Altered expression and insulin-induced trafficking of Na\(^+\)-K\(^+\)-ATPase in rat skeletal muscle: effects of high-fat diet and exercise

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Galuska D, Kotova O, Barrès R, Chibalina D, Benziane B, Chibalin AV. Altered expression and insulin-induced trafficking of Na\(^+\)-K\(^+\)-ATPase in rat skeletal muscle: effects of high-fat diet and exercise. Am J Physiol Endocrinol Metab 297: E38–E49, 2009. First published April 14, 2009; doi:10.1152/ajpendo.90990.2008.—Skeletal muscle Na\(^+\)-K\(^+\)-ATPase plays a central role in the clearance of K\(^+\) from the extracellular fluid, therefore maintaining blood [K\(^+\)]. Na\(^+\)-K\(^+\)-ATPase activity in peripheral tissue is impaired in insulin resistant states. We determined effects of high-fat diet (HFD) and exercise training (ET) on skeletal muscle Na\(^+\)-K\(^+\)-ATPase subunit expression and insulin-stimulated translocation. Skeletal muscle expression of Na\(^+\)-K\(^+\)-ATPase isosforms and transcription factor DNA binding was determined before or after 5 days of swim training in Wistar rats fed chow or HFD for 4 or 12 wk. Skeletal muscle insulin resistance was observed after 12 wk of HFD. Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit protein expression was increased 1.6-fold \((P < 0.05)\), whereas \(\alpha_2\)- and \(\beta_1\)-subunits and protein expression were decreased twofold \((P < 0.01)\) in parallel with decrease in plasma membrane Na\(^+\)-K\(^+\)-ATPase activity after 4 wk of HFD. Exercise training restored \(\alpha_1\)-, \(\alpha_2\)-, and \(\beta_1\)-subunit expression and Na\(^+\)-K\(^+\)-ATPase activity to control levels and reduced \(\beta_2\)-subunit expression 2.2-fold \((P < 0.05)\). DNA binding activity of the \(\alpha_1\)-subunit-regulating transcription factor ZEB (AREB6) and \(\alpha_1\) mRNA expression were increased after HFD and restored by ET. DNA binding activity of Sp-1, a transcription factor involved in the regulation of \(\alpha_2\)- and \(\beta_1\)-subunit expression, was decreased after HFD. ET increased phosphorylation of the Na\(^+\)-K\(^+\)-ATPase regulatory protein phospholemman. Phospholemman mRNA and protein expression were increased after HFD and restored to control levels after ET. Insulin-stimulated translocation of the \(\alpha_2\)-subunit to plasma membrane was impaired by HFD, whereas \(\alpha_1\)-subunit translocation remained unchanged. Alterations in sodium pump function precede the development of skeletal muscle insulin resistance. Disturbances in skeletal muscle Na\(^+\)-K\(^+\)-ATPase regulation, particularly the \(\alpha_2\)-subunit, may contribute to impaired ion homeostasis in insulin-resistant states such as obesity and type 2 diabetes.

Na\(^+\)-K\(^+\)-ATPase is an integral membrane protein critically involved in maintenance of intracellular sodium and potassium concentrations, cell volume, membrane potential, and electrochemical gradients (5, 34). In excitable tissues, including skeletal muscle, Na\(^+\)-K\(^+\)-ATPase promotes energization of the resting potential and reuptake of extracellular potassium. Na\(^+\)-K\(^+\)-ATPase is a heteromeric enzyme composed of two polypeptide subunits: a 112-kDa \(\alpha\)-subunit and a 35- to 60-kDa glycosylated \(\beta\)-subunit (5, 34). Multiple isoforms of \(\alpha\)- and \(\beta\)-subunits are expressed by and each is encoded by a different gene (34). Adult skeletal muscle expresses two \(\alpha\)-subunits (\(\alpha_1\) and \(\alpha_2\)) and two \(\beta\)-subunits (\(\beta_1\) and \(\beta_2\)), and the relative abundance of each isoform and Na\(^+\)-K\(^+\)-ATPase activity is regulated in a fiber type-specific manner (26, 48, 50, 51). Following insulin exposure or contraction, Na\(^+\)-K\(^+\)-ATPase unit abundance in the plasmalemma and T-tubules of skeletal muscle is increased (4). The phospholemman (PLM, FXYD1) forms a complex with \(\alpha\beta\) dimers in heart and skeletal muscle. Interaction of PLM with sodium pump units modulates activity of the Na\(^+\)-K\(^+\)-ATPase (7). Skeletal muscle contains one of the largest pools of Na\(^+\)-K\(^+\)-ATPase and therefore plays a central role in the extrarenal clearance of K\(^+\) from the blood during ingestion or infusion of K\(^+\) (11, 35). With major hyperkalemia, skeletal muscle can rapidly accumulate significant amounts of plasma K\(^+\) (35). Hyperkalemia and impaired K\(^+\) tolerance frequently occurs in people with diabetes mellitus (39). Moreover, the Na\(^+\)/K\(^+\) ratio is increased in skeletal muscle from insulin-resistant patients (31), suggesting reduced Na\(^+\)/K\(^+\) pumping capability. In streptozocin-induced diabetic rats, the decreased Na\(^+\)-K\(^+\)-ATPase content in skeletal muscle, heart ventricular muscle, and peripheral nerves (29, 44) can be prevented by insulin treatment (29). Furthermore, in type 2 diabetic patients, Na\(^+\)-K\(^+\)-ATPase expression in skeletal muscle (15) and erythrocytes (36) is decreased, and restored by insulin treatment (44).

