner.

**RNA isolation and microarray analysis.** A portion of the soleus muscle taken from rats under resting conditions or 3 h after an acute bout of treadmill running exercise was preserved and frozen using RNAlater (Qiagen). RNA was extracted using QIAzol lysis reagent (Qiagen) and purified using RNeasy cleanup kit (Qiagen). RNA quality and integrity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The Ambion WT Expression Kit (PN 4425209C) was used and labeling of cDNA was performed using the Affymetrix GeneChip WT Terminal Labeling Kit (PN 900671). Hybridization, washing, and staining were performed on Affymetrix GeneChip Rat Gene-ST 1.0 arrays. Low-level processing and significance analysis of microarrays analysis were undertaken as previously described (24,25) to generate a list of regulated genes. The gene list was then subject to ingenuity pathway analysis–based pathway analysis using the Upstream Analysis tool.

**Statistical Analysis.** Differences between groups were identified using a two-way ANOVA using phenotype and exercise as independent variables. When appropriate, Tukey post hoc testing was performed to assess differences between individual groups. Results are expressed as mean  $\pm$  SEM and statistical significance was accepted at  $P < 0.05$ .

## RESULTS

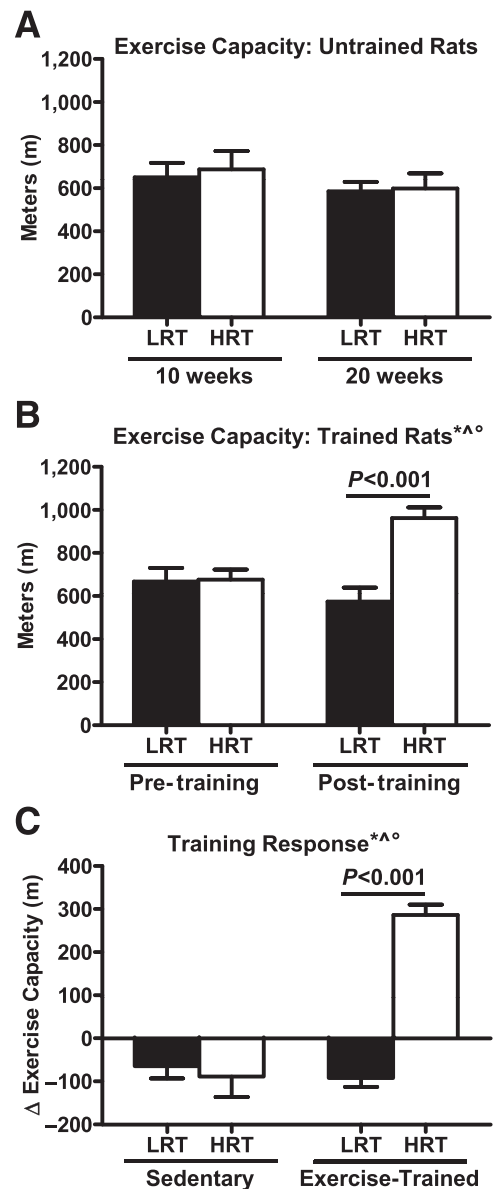
**Selective breeding for LRT and HRT.** Based on evidence that the ability to increase aerobic capacity with exercise training is determined by inherited factors (26), we aimed to create animal models of LRT and HRT via the process of selective breeding. Before selective breeding (generation 0), the average response of the rat population to this exercise training protocol resulted in  $140 \pm 15$  m increase in exercise capacity. After 15 generations of selective breeding, rats bred as HRT improved endurance running capacity by  $223 \pm 20$  m, whereas rats bred as LRT declined  $-65 \pm 15$  m in response to treadmill training. We closely evaluated 40 females (20 LRT and 20 HRT) by comparing groups that underwent

8 weeks of treadmill running exercise (exercise-trained) with untrained (sedentary) control groups. At 10 weeks of age, rats underwent exercise capacity testing, and it was determined that intrinsic exercise capacity (that which is present in the absence of training) was the same between LRT and HRT (Fig. 1A and B). After exercise training, exercise capacity was 67% higher in HRT compared with LRT (Fig. 1B). When the data were expressed as training response ( $\Delta$  exercise capacity), LRT failed to improve exercise capacity and HRT had 54% increase in exercise capacity because of training (Fig. 1C; exercise-trained). Therefore, we have successfully created populations of nonresponders (LRT) and HRT through divergent selective breeding.

**LRT display whole-body metabolic dysfunction.** Clinical data demonstrate a strong correlation between low exercise capacity and metabolic disease (8). As such, we hypothesized that selection for LRT would segregate with higher metabolic disease risk. In support of this hypothesis, LRT displayed impaired whole-body insulin sensitivity (homeostasis model of insulin resistance; Fig. 2A) and higher circulating insulin concentrations under sedentary and exercise-trained conditions (Table 1). Consistent with training-independent insulin resistance, sedentary LRT had impaired glucose tolerance as assessed by the glucose area under the curve after intraperitoneal injection of 2 g/kg glucose (Fig. 2B) and blood glucose levels were higher in LRT 90 min after intraperitoneal injection of 0.75 units/kg insulin (Fig. 2C). LRT also had higher body (Fig. 2D) and gonadal fat pad (Fig. 2E) weights, higher plasma triglycerides (Fig. 2F), and higher circulating leptin (Table 1). Liver triglyceride content was 28% higher in LRT after exercise training (Fig. 2I), indicating metabolic dysfunction in this tissue. Notably, insulin resistance and adiposity in LRT were present in the absence of exercise training (sedentary condition), at a relatively young age (20 weeks), and under normal diet conditions (chow-fed, 13.5% calories from fat), demonstrating that primary metabolic defects co-selected with the genes that contribute to low aerobic training response.