Na\(^+\)-K\(^+\)-ATPase protein expression is differently regulated by exercise training, starvation, hypokalemia and type 1 and type 2 diabetes mellitus (15, 44, 50, 51). Physical activity and exercise training reduces the risk of developing type 2 diabetes and obesity (52, 60), in part by increased skeletal muscle protein expression of key genes important for glucose uptake and utilization (9, 43). Endurance exercise training also increases Na\(^+\)-K\(^+\)-ATPase content, as measured by \[^{3}H\]ouabain binding in skeletal muscle from either humans or rodents (for review see Ref. 11). Long-term exercise training in diabetic rodents (10 wk treadmill training) restores the decreased skeletal muscle \[^{3}H\]ouabain binding caused by untreated diabetes induced by partial pancreatectomy (44). Acute exercise increases mRNA expression of Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)- and \(\beta_2\)-subunit isoforms without changing protein expression (53). Despite these correlative observations, the molecular mechanism regulating the exercise-mediated effect on the genes encoding Na\(^+\)-K\(^+\)-ATPase is incompletely understood. Several transcription factor consensus sequences have been identified in the promoters of genes encoding Na\(^+\)-K\(^+\)-ATPase subunits; however, the role in skeletal muscle Na\(^+\)-K\(^+\)-ATPase regulation is unknown.
We hypothesize that Na\(^{+}\)-K\(^{-}\)-ATPase expression and function may be altered by lifestyle factors such as diet and training. In the present study, we determined the effects of high-fat diet and short-term moderate exercise training on skeletal muscle Na\(^{+}\)-K\(^{-}\)-ATPase expression and activity and plasma membrane abundance. Furthermore, we assessed the effect of high-fat diet and exercise training on the DNA binding activity of transcription factors involved in skeletal muscle remodeling and, potentially, the regulation of Na\(^{+}\)-K\(^{-}\)-ATPase subunit isoform gene expression.

**MATERIALS AND METHODS**

**Materials.** Specific monoclonal antibodies against α\(_2\)-subunit of Na\(^{+}\)-K\(^{-}\)-ATPase (clone 6H) were generously provided by Dr. M. Caplan (Yale University, New Haven, CT) and monoclonal antibodies against α\(_2\)-subunit (clone McB2) were kindly provided by Dr. K. Sweadner (Massachusetts Central Hospital, Boston, MA). Polyclonal antibodies against β\(_i\) and β\(_o\)-subunits of Na\(^{+}\)-K\(^{-}\)-ATPase were kindly provided by Dr. P. Martin-Vasallo (University of La Laguna, Tenerife, Spain). Polyclonal antibodies against phospholemman phosphorylated at Ser68 (47) were kindly provided by Dr. J Cheung (Thomas Jefferson University, Philadelphia, PA). Polyclonal antibodies used for detection of hexokinase II were a generous gift from Dr. O. Pedersen (Steno Memorial Hospital, Gentofte, Denmark). The GLUT4 polyclonal antibody was from Biogenes (Poole, UK). UCP3 polyclonal antibody was from Millipore (Brdford, MA). Mouse monoclonal antibodies against succinate-ubiquinol oxidoreductase 70-kDa subunit (SUO), and cytochrome oxidase I (COX I) were obtained from Molecular Probes, (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit and antimouse IgG were from Bio-Rad (Hercules, CA). Reagents for enhanced chemiluminescence (ECL) were from Amersham Biotech (Arlington Heights, IL). Antibodies to COX I, COX II, cytochrome c oxidase, and cytochrome c were from Novus (Littleton, CO). Monoclonal antibodies to phospholipidosis and to phospholemman were kindly provided by Dr. J. Cheung (Thomas Jefferson University, Philadelphia, PA).

**Animals, diets, and exercise protocol.** Female Wistar rats (120–130 g) were purchased from B & K Universal (Sollentuna, Sweden) and housed in the animal facility at the Department of Physiology and Pharmacology, Karolinska Institutet, for 5–12 wk prior to use. Rats were kept on a 12:12-h light-dark cycle. Animals received either standard rodent chow (Lactamin R34; AnalyCen, Lindköping, Sweden) or a high-fat diet (55% of calories from fat, TD93075; Harlan Teklad, Madison, WI) and had free access to water. After 3 or 11 wk, chow-fed and fast-fed rats were randomly assigned to two subgroups: exercise trained or sedentary control. Exercise-trained rats were acclimated to swimming for 10 min per day for 2 days and thereafter subjected to two 3-h swimming exercise bouts, separated by one 45-min rest period per day for 5 days (9). Rats swam in groups of six, in plastic barrels measuring 45 cm in diameter, filled to a depth of ~40 cm. Water temperature was maintained at 34–35°C. After the last exercise bout, animals were fed ad libitum. Approximately 16 h after the last exercise bout, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Fast-twitch glycolytic muscles were dissected. Extensor digitorum longus (EDL) muscle was used for glucose transport measurements. Epitrophiear muscle muscles were used for determination of Na\(^{+}\)-K\(^{-}\)-ATPase cell surface abundance. White gastrocnemius muscles were removed and immediately frozen for further analysis as described below. Omental fat pads were removed and weighed. The ethics committee for North Region of Stockholm approved the study protocol.

**Blood chemistry analysis.** Plasma insulin level was measured by enzyme immunoassay using commercial kit (Mordcia, Uppsala, Sweden). Nonesterified fatty acids in serum were quantitated by enzymatic colorimetric method (NEFA C test kit; Wako Chemicals). Plasma [K\(^{+}\)] measurements were performed with an FLM flame photometer (Radiometer, Brønshøj, Denmark).

**Muscle incubations and glucose transport.** Medium was prepared from a preaggregated (95% O\(_{2}\)-5% CO\(_{2}\)) Krebs-Henseleit buffer (KHB) containing 5 mM HEPES and 0.1% BSA (RIA grade). EDL muscles were preincubated in a shaking water bath (30°C) for 20 min in 2 ml of KHB containing 5 mmol/l glucose and 15 mmol/l mannitol without (basal) or with insulin (100 μU/ml and 20,000 μU/ml). After preincubation, muscles were incubated at 30°C for 10 min in glucose-free KHB containing 20 mmol/l mannitol with and without insulin. Thereafter, muscles were transferred to KHB containing 8 mM 3-O-methyl-[\(^{3}H\)]glucose (438 Ci/mmol) and 12 mM [\(^{14}C\)]mannitol (42 μCi/ml) and incubated (10 min) with or without insulin. Glucose transport activity was assessed as described (56) and expressed as micromoles per milliliter of intracellular water per hour.