Altered systemic inflammatory mediators are postulated to be contributing factors to the metabolic syndrome (27,28). Therefore, we measured the circulating levels of key inflammatory markers in LRT and HRT. Plasma cytokine levels were not different between untrained LRT and HRT rats (Fig. 2G and H, Table 1). However, after exercise training (samples taken 48 h after the last exercise bout), plasma concentrations of the inflammatory cytokine TNF- $\alpha$  were 85% higher in LRT (Fig. 2G), demonstrating that an altered inflammatory response to training accompanies the LRT phenotype. In contrast, plasma concentrations of the “anti-inflammatory” cytokine TGF- $\beta$  were 50% lower in LRT compared with HRT after exercise training (Fig. 2H). Thus, TGF- $\beta$  and TNF- $\alpha$  were divergently regulated by exercise training in LRT and HRT, indicating that these pleiotropic cytokines may contribute to the training response phenotype.

**LRT do not have impaired mitochondrial function in skeletal muscle.** Although other organs (e.g., the heart) may contribute to the exercise capacity phenotypes in LRT and HRT, because of its significant contribution to total body mass, skeletal muscle has greater potential to influence both exercise capacity and whole-body metabolic health (29). Therefore, we measured several characteristics of skeletal muscle that have the potential to influence exercise capacity and metabolic health in LRT and HRT.



**FIG. 1.** Exercise capacity in rats bred for LRT and HRT. Exercise capacity (m) was measured using an incremental treadmill running test to exhaustion in (A) sedentary rats at 10 and 20 weeks of age and in (B) a separate group of age-matched trained rats before (pretraining) and after (posttraining) 8 weeks (3 days/week) of treadmill running exercise. C: Exercise response was calculated as the difference in exercise capacity before and after exercise training (exercise-trained) for each rat. The change in exercise capacity between 10 and 20 weeks of age is shown for animals in the control group (sedentary). \* $P < 0.05$  for phenotype main effect; <sup>^</sup> $P < 0.05$  for exercise main effect; <sup>o</sup> $P < 0.05$  for phenotype-exercise interaction by two-way ANOVA.  $P$  values obtained by Tukey post hoc testing are shown.  $n = 10$ –12/group.

Impaired mitochondrial capacity has been proposed as a mechanism contributing to the metabolic syndrome (30,31), and increased mitochondrial capacity contributes to improved aerobic exercise capacity with training (32). However, there was no difference in markers of mitochondrial density (citrate synthase activity in whole-muscle lysates) or mitochondrial function (maximal ATP production rates in isolated mitochondria) between LRT and HRT in the sedentary state (Table 2). Furthermore, LRT had normal increases in mitochondrial density and function in response to the moderate exercise training regimen (Table 2).