**Citrine synthesis activity was measured spectrophotometrically in muscle homogenates, as described (3).** Muscle samples were homogenized in KCL-EDTA buffer, pH 7.4, 1.50 wt/vol ratio and thereafter analyzed. Homogenates were diluted in assay buffer [final concentration 50 mM Tris·HCl, pH 7.5, 0.2 mM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.1 mM acetyl-CoA, 0.05% Triton X]. Oxaloacetate was used as substrate.

**[\(^{3}H\)]Ouabain binding.** Measurements of gastrocnemius muscle [\(^{3}H\)]Ouabain binding site concentration were performed as described (29, 44). Samples (3–5 mg wet wt) were cut from white gastrocnemius muscle and washed twice for 10 min at 4°C in a buffer containing 10 mM Tris·HCl, 250 mM sucrose, 3 mM MgSO\(_{4}\), and 1 mM sodium vanadate, pH 7.4. Subsequently, specimens were equilibrated twice at 37°C for 60 min in the same buffer containing [\(^{3}H\)]ouabain (2 μCi/ml) and unlabeled ouabain to a final concentration of 1 μM. After incubations, a washout in ice-cold unlabeled buffer for 2 h with a change of medium every 30 min was performed to eliminate nonspecific [\(^{3}H\)]Ouabain binding. Samples were then blotted on dry filter paper, weighed, and soaked with 0.5 ml of 5% trichloracetic acid overnight in minivials. The next day, 3 ml of scintillation fluid was added, and samples were counted in a tritium channel. The amount of [\(^{3}H\)]Ouabain taken up and retained by samples was calculated on the basis of sample wet weight and the specific radioactivity of incubation medium and samples and was expressed as picomoles per gram wet weight. [\(^{3}H\)]Ouabain binding site concentration was measured in triplicates for each muscle.

**Membrane fractionation.** Crude membrane fractions were isolated by differential centrifugation. Approximately 350 mg of white gastrocnemius muscle was weighed, minced, and initially homogenized with a Polytron at a low speed (setting 4, 2 × 10 s) in homogenization buffer (20 mM Tris·HCl, 0.25 M sucrose, 1 mM EDTA, 1 μM okadaic acid, 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). The resulting homogenate was centrifuged for 10 min at 3,000 g. The supernatant was collected and kept on ice. The pellet was resuspended in 100 μl of homogenization buffer and centrifuged again for 10 min at 3,000 g. Only negligible amounts of Na\(^{+}\)-K\(^{-}\)-ATPase subunits (<1% of initial homogenate) were detected in the final 3,000-g pellet by Western blot. The separation was equally efficient for all muscles regardless of the treatment group, indicating that fractionation of the muscles was unaffected by the different dietary or exercise treatments. To obtain the crude membrane fraction, the pooled 3,000-g supernatants were collected and then pelleted for 1 h at 150,000 g. Crude membrane pellets were resuspended in 500 μl of homogenization buffer and stored at –70°C. Protein concentration was determined with a BCA protein assay (Pierce, Rockford, IL). To facilitate detection of the sodium pump β\(_{1}\) and β\(_{2}\)-subunits, sugar residues were removed from crude membrane fraction with peptide-N-glycosidase F, as described (50). Na\(^{+}\)-K\(^{-}\)-ATPase subunits were undetected in the 150,000-g supernatant.

**Plasma membrane (PM) fractions were isolated from crude membrane fractions by differential centrifugation and discontinuous sucrose gradients (2, 8).** After centrifugation of the discontinuous sucrose gradients, the fraction on top of the 25% layer that corresponds to a membrane fraction enriched with PM was collected (23).
Protein concentration was determined using the BCA assay (Pierce). Protein recovery yield (calculated as %protein content in crude membranes in 5 membrane preparations) was 4.4 ± 0.3% for PM.

**Determination of Na^+^-K^-ATPase activity.** Na^+^-K^-ATPase activity in membrane fractions was measured at maximum velocity ($V_{max}$) as described previously (1). Aliquots of the isolated membrane fractions (protein content 3 μg for crude membrane and 1 μg for PM, respectively) were transferred to the Na^+^-K^-ATPase assay medium (final volume 100 μl), containing 50 mM NaCl, 5 mM KCl, 10 mM MgCl$_2$, 1 mM EGTA, 50 mM Tris-HCl, 10 mM Na$_2$ATP, and [γ-32P]ATP (NEN Life Science Products; specific activity, 3,000 Ci/mmol) in tracer amounts (3.3 μCi/μl) at 4°C. The samples were then incubated at 37°C for 15 min. The reaction was terminated by rapid cooling to 4°C and addition of a mixture of trichloroacetic acid-charcoal (5%-10%). After separating the charcoal phase (12,000 g for 5 min) containing the nonhydrolyzed nucleotide, the 32P liberated in the supernatant was counted. Na^-K^-ATPase activity was calculated as the difference between test samples (total ATPase activity) and samples assayed in a medium devoid of Na^+^ and K^+^ and in the presence of 2 mM ouabain (ouabain-insensitive ATPase activity).

**Real-time PCR.** Quantification of the mRNA expression of Na^-K^-ATPase subunits from rat white gastrocnemius muscle was performed using quantitative real-time PCR with the ABI PRISM 7000 Sequence Detector System and fluorescence-based SYBR Green technology (Applied Biosystems, Warrington, UK). Total RNA was prepared from the tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Synthesis of cDNA was performed with random hexamer primers using SuperScript First Strand Synthesis System (Invitrogen). Real-time PCR reactions contained 400 ng of each PCR primer and 1X SYBR Green PCR Master Mix (Applied Biosystems). The forward (F) and reverse (R) primer sequences were as follow: for Atpl1a (GenBank NM012504.1) GGCTGTCATCCTCTCATTG (F) and CGGTTG-GGCCAGAACC (R); Atpl1a2 (GenBank NM012505.1) GGTGGCC-CCTCGGAATGTGAC (F) and ATGAGATGAGGAGACTGTTAGG-GAAA (R); Atplb1 (GenBank NM013113.1) GCCCGAGAAGGC-GACATGAT (F) and CCCGCTCCTGGTGTTCA (R); Atplb2 (GenBank NM012502.2) GGTTGTTG-GAGATTTGGAAGGA (F) and GTTGGGCCCATGGAACCTG (R); phospholemman (Fsydl1; GenBank NM013648.1) CATGGCCATGCGACAGCT (F) and CGCAGGGTGTTGTAACGT (R). All samples were run in duplicate, and the relative quantities of different mRNA transcripts were calculated after normalization of the data against actin-β, as endogenous controls, using relative quantification method.

**Cell surface biotinylation.** Epitrochlearis muscles were incubated (30 min) in a shaking water bath (30°C) in 2 ml of KHB supplemented with 5 mM glucose and 15 mM mannitol with or without 120 nM insulin. After incubation, muscles were washed three times with ice-cold PBS and incubated with biotinylated reagent as described previously (2). Epitrochlearis muscles were exposed to EZ-link Sulfo-NHS-SS-biotin at a final concentration 1.5 mg/ml in PBS at 4°C for 60 min with gentle shaking. Thereafter, medium was aspirated, and unreacted Sulfo-NHS-SS-biotin was quenched with PBS containing 100 mM of glycine. Isolated skeletal muscle was washed three times with ice-cold PBS, and excess PBS was aspirated. Before solubilization, epitrochlearis muscles were frozen and pulverized in liquid nitrogen. Samples were solubilized in 1 ml of ice-cold lysis buffer containing 20 mM Tris, pH 8.0, 135 mM NaCl, 1 mM MgCl$_2$, 2.7 mM KCl, 10 mM Na$_2$PO$_4$, 10 mM NaF, 1 mM Na$_2$V$_3$O$_5$, 1 μM okadaic acid, 1% Triton X-100, 10% vol/vol glycerol, 0.2 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. Western blot analysis. Aliquots of muscle lysate (50 μg of protein) and crude membrane fractions (40 μg of protein) were resuspended in Laemmli sample buffer. Proteins were separated by SDS-PAGE, and Western blot analysis. Oligonucleotides for the consensus binding sites for myocyte enhancer factor 2 (MEF2), zinc finger/homeodomain protein (ZEB), Sp-1, AP-1, and NF-1 transcription factors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides were end-labeled with T4 polynucleotide kinase. Labeled oligonucleotide probes (0.5 ng) were incubated with 2 μg of nuclear extract in a 20-μl reaction containing 1 μg poly(dI-dC), 40 mM KCl, 5 mM MgCl$_2$, 15 mM Hepes, pH 7.9, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol for 20 min at room temperature. For competition studies, extracts were preincubated with a 40-fold molar excess of unlabeled oligonucleotide for 10 min before addition of the radiolabeled probe. Samples were analyzed on a PhosphoImager. Statistics. Data are presented as means ± SE. Student’s t-test was used to assess differences between two treatments within a group. All other differences were determined by repeated-measures ANOVA. The Fischer’s LSD post hoc analysis was used to identify specific differences occurred between results, with significance taken when $P < 0.05$.

**RESULTS**

**Animal characteristics and validation of the efficiency of the diet intervention and exercise training protocol.** The 5-day swimming exercise protocol used in the present study increases GLUT4 expression and leads to a coordinated change in the

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expression of proteins involved in insulin signaling in rat epitrochlearis muscle (9). To fulfill the aim of the study, in addition to the epitrochlearis muscle we also utilized the EDL and the white gastrocnemius (WG) muscle, because they have similar fast-twitch glycolytic phenotype and fiber type characteristics (14, 22).

Regardless of diet, exercise training increased protein expression of GLUT4 and HKII 2-fold in white gastrocnemius muscle ($P < 0.01$, Fig. 1, A and B), a tissue utilized for the most of assays performed in the study. Expression of these proteins was unaltered by high-fat diet in sedentary rats. Thus, high-fat diet alone does not affect either the expression or the exercise-mediated upregulation of these proteins, which are believed to be important for glucose transport and the initial steps of glucose metabolism in skeletal muscle.

To further characterize the effects of diet and exercise training intervention, we assessed the expression of mitochondria markers UCP3, succinate ubiquinol oxidoreductase (SUO), and COX I and measured citrate synthase activity in white gastrocnemius muscle. High-fat diet or exercise training alone increased the expression of UCP3 twofold ($P < 0.01$); however, UCP3 expression was further increased 1.5-fold after exercise training ($P < 0.05$; Fig. 1C). Importantly, high-fat diet alone tends to increase the mitochondria oxidative potential markers in skeletal muscle (Fig. 1, C–F), as previously reported (18). However, significant increases in the expression of the mitochondria markers SUO and COX I, as well as citrate synthase activity, was noted only in response to exercise training regardless of high-fat diet (Fig. 1, D–F).