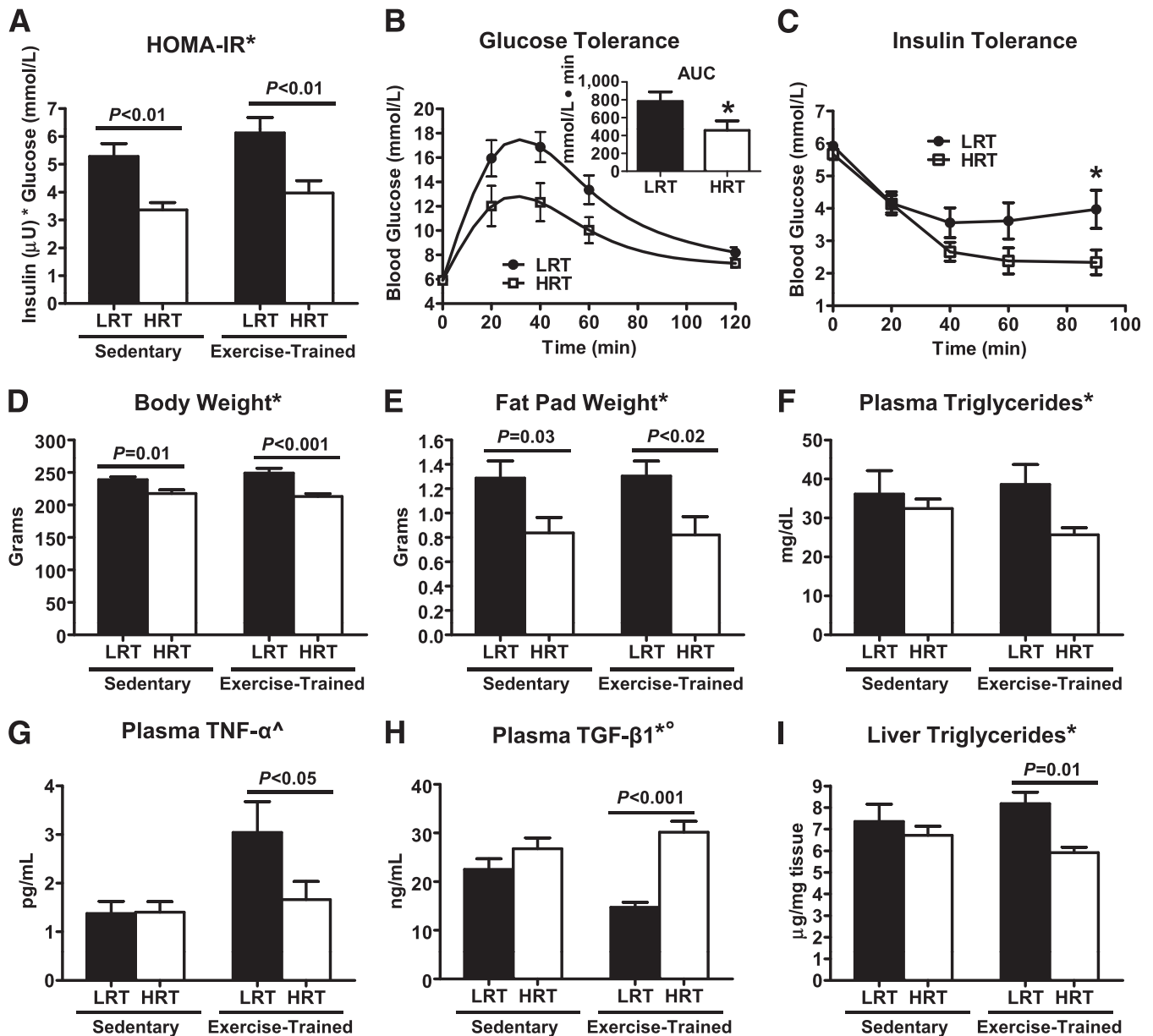


FIG. 2. Whole-body metabolic dysfunction in LRT. Fasting blood samples were collected from sedentary and exercise-trained LRT/HRT. **A:** Plasma glucose and insulin values were used to calculate the homeostasis model of insulin resistance (HOMA-IR). **B:** Glucose tolerance was assessed in sedentary rats after an intraperitoneal (IP) injection of 2 g/kg glucose and the area under curve (AUC) was calculated. **C:** Insulin tolerance was assessed after IP injection of 0.75 units/kg insulin. Body weight (**D**) and gonadal fat pad weight (**E**) were measured in sedentary and exercise-trained LRT and HRT. Plasma triglycerides (**F**), TNF- $\alpha$  (**G**), and TGF- $\beta$ 1 (**H**) concentrations were analyzed by ELISA. **I:** Liver triglycerides were estimated from total liver glycerol content. \* $P < 0.05$  for phenotype main effect;  $^{\wedge}P < 0.05$  for exercise main effect;  $^{\circ}P < 0.05$  for phenotype-exercise interaction by two-way ANOVA.  $P$  values obtained by Tukey post hoc testing are displayed.  $n = 10$ –12/group.

Therefore, our results indicate that impaired mitochondrial function is not responsible for metabolic dysfunction or the failure to improve exercise capacity with training in LRT.

**LRT have altered skeletal muscle fiber type.** Endurance athletes often have a higher proportion of type I (oxidative) fibers in skeletal muscle (33), and low type I fiber content is associated with insulin resistance, obesity, and type 2 diabetes (34–36). Consistent with a proposed link between lower proportion of type I fibers and metabolic dysfunction, type I fibers accounted for ~7% of the total fibers in LRT compared with >20% in HRT (Fig. 3A). Therefore, higher type I fiber content may contribute to improved insulin sensitivity in HRT. However, fiber-type differences between LRT and HRT were independent of

exercise training and therefore cannot explain posttraining differences in exercise capacity. No differences were observed in type IIA (oxidative/glycolytic) fiber content (Supplementary Fig. 1).

**LRT have impaired exercise-induced angiogenesis in skeletal muscle.** Exercise training stimulates angiogenesis in skeletal muscle, and this may contribute to improved exercise capacity and metabolic health (10,37). Capillary density was similar in skeletal muscle from LRT and HRT under untrained conditions (Fig. 3B). In contrast, capillary density was 50% higher in HRT after exercise training (Fig. 3B). The failure of LRT to stimulate angiogenesis in response to training may contribute to divergent responses to training in these models. Overall, the skeletal muscle

TABLE 1  
Plasma measurements taken from sedentary and exercise-trained LRT and HRT responders to exercise training

	LRT SED	LRT EXT	HRT SED	HRT EXT	Phenotype <i>P</i>	Exercise <i>P</i>
Insulin, ng/mL	1.04 ± 0.10	1.01 ± 0.10	0.69 ± 0.06*	0.78 ± 0.08*	0.002	0.75
Glucose, mmol/L	4.93 ± 0.10	4.94 ± 0.25	4.74 ± 0.16	4.89 ± 0.24	0.55	0.71
Leptin, pg/mL	5,760 ± 592	7,115 ± 734	3,725 ± 540*	4,058 ± 723*	<0.001	0.20
IL-1β, pg/mL	4.27 ± 0.79	3.32 ± 0.60	3.45 ± 0.85	3.49 ± 0.88	0.29	0.95
IL-6, pg/mL	17.77 ± 4.69	14.63 ± 2.37	29.60 ± 8.23	16.05 ± 2.40	0.2	0.11

*n* = 10/group. EXT, exercise-trained; IL, interleukin; SED, sedentary. \**P* < 0.05 vs. corresponding LRT value by Tukey post hoc testing.

characteristics of low type I fiber content in conjunction with impaired exercise-induced angiogenesis demonstrate a general alteration in muscle phenotype and impaired tissue remodeling in LRT.

**Response to a single bout of treadmill running in LRT and HRT.** It has been hypothesized that each single bout of exercise initiates signaling and transcriptional events that accumulate with repeated bouts to produce exercise training adaptations (38). However, a direct link between the acute molecular responses to exercise and the molecules that regulate long-term adaptation remains elusive (39) and our unique model affords an opportunity to provide new insight into this question. To determine differences in the molecular response to a single bout of exercise, LRT and HRT rats remained sedentary for 20 weeks after phenotyping to wash-out the effects of training. The detrained rats then performed a single bout of moderate-intensity treadmill running for 25 min at a speed of 15 m/min. Oxygen consumption, the respiratory exchange ratio, muscle glycogen concentrations, and serum free fatty acid concentrations were similarly altered by exercise in LRT and HRT, indicating that the intensity of the exercise bout was not different for the LRT and HRT rats (Supplementary Fig. 2). To test the hypothesis that LRT had altered exercised-induced signaling, we measured AMPK and Akt phosphorylation-molecular signals that have been proposed to be critical mediators of exercise training-induced adaptations in skeletal muscle (40,41). Exercise increased AMPK and Akt signaling in the muscle; however, there was no difference between LRT and HRT (Supplementary Fig. 3). These data suggest that as-yet-unidentified pathways mediate the adaptive response to exercise training.