Fasting plasma insulin, FFA, and muscle triglycerides for these animals have been previously reported in (27). Body weight was unaltered by either exercise training or high-fat feeding. However, omental fat pad weight was increased threefold after 4 wk of high-fat feeding ($P < 0.01$). Exercise training reduced omental fat pad weight in high-fat-fed rats to levels observed in chow-fed sedentary rats (27). Glucose tolerance was impaired in rats fed a high-fat diet for 3 wk (Fig. 2A). After 11 wk of high-fat diet, the glucose intolerance was more pronounced, with significantly elevated fasting blood glucose levels compared with chow-fed rats (Fig. 2B). Plasma $[K^+]$ was slightly elevated after high-fat diet [4.0 ± 0.1 meq/l for chow-fed sedentary rats vs. 4.4 ± 0.1 meq/l for high-fat-fed rats ($n = 6–8$, $P < 0.05$) after 3 wk on the high-fat diet and 4.1 ± 0.1 meq/l for chow-fed sedentary rats vs. 4.5 ± 0.2 meq/l for high-fat-fed rats after 11 wk on high-fat diet ($n = 6–7$, $P = 0.08$).

**Insulin-stimulated glucose transport in isolated EDL muscle.** Glucose transport was assessed in the presence of submaximal or maximal concentrations of insulin (100 and 20,000 μU/ml, respectively) in isolated EDL muscle (Fig. 2, C and D). Rats were subjected to chow or high-fat diet for 4 (Fig. 2C) or 12 wk (Fig. 2D). After 4 wk of high-fat diet, insulin-stimulated glucose transport in EDL muscle was unaltered between chow- and fat-fed sedentary rats. Exercise training tended to increase insulin-stimulated glucose transport activity in chow- and fat-fed rats, but these differences were not significant (Fig. 2C). After 12 wk of high-fat diet, insulin responsiveness was impaired, as evidenced by a profound (32%) decrease in glucose uptake in isolated EDL muscle (Fig. 2D). Exercise training restored insulin action on glucose transport fat-fed rats (Fig. 2D).

![Fig. 1. Effect of high-fat diet and/or exercise training on glucose metabolism and mitochondria marker expression and citrate synthase activity in rat gastrocnemius muscle. A–E: representative immunoblot of GLUT4 (A), hexokinase II (HKII; B), UCP3 (C), succinate-ubiquinol oxidoreductase 70-kDa subunit (SUO; D), and cytochrome oxidase I (COX I; E) protein expression. Bottom: data quantification by densitometry. F: citrate synthase activity in muscle homogenates from gastrocnemius muscle. Values are reported as means ± SE for 6–8 rats. *$P < 0.05$ vs. chow-fed sedentary (CS) rats.](http://ajpendo.physiology.org/
Effect of diet and exercise training on [3H]ouabain binding and Na\(^{+}\)K\(^{+}\)-ATPase activity in skeletal muscle. Since the rodent \(\alpha_{1}\)-subunit isoform has a very low affinity to ouabain, [3H]ouabain binding largely reflects the \(\alpha_{2}\)-subunit content (11). Specific binding of [3H]ouabain was significantly reduced in skeletal muscle from sedentary rats after 4 wk of high-fat diet (Fig. 3A). Exercise training alone did not affect [3H]ouabain binding, although in fat-fed exercise-trained rats binding was restored to levels observed in chow-fed sedentary rats. Due to low ouabain-sensitive ATPase activity in homogenates of gastrocnemius muscle, Na\(^{+}\)K\(^{+}\)-ATPase activity was measured under maximum velocity (\(V_{\text{max}}\)) conditions in crude membrane fractions prepared from white gastrocnemius muscle, as described in MATERIALS AND METHODS. Na\(^{+}\)-K\(^{+}\)-ATPase activity was slightly altered by high-fat diet or exercise (Fig. 3B), with a trend toward decreased Na\(^{+}\)-K\(^{+}\)-ATPase activity (16%, \(P = 0.08\)) noted in fat-fed sedentary rats. Furthermore, in chow-fed rats, exercise training led to a slight increase in Na\(^{+}\)-K\(^{+}\)-ATPase activity (14%, \(P = 0.07\)). In the further-purified plasma membrane fraction, Na\(^{+}\)-K\(^{+}\)-ATPase activity was significantly reduced (41%, \(P < 0.05\)) in the high-fat-fed sedentary rats (Fig. 3C), in parallel with results of ouabain binding (Fig. 3A). Exercise training resulted in a significant increase in the plasma membrane fraction Na\(^{+}\)-K\(^{+}\)-ATPase activity regardless of dietary treatment (Fig. 3C). Similar effects of diet and exercise training on [3H]ouabain binding and Na\(^{+}\)-K\(^{+}\)-ATPase activity in skeletal muscle were observed after 12 wk of high-fat feeding (data not shown).

Skeletal muscle expression of Na\(^{+}\)-K\(^{+}\)-ATPase subunits.

High-fat diet for 4 wk increased skeletal muscle mRNA content of the \(\alpha_{2}\)-subunit 49% compared with chow-fed rats (Fig. 4A), and this effect was normalized by exercise training. In contrast, neither high-fat diet nor exercise altered mRNA content of the \(\alpha_{1}\)-subunit (Fig. 4B). Na\(^{+}\)-K\(^{+}\)-ATPase \(\beta_{1}\)-subunit mRNA content was unaltered between skeletal muscle from sedentary fat-fed and chow-fed rats (Fig. 4C) but increased in chow-fed rats in response to exercise training (Fig. 4C). \(\beta_{2}\)-Subunit mRNA content was unaltered by high-fat diet but decreased in response to exercise training (Fig. 4D). mRNA expression of PLM (FXYD1), a Na\(^{+}\)-K\(^{+}\)-ATPase-interacting regulatory protein, increased in response to high-fat diet (Fig. 4E), in parallel with changes in expression of the \(\alpha_{1}\)-subunit. Exercise training restored mRNA expression of the \(\alpha_{1}\)-subunit and PLM to levels observed in chow-fed sedentary rats (Fig. 4, A and E).

Immunoblot analysis was used to determine whether high-fat diet or exercise training alters Na\(^{+}\) pump expression in crude membranes prepared from gastrocnemius muscle. Protein expression of the \(\alpha_{1}\)-subunit in the crude membrane fractions was increased in fat-fed vs. chow-fed sedentary rats (Fig. 5A), similarly to the results for \(\alpha_{1}\) mRNA expression (Fig. 4A). High-fat diet decreased \(\alpha_{2}\)-subunit protein expression 50% (\(P < 0.01\)) compared with chow-fed sedentary rats (Fig. 5B), contrary to the \(\alpha_{2}\)-subunit mRNA expression (Fig. 4B). The magnitude of decrease in \(\alpha_{2}\)-subunit protein expression as determined by immunoblot analysis corresponded with the reduction in [3H]ouabain binding (Fig. 3A). In fat-fed rats, exercise training completely restored \(\alpha\)-subunit expression to levels observed in chow-fed sedentary rats (Fig. 5, A and B). Exercise training was without effect on Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit protein expression.

Na\(^{+}\)-K\(^{+}\)-ATPase \(\beta_{1}\)-subunit protein level was decreased (52%, \(P < 0.05\)) in skeletal muscle crude membrane fractions from sedentary fat-fed rats vs. all other groups (Fig. 5C). Exercise training restored protein expression of the Na\(^{+}\)-K\(^{+}\)-ATPase \(\beta_{1}\)-subunit.

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ATPase β1-subunit in fat-fed rats. Na⁺-K⁺-ATPase β2-subunit protein level was unaltered by high-fat diet but was decreased 49% in response to exercise training in skeletal muscle crude membranes from either chow- or high-fat-fed rats (Fig. 5D), similarly to β2 mRNA expression. The pattern of skeletal muscle expression of Na⁺-K⁺-ATPase isoforms was unchanged between 4 and 12 wk of high-fat diet (Fig. 5, E–H).

High-fat diet increased PLM protein content in skeletal muscle (Fig. 6A). Exercise training restored the PLM protein content to sedentary chow-fed levels (Fig. 6A). PLM phosphorylation at Ser68 was unaltered by high-fat diet despite the increase in protein expression, suggesting that relative PLM phosphorylation at Ser68 per protein was decreased (Fig. 6, A and B). Exercise training increased PLM phosphorylation at Ser68 102% in either chow- or high-fat-fed rats (Fig. 6B).

**Na⁺-K⁺-ATPase cell surface abundance.** We utilized a cell surface biotinilation technique to evaluate the effect of high-fat diet or exercise on plasma membrane Na⁺-K⁺-ATPase abundance in epitrochlearis muscle under basal or insulin-stimulated conditions. Insulin markedly increased cell surface abundance of α1- and α2-subunits in isolated rat epitrochlearis muscle (Fig. 7, A and B). The α1-subunit abundance was similar among all treatment groups (Fig. 6A), contrary to total α2-subunit expression profile (Fig. 3A). Although the measurement of the Na⁺-K⁺-ATPase cell surface abundance may not reflect the activity of functionally operating sodium pumps, the α2-subunit cell surface abundance was decreased in skeletal muscle from high-fat-fed vs. chow-fed sedentary rats (Fig. 6B), in parallel with the profile for total α2-subunit protein expression and the plasma membrane Na⁺-K⁺-ATPase activity. Exercise training completely restored α2-subunit cell surface abundance to levels observed in chow-fed sedentary rats (Fig. 7B). Exercise training had no effect on Na⁺-K⁺-ATPase cell surface abundance.

**Effect of diet and exercise training on MEF2, ZEB, and Sp-1 DNA-binding activity.** To evaluate a possible effect of high-fat diet or exercise training on transcription factor activity, the functional binding properties of AP-1, ZEB (AREB6), MEF2, NF-1, and Sp-1 transcription factors were determined in nuclear extracts from gastrocnemius muscle. The MEF2-binding element is required for tissue-specific metabolic regulation of glucose transporter GLUT4 (49). In nuclear extracts isolated from gastrocnemius muscle from exercise-trained rats, MEF2 DNA binding (Fig. 8) was increased 3.5-fold (P < 0.01) regardless of diet. MEF2 DNA binding was unaffected by high-fat diet (Fig. 8). ZEB (AREB6) is a transcription factor essential for differentiation of myoblasts into myotubules (41). ZEB forms a heterodimer with MEF2 (40) and has a consensus sequence located in the promoter of the rat α1-subunit of the Na⁺-K⁺-ATPase gene (57). High-fat diet was associated with a twofold increase (P < 0.05) in DNA binding to the ZEB recognition sequence in nuclear extracts from gastrocnemius muscle (Fig. 8). Exercise training had no effect on ZEB DNA binding. The transcription factor Sp-1 serves as a ubiquitous housekeeping transcription factor, and Sp-1-binding elements are present in several copies in the promoters of the genes encoding the α1-, α2-, and β1-subunits of Na⁺-K⁺-ATPase (45, 46, 57). High-fat diet was associated with a two- to fivefold reduction in Sp-1 DNA binding in nuclear extracts from gastrocnemius muscle of sedentary rats (P < 0.05). Exercise training restored Sp-1 DNA-binding levels to that of the chow-fed sedentary state. DNA-binding activity of AP-1 and NF-1, two other ubiquitous housekeeping transcription factors with consensus binding sites in α-subunits promoters (45, 46, 57), was determined. AP-1 and NF-1 DNA binding was unaltered by either diet or exercise (data not shown).