**Contrasting transcriptional responses to exercise in LRT and HRT.** As a framework to identify the gene networks that regulate training response, a microarray

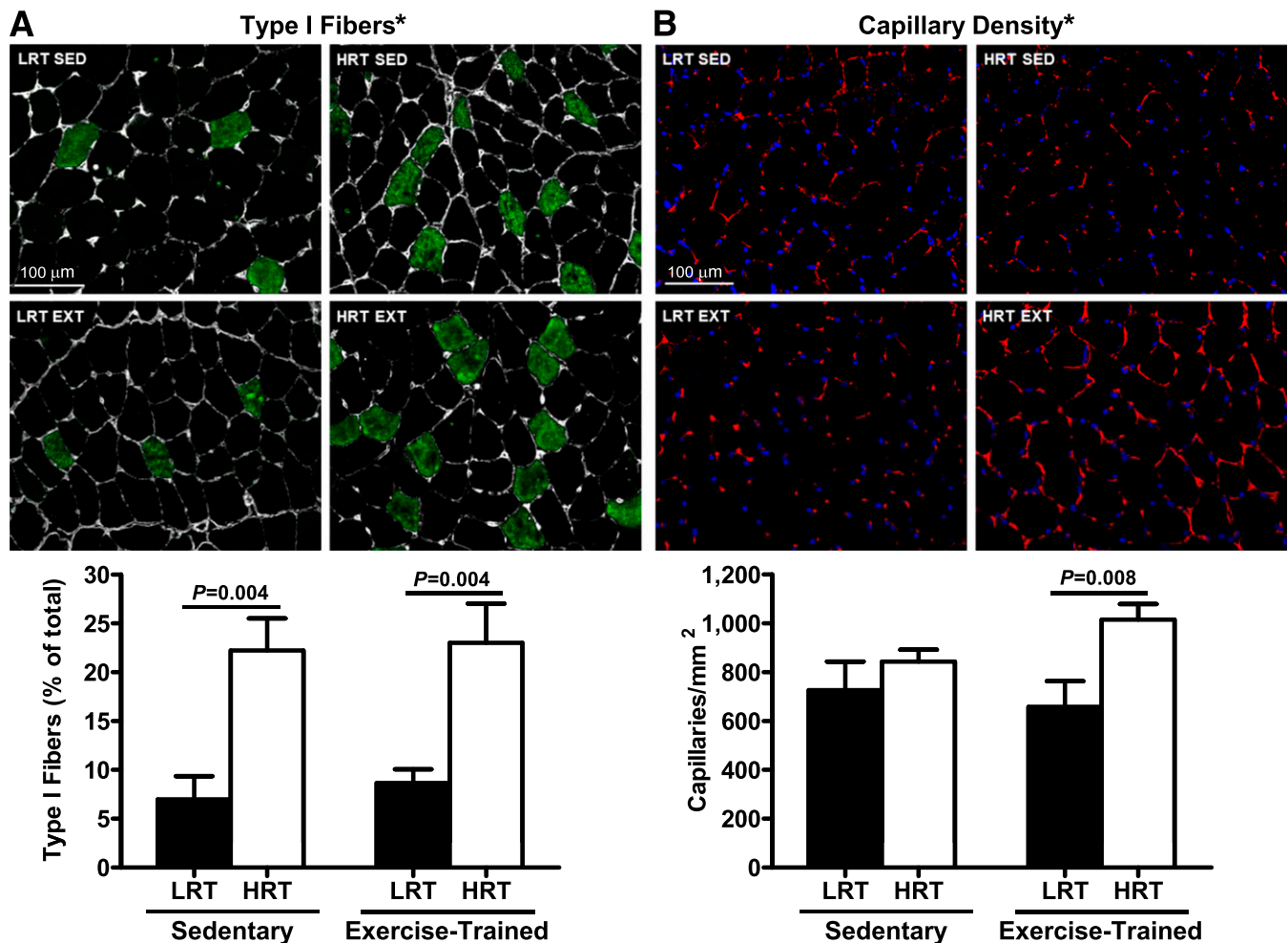
experiment was performed to establish the exercise-responsive networks that were differentially regulated in LRT and HRT. Microarray analysis of RNA extracted from soleus muscles in the basal state and 3 h after a single bout of exercise identified subsets of genes that were exclusively upregulated in response to exercise in LRT (*n* = 130 genes) and HRT (*n* = 59 genes; Supplementary Fig. 4), whereas 133 genes were downregulated in response to exercise exclusively in LRT (Supplementary Fig. 4). Analysis using ingenuity pathway analysis revealed that the LRT and HRT exercise-regulated transcriptomes shared no common ontological overlap, indicating a striking contrast in transcriptional responses to the same exercise bout in these models. Genes differentially regulated by exercise in LRT and HRT belonged to the functional categories of gene expression, development, cell-cycle regulation, cellular growth, proliferation, and movement (Supplementary Fig. 4D). This unbiased analysis of the global molecular responses to acute exercise demonstrates that inherent differences in the muscle remodeling response may contribute to the exercise adaptation phenotype.

**SMAD3, CREB1, and histone deacetylase target genes are dysregulated in LRT.** To identify the upstream processes underlying differential exercise-induced gene network modulation between LRT and HRT, the ingenuity pathway analysis upstream analysis tool was used on their respective exercise-regulated transcriptomes. This analysis identified activation of SMAD3 and cAMP-responsive element-binding 1 (CREB1) target genes and inhibition of histone deacetylase (HDAC)-regulated genes after exercise in LRT (Fig. 4; *P* <  $1 \times 10^{-5}$ ). No such gene network nodes were found in the HRT exercise-regulated gene list, demonstrating that LRT activate a unique molecular response to exercise. Based on these data, we hypothesized that exercise-stimulated

TABLE 2  
Skeletal muscle mitochondrial capacity is similar in LRT and HRT rats

	LRT SED	LRT EXT	HRT SED	HRT EXT	Phenotype <i>P</i>	Exercise <i>P</i>
Citrate synthase activity (nmol/min/mg muscle protein)						
Muscle						
Soleus	316.9 ± 3.8	343.5 ± 15.0	337.1 ± 18.1	368.7 ± 11.6	0.1	0.04
Maximal ATP production rate (nmol/min/min mitochondrial protein)						
Substrate						
Pyruvate/ malate	3.19 ± 0.55	5.26 ± 0.77*	3.29 ± 0.50	4.20 ± 0.84	0.49	0.03
Glutamate/ malate	3.84 ± 0.62	7.39 ± 1.22*	3.44 ± 0.67	5.65 ± 1.13	0.27	0.004
Glutamate/ succinate	6.01 ± 0.87	9.33 ± 1.45	5.56 ± 0.94	6.77 ± 1.40	0.22	0.06

Citrate synthase activity was measured in whole-muscle homogenates from SED and EXT rats (*n* = 6/group). Mitochondrial ATP production in response to various substrates was measured in mitochondria isolated from the soleus muscle of sedentary or exercise-trained rats (*n* = 12/group). EXT, exercise-trained; SED, sedentary. \**P* < 0.05 vs. corresponding SED value by Tukey post hoc testing.