**DISCUSSION**

High-fat diet causes mild insulin resistance in rodents (17, 20, 59). Here, we demonstrate that 4 wk of high-fat diet in Wistar rats leads to coordinated alterations in skeletal muscle expression of Na⁺-K⁺-ATPase subunits. Importantly, in glucose-intolerant rats, these changes precede the development of
skeletal muscle insulin resistance. In response to high-fat diet, skeletal muscle protein expression of the Na\(^{+}\)/K\(^{+}\)-ATPase \(\alpha_1\)-subunit is increased, and expression of the \(\alpha_2\)- and \(\beta_1\)-subunits is decreased. Interestingly, these coordinated changes in mRNA and protein expression were observed only for the \(\alpha_1\) and \(\beta_2\)-subunits and PLM, the Na\(^{+}\)/K\(^{+}\)-ATPase-interacting regulatory protein. Increase in total \(\alpha_1\)-subunit protein expression was not reflected in the \(\alpha_1\)-subunit cell surface abundance and plasma membrane Na\(^{+}\)/K\(^{+}\)-ATPase activity, suggesting that an excess of \(\alpha_1\)-subunits could be sequestered in intracellular compartments where it is not involved in active ion transport. High-fat diet decreased ouabain binding and Na\(^{+}\)/K\(^{+}\)-ATPase activity in skeletal muscle. Notably, the \(\alpha_2\)-subunit protein expression, the cell surface abundance, and the insulin-stimulated translocation to the plasma membrane were reduced by high-fat feeding, concomitant with a decrease in plasma membrane Na\(^{+}\)/K\(^{+}\)-ATPase activity. Our data provide evidence to suggest that the \(\alpha_2\)-subunit of Na\(^{+}\)/K\(^{+}\)-ATPase plays a specific role in skeletal muscle and the adaptive response to metabolic challenges.

Previous reports indicate that K\(^{+}\) depletion is associated with reduced Na\(^{+}\)/K\(^{+}\)-ATPase \(\alpha_2\)-subunit expression in skeletal muscle (51), furthermore that high-fat feeding is sometimes associated with decreased K\(^{+}\) intake due to a low K\(^{+}\) content in some commercial high-fat diets (10), leading to decreased insulin-stimulated K\(^{+}\) flux into skeletal muscle. Therefore, there is the possibility that some of the changes in Na\(^{+}\)/K\(^{+}\)-ATPase subunit expression in high-fat-fed rats in the present study may have resulted from reduced K\(^{+}\) intake. However, in contrast to Choi et al. (10), the high-fat diet and

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**Fig. 4.** mRNA content of Na\(^{+}\)/K\(^{+}\)-ATPase of \(\alpha_1\)- (A), \(\alpha_2\)- (B), \(\beta_1\)- (C), and \(\beta_2\)- (D) subunits and phospholemman (PLM; E) in gastrocnemius muscle from CS, FS, CT, and FT rats after 4 wk of HFD. Values are reported as means ± SE for 6–7 rats. *P < 0.05 vs. CS rats.
the chow diet used in the current study had similar K\(^{+}\) content. Furthermore, in the current study, skeletal muscle \(\alpha_1\)-subunit expression was increased and \(\beta_2\)-subunit expression was unaltered in fat-fed rats, which is contrary to effects noted with K\(^{+}\) depletion (51). Moreover, plasma FFA levels were elevated after 4 wk of high-fat diet (27), in contrast to Choi et al. Thus, the observed effects of high-fat diet noted in the present study are likely to be directly attributable to the increased fat content and not due to K\(^{+}\) depletion.

In the present study, the 4-wk high-fat diet increased omental fat pad mass, plasma FFA concentration, and skeletal muscle triglyceride content (27). Previous studies have shown that an accumulation of central-visceral fat strongly correlates with features of the metabolic syndrome and development of insulin resistance (28) in rodents and humans. Increased skeletal muscle triglyceride content also associates with the metabolic syndrome. Thus, the high-fat-feeding protocol utilized in the present study mimics the style-induced presymptomatic stage of metabolic syndrome. In rats maintained on a high-fat diet for 12 wk, a profound decrease in insulin-stimulated glucose uptake in isolated EDL muscle was noted. The pattern of skeletal muscle expression of Na\(^{+}\)-K\(^{+}\)-ATPase isoforms was not further altered between 4 and 12 wk of high-fat diet. Thus, alterations in the expression of Na\(^{+}\)-K\(^{+}\)-ATPase iso-
forms in skeletal muscle precede the development of insulin resistance on glucose transport. Interestingly, an increase in α1-subunit and a decrease in α2-subunit expression have been detected in skeletal muscles of aged rats (48), concomitant with an increase in interstitial K+ concentrations after contractions (33). Aging is associated with an impaired glucose tolerance, insulin resistance, and diabetes. Whether changes in Na\(^+-\)K\(^+\)-ATPase subunit expression directly contributes to the development of skeletal muscle insulin resistance for glucose transport remains to be established.

The functional relevance and physiological implications of multiple Na pump isoforms expressed in skeletal muscle are unclear. Different functional roles for α1- and α2-isoforms may influence skeletal muscle contractility. Consistent with this notion, force production in EDL muscle is less in α1\(^{-/-}\) mice and greater in α2\(^{-/-}\) mice compared with wild-type mice (21). The α2-subunit corresponds to 70–80% of total skeletal muscle Na\(^+-\)K\(^+\)-ATPase isoform expression (11, 19). Here, we show that high-fat diet is associated with a 50% reduction in α2-subunit expression in gastrocnemius muscle, with a parallel increase in α1-subunit expression. This finding is consistent with a similar compensatory upregulation of α1-subunit expression in the α2\(^{-/-}\) mouse (21). However, it is unlikely that increase in α1-subunit expression provides a compensatory mechanism under basal or insulin-stimulated conditions, since Na\(^+-\)K\(^+\)-ATPase activity was decreased in plasma membrane from high-fat-fed rats, concomitant with a decrease in α2 cell surface abundance. However, we cannot exclude the possibility that stimuli different from insulin (i.e., contraction, hypoxia, or other hormones) may promote compensatory translocation of α1 to plasma membrane.