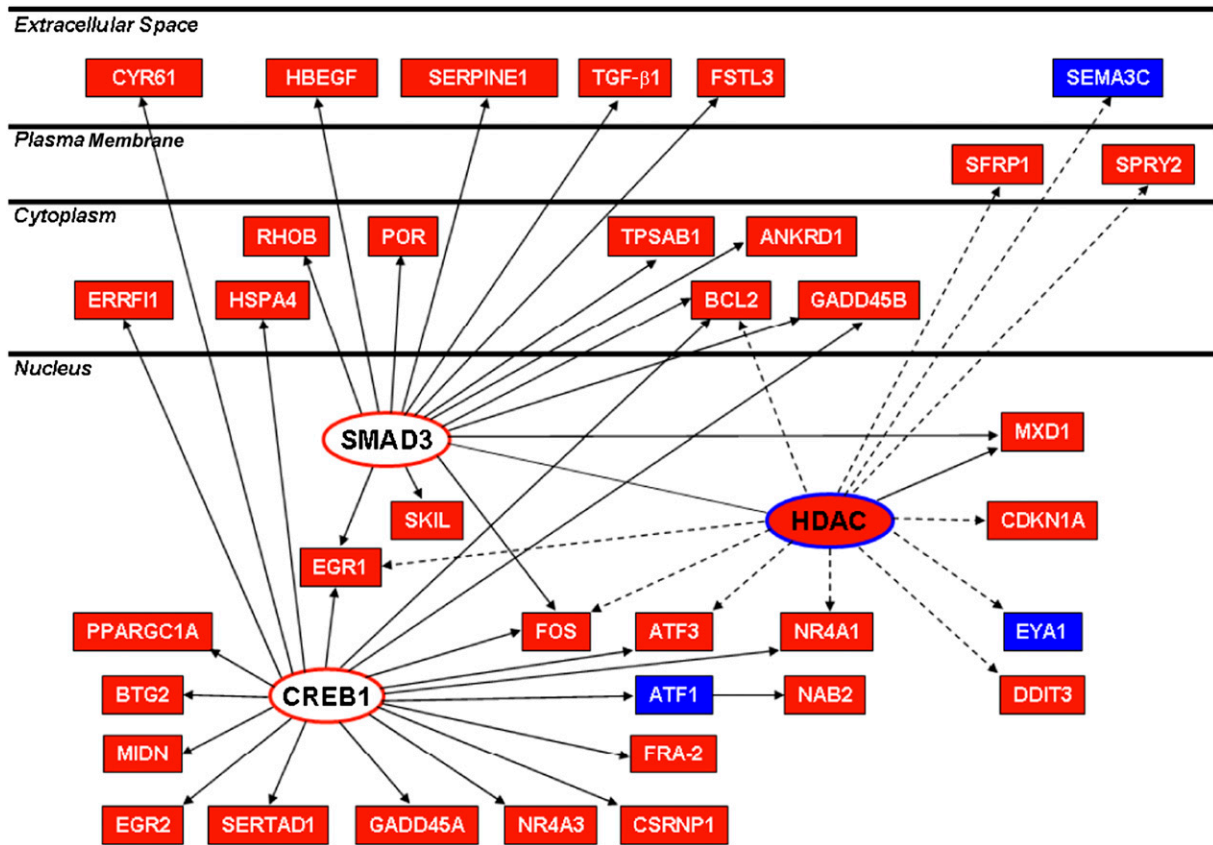
**FIG. 3.** HRT have fewer oxidative muscle fibers and impaired exercise-induced angiogenesis. Plantaris muscles from sedentary (SED) and exercise-trained (EXT) rats were frozen in N<sub>2</sub>-cooled isopentane and cut into 6- $\mu$ m cross-sections. **A:** Sections were stained with antibodies against laminin (white) and myosin heavy chain I (green) and visualized using fluorescent secondary antibodies under 100 $\times$  magnification. Type I fiber content was expressed as % of total muscle fibers counted. **B:** Capillary density (capillaries/mm<sup>2</sup>) was calculated in sections stained with an antibody against the endothelial marker CD31 (red). Nuclei were visualized with DAPI stain (blue). *n* = 4–5/group. \**P* < 0.05 for phenotype main effect by two-way ANOVA. *P* values obtained by Tukey post hoc testing are displayed.

protein signaling events that control SMAD, CREB, and HDAC transcription are dysregulated in LRT.

**Muscle signal transduction is altered in LRT.** CaMKII is an important mediator of intracellular calcium homeostasis and skeletal muscle plasticity (42,43), and is a key feature of the human exercise training transcriptome (44). CaMKII is a negative regulator of HDAC (43) that can mediate transcription via direct regulation of SMAD3 (45) and CREB1 (46), which were shown to contribute to the LRT exercise-responsive transcriptome, making altered CaMKII regulation a feasible mediator of dysregulated transcription in LRT. Phosphorylation of CaMKII $\delta$  at its autoregulatory site, Thr286, was higher in LRT, both in the basal state and after exercise, indicating constitutive activation of the enzyme (Fig. 5B). Constitutive activation of CaMKII in LRT was specific to the  $\delta$  isoforms of the enzyme (~55–60 kDa), because phosphorylation of the  $\beta$ m isoform (~70 kDa) was similar in LRT and HRT (Fig. 5A), which may reflect the different subcellular localization of these isoforms (47). Given the importance of CaMKII in many aspects of muscle signal transduction and remodeling processes (42,46), this chronic increase in CaMKII

activation likely contributes to altered transcription and impaired muscle plasticity in LRT.

SMAD3 is a primary mediator of TGF- $\beta$  signaling and transcription in conjunction with its binding partner SMAD2 and molecular chaperone SMAD4 (48). To determine the mechanism behind altered exercise-induced SMAD3 transcription in LRT, we assessed canonical receptor-mediated TGF- $\beta$  signaling by measuring the phosphorylation of SMAD2/3 at COOH terminal residues but found no phosphorylation of these sites in LRT and HRT skeletal muscle. Furthermore, exercise did not increase TGF- $\beta$ -activated kinase phosphorylation in LRT or HRT (Fig. 5A), confirming that canonical TGF- $\beta$  signaling is not activated by exercise. TGF- $\beta$ -mediated transcription also can be regulated by an alternative pathway involving phosphorylation of SMADs in their linker region (49). Exercise had no effect on SMAD3 and SMAD4 linker phosphorylation in both phenotypes (Fig. 5A). In contrast, exercise robustly increased SMAD2 linker region phosphorylation (Ser245/250/255), an effect that was three-fold greater in LRT (Fig. 5C). Exercise-induced SMAD2 linker phosphorylation in skeletal muscle has not been previously reported; therefore, its functional role is



**FIG. 4.** Analysis of gene transcription in response to an acute bout of exercise identifies dysregulation of SMAD, CREB, and HDAC activity in LRT. RNA was extracted from the soleus muscles of rats under resting conditions or 3 h after an acute bout of treadmill running exercise. Genes that were significantly upregulated (red) or downregulated (blue) in response to exercise in LRT/HRT were identified using Affymetrix Rat ST 1.0 chips and analyzed using ingenuity pathway analysis (IPA; false discovery rate [FDR] = 5%, no fold-change filter). Transcription factor analysis in IPA clearly identified activation of SMAD3 ( $Z$  score = 2.3;  $P = 1.1 \times 10^{-7}$ ) and CREB1 ( $Z$  score = 2.1;  $P = 6.7 \times 10^{-6}$ ) target genes, whereas HDAC-regulated genes were inhibited ( $Z$  score = -2.8;  $P = 1.1 \times 10^{-7}$ ) in response to exercise in LRT. Direct transcription factor/target gene relationships are indicated by solid arrows, and indirect relationships are indicated by broken arrows. No transcription factor enrichment was found in the HRT-regulated gene list, which was a set of entirely upregulated genes ( $n = 156$ , FDR = 5%, no fold-change filter) that bore no ontological or pathway overlap with the LRT dataset in IPA.  $n = 5-6$  chips/group.

not yet understood. However, investigations of cultured fibroblasts indicate that phosphorylation of the SMAD linker region inhibits canonical TGF- $\beta$  signaling (49) and may shift transcription toward target genes involved in extracellular matrix synthesis (50). Thus, we postulate that this novel phosphorylation site may contribute to impaired muscle remodeling in response to exercise in LRT.

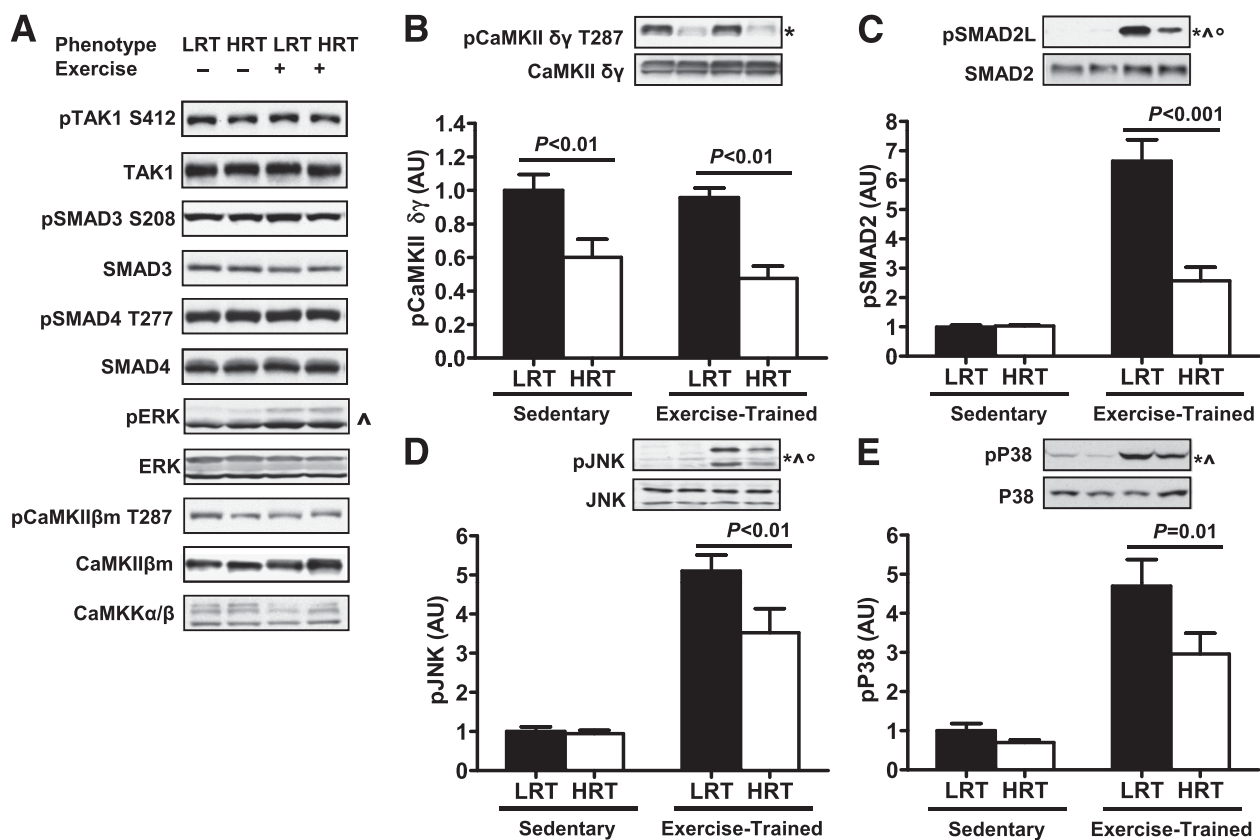
Exercise increases JNK and p38 MAPK activity (51), and both JNK and p38 MAPK are upstream kinases for SMAD linker phosphorylation in vitro (50). Exercise increased JNK and p38 MAPK phosphorylation in both LRT and HRT; however, the increase was  $\sim 50\%$  greater in LRT demonstrating hyperactivation of these signaling proteins (Fig. 5D and E). Based on our signal transduction and microarray analysis, we propose that the mechanism for the LRT phenotype involves the exercise-induced hyperactivation of JNK and p38 MAPK, leading to increased phosphorylation of their target, SMAD2, thereby resulting in increased transcription of SMAD3 target genes in LRT (Fig. 6).

## DISCUSSION

We demonstrate that two-way selective breeding based on the aerobic response to exercise training generates rat models of LRT and HRT. Our results parallel clinical data in humans indicating that in response to standardized

aerobic exercise training, some individuals fail to improve their exercise capacity (nonresponders), whereas others achieve great gains (high responders) (14,15,52). The ability to enrich the trait of exercise response through selective breeding illustrates conclusively that inherited factors (genetic and epigenetic) determine this phenotype and validates our model for the study of this complex trait. Furthermore, despite strong clinical associations (8), previous research has not uncovered causative or mechanistic links between exercise training responsiveness and metabolic disease. We now demonstrate that selective breeding for the trait of LRT leads to whole-body metabolic dysfunction, including insulin resistance, increased adiposity, dyslipidemia, and inflammation. Remarkably, metabolic dysfunction in low-responders occurred even in the absence of training, suggesting an intrinsic metabolic defect segregates with the training response phenotype. These data are the first to establish a causative relationship between training response and metabolic disease risk, providing mechanistic validation for the numerous epidemiological studies that link aerobic exercise capacity and health.

To identify the molecular mechanisms that contribute to the LRT phenotype, we designed a novel multilevel approach using bioinformatic analysis of exercise-induced alterations in RNA networks to identify signal transduction



**FIG. 5.** Hyperphosphorylation of CaMKII, SMAD2, and MAPK in LRT. *A–E*: Phosphorylation of proteins involved in calcium, MAPK, and TGF- $\beta$  MAPK signaling were measured by Western blotting in lysates from gastrocnemius muscle under resting conditions (basal) or immediately after an acute bout of exercise (exercise). ERK, extracellular signal-regulated kinase; P38, p38 mitogen-activated protein kinase; SMAD, mothers against decapentaplegic homolog.  $n = 6–7/\text{group}$ . \* $P < 0.05$  for phenotype main effect;  $^{\wedge}P < 0.05$  for exercise main effect;  $^{\circ}P < 0.05$  for phenotype-exercise interaction by 2-way ANOVA.  $P$  values obtained by Tukey post hoc testing are displayed. AU, arbitrary units.

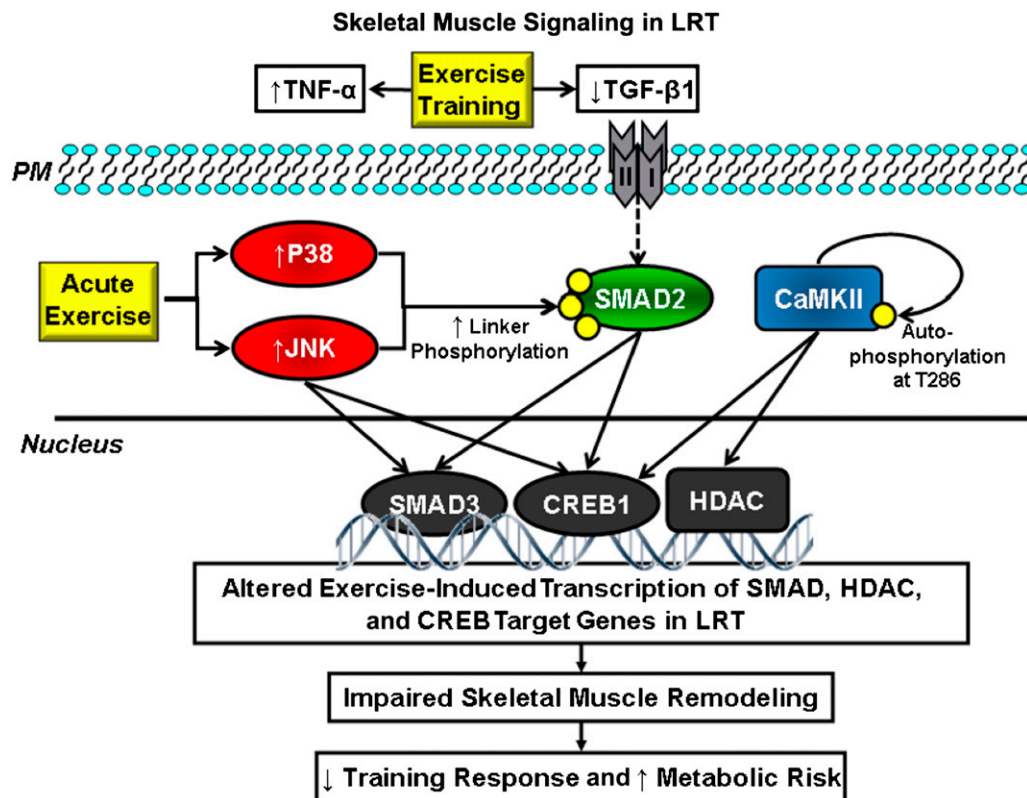
networks that regulate the molecular response to exercise. Our analysis revealed that gene networks involved in tissue remodeling are divergently regulated in LRT and HRT, a result that is consistent with analysis of gene networks regulated by exercise training in high-responding and low-responding humans (44,53). Specifically, we found that altered signaling via calcium, MAPK, and TGF- $\beta$  pathways led to markedly different exercise-induced transcriptional networks in LRT. The involvement of multiple interacting pathways leading to hundreds of differentially regulated genes in LRT highlights the complexity of the exercise training response. The ability to study complex networks that more closely resemble human disease represents an advantage of using selective breeding as a tool over more traditional animal models based on single-gene modifications. Furthermore, our initial investigation examining the response to moderate aerobic training sets the stage for future studies using selective breeding models to investigate adaptations resulting from different exercise training modes (i.e., resistance vs. endurance) or intensities, which also have been associated with improved metabolic health.

Akt and AMPK, which represent signaling networks that have been extensively studied in the exercise and metabolism fields, were normally activated by exercise in LRT, suggesting they are insufficient to induce training adaptations. Therefore, using an unbiased approach based on bioinformatics analysis of skeletal muscle training response in humans (44,53) and the LRT/HRT models, we tested the hypothesis that TGF- $\beta$  signaling regulates the training

response phenotype. The finding that exercise induces phosphorylation of SMAD2 in the linker region represents a novel exercise-regulated residue in skeletal muscle. Furthermore, exercise-induced SMAD2 linker phosphorylation was three-fold higher in LRT, suggesting altered TGF- $\beta$  signaling contributes to the exercise-resistant phenotype. Because of its novelty, the role of SMAD2 linker phosphorylation in skeletal muscle is not known. However, investigations using cancer cell models demonstrate that SMAD linker region phosphorylation antagonizes canonical TGF- $\beta$  signaling and may shift transcription of target genes toward those related to the extracellular matrix and tissue remodeling (49,50). At the whole-body level, LRT had two-fold lower levels of circulating TGF- $\beta$ 1 after exercise training compared with HRT, providing further evidence that altered TGF- $\beta$  signaling is a key feature of the LRT phenotype. TGF- $\beta$  is a potent stimulator of angiogenesis (54) and therefore represents a plausible mechanism for impaired skeletal muscle angiogenesis in LRT.

Dysregulated interactions between the MAPK and TGF- $\beta$  signaling pathways are thought to be responsible for many disease states, including fibrosis and metastatic carcinoma (49,50). We now identify hyperactivation of JNK and P38 MAPK in response to exercise as a likely mechanism for enhanced SMAD2 linker phosphorylation in LRT (Fig. 6). MAPK activation is considered to be a normal response to acute exercise (51). However, chronic hyperactivation of JNK and P38 MAPK independent of exercise are





**FIG. 6.** Muscle signaling in LRT. A proposed sequence of signaling and transcriptional regulatory events that occurred in LRT was generated based on bioinformatic analysis of exercise-stimulated transcription and Western blotting analysis of skeletal muscle samples. In response to acute exercise, LRT have hyperactivation of JNK and P38 MAPK, leading to elevated phosphorylation of SMAD2 in its linker region at Ser245/250/255. Increased exercise-induced SMAD2 linker region phosphorylation results in altered gene expression by its binding partners SMAD3 and CREB1. Constitutive activation of CaMKII by phosphorylation of its autoregulatory site Thr286 also may contribute to altered transcription in LRT via its regulatory effects on HDAC and CREB1. Altered signal transduction and gene transcription likely lead to impaired remodeling of skeletal muscle in LRT, which, in turn, may contribute to decreased exercise capacity and whole-body metabolic dysfunction. ERK, extracellular signal-regulated kinase; P38, p38 mitogen-activated protein kinase; PM, plasma membrane; SMAD, mothers against decapentaplegic homolog.

associated with obesity, inflammation, and insulin resistance (28,55), all of which are consistent with the LRT disease risk phenotype. TNF- $\alpha$  was two-fold higher in LRT after exercise training and is a potent mediator of inflammation and a known activator of MAPK in muscle (27). Therefore, it is feasible that an exercise-induced inflammatory response interferes with TGF- $\beta$  signaling and impairs muscle remodeling after exercise, contributing to the LRT phenotype. In line with this assertion, we found altered target gene expression of CREB1 and SMAD3 in response to acute exercise in LRT skeletal muscle, which are common mediators of the MAPK and TGF- $\beta$  pathways (56,57).

In summary, using artificial selective breeding as a tool, we have generated animal models that establish physiological, tissue-specific, and molecular links between resistance to aerobic exercise training and metabolic disease. At the whole-body level, selective breeding for LRT caused significant metabolic dysfunction, including increased adiposity, insulin resistance, and inflammation. In skeletal muscle, LRT displayed impaired exercise-induced angiogenesis but a normal increase in mitochondrial capacity, indicating that the “supply” side, rather than the “demand” side, of aerobic energy transfer is limiting to exercise capacity. At the molecular level, increased stress and mitogenic signaling in response to acute exercise resulted in altered exercise-induced gene transcription in LRT. Furthermore, we identified a potentially novel role for TGF- $\beta$ 1 in exercise training adaptations and discovered that

SMAD2 linker phosphorylation is regulated by exercise and associated with the exercise-resistant phenotype of LRT. Using this powerful biological model system, we have discovered signaling networks that can be investigated as therapeutic targets for enhancing the improvement of aerobic capacity with exercise and thus the attenuation of metabolic disease in humans.

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S.J.L. designed experiments, performed experiments, analyzed the data, and wrote and edited the manuscript. D.A.R. performed experiments and analyzed the data. A.B.A.-W., M.F.H., I.J.G., R.A., N.R.Q., and T.G. performed experiments. D.C.-T. performed experiments and analyzed the data. P.L.G., R.A.F., S.L.B., L.G.K., and L.J.G. designed experiments. J.A.T. analyzed the data. L.G.K. designed experiments, analyzed the data, and wrote and edited the manuscript. L.J.G. designed experiments and wrote and edited the manuscript. L.J.G. 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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