Long-term intensive exercise training increases Na\(^+-\)K\(^+\)-ATPase content in skeletal muscle (reviewed in Ref. 11). Previous studies have utilized longer exercise regimens or have been based on analysis of rodent skeletal muscle with [\(^3\)H]ouabain binding, thus only assessing the expression of the ouabain-sensitive α2-isofrom (11, 44). In the present study, exercise training had no effect on the expression of α1-, α2-, and β1-subunit isoforms, whereas expression of the β2-subunit was reduced. In skeletal muscle, α2 interacts with β1 preferably (32). The dissociation between mRNA and protein expression for α2 and β1 suggest that the high-fat diet induces posttranslational regulation of α2 and β1 proteins. Conversely, α1 and β2 showed a tight association between mRNA and protein levels. Taken together, these results indicate that Na\(^+-\)K\(^+\)-ATPase isoforms are regulated at both mRNA and protein levels.

Although exercise training has no effect on α1-, α2-, and β1-subunit expression, the high-fat feeding-induced changes in the expression pattern of these subunits was restored to levels observed in chow-fed rats. The mechanism of this restoration of Na\(^+-\)K\(^+\)-ATPase subunit content in skeletal muscle by exercise is unclear but may be associated with a normalization...
of the physiological milieu induced by high-fat diet. Notably, the Na\(^+\)-K\(^+\)-ATPase expression does not always correlate with muscle oxidative capacity. Alternatively, the normalization of Na\(^+\)-K\(^+\)-ATPase subunit content may be regulated through changes in transcription factor activity.

The expression of the \(\beta_2\)-subunit was reduced on mRNA and protein levels by training regardless of diet. The \(\beta_2\)-subunit has been revealed to be an adhesion molecule in the glia (16). Whether the \(\beta_2\)-subunit is involved in cell adhesion in skeletal muscle is unknown. Moreover, we cannot exclude the possibility that the exercise-induced decrease in \(\beta_2\) expression may be related to muscle damage and remodeling after exercise.

Previous studies have reported increased PLM expression in response to prolonged treadmill exercise training (42). The discrepancy between the present and previously published data could be due to differences in the duration and intensity of the exercise training protocol. We did not find an increase in PLM expression with exercise training but did observe increased PLM expression and decreased PLM phosphorylation with high-fat diet. We found that exercise training leads to an increase in Na\(^+\)-K\(^+\)-ATPase activity in skeletal muscle, concomitant with increased phosphorylation of PLM at Ser\(^{68}\). In the unphosphorylated state, PLM binds to the Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunits and inhibits the pump activity by decreasing Na\(^+\) affinity or \(V_{\text{max}}\) (7, 12). In rat skeletal muscle, PLM associates with the \(\alpha_1\) and \(\alpha_2\)-isoforms (12, 42). Thus, the high-fat diet-induced increase in PLM expression may partially contribute to a decrease in Na\(^+\)-K\(^+\)-ATPase activity. PLM phosphorylation disrupts the PLM-\(\alpha\)-subunit interaction and stimulates the pump. The increase in plasma membrane fraction Na\(^+\)-K\(^+\)-ATPase activity in response to training without increase in Na\(^+\)-K\(^+\)-ATPase cell surface abundance in parallel with increased PLM phosphorylation provides evidence to suggest that the phosphorylation of PLM in response to exercise training may play a role in increasing the \(V_{\text{max}}\) of the Na\(^+\)-K\(^+\)-ATPase and restoration of the pump activity. The difference noted between the magnitude of increase in PLM phosphorylation and the increase in Na\(^+\)-K\(^+\)-ATPase activity in crude membrane fraction compared with the plasma membrane may be due to limitations in our detection of Na\(^+\)-K\(^+\)-ATPase activity. PLM is located mostly in the plasma membrane, whereas a total crude membrane fraction was used for assessment of Na\(^+\)-K\(^+\)-ATPase activity. Thus, enhanced Na\(^+\)-K\(^+\)-ATPase activity in plasma membrane specifically may have been diluted in our assay.

Interestingly, insulin induces phosphorylation of PLM (55), and blocking of PLM phosphorylation at Ser\(^{68}\) reduced insulin-stimulated GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes (54). Thus, PLM phosphorylation may be important for restoring glucose uptake in response to exercise training.

To explore the molecular mechanisms by which high-fat diet and exercise regulates skeletal muscle Na\(^+\)-K\(^+\)-ATPase isoform expression, we determined transcription factor activity by using a DNA-binding assay. Apart from classical regulation by steroid and thyroid hormones (38), the molecular regulation of genes encoding Na\(^+\)-K\(^+\)-ATPase subunits under physiological conditions where Na\(^+\)-K\(^+\)-ATPase expression is altered, such as hypokalemia (50, 51), diabetes (15, 44), or exercise training (11, 30, 44), is largely unknown. The Na\(^+\)-K\(^+\)-ATPase \(\alpha_2\)-subunit gene promoter contains consensus sequences for several transcription factors, including AP-1, AP-2, NF-1, and Sp-1 (45). An Sp-1 consensus sequence has been identified as a positive regulatory element in the rat \(\alpha_2\) and \(\beta_1\)-subunit gene promoter (25). Sp-1 DNA-binding activity was markedly suppressed in nuclear extracts from skeletal muscle obtained from sedentary fat-fed rats, suggesting that Sp-1 may regulate \(\alpha_2\) and \(\beta_1\)-subunit expression. However, Sp-1 DNA-binding activity did not correlate with \(\alpha_2\) and \(\beta_1\)-subunit mRNA content. Thus, the regulation of \(\alpha_2\) and \(\beta_1\)-subunit protein expression by high-fat diet may occur on a translational or mRNA degradation level, rather than a transcriptional level.

ZEB (AREB6) is expressed in heart and skeletal muscle and has been identified as a specific transcription factor regulating the rat \(\alpha_1\)-subunit gene (24, 57). Interestingly, we found ZEB-biding sites on the FXYD1 promoter at positions −270 to −295 and −329 to −354, relative to the +1 transcription site. High-fat diet increased skeletal muscle ZEB DNA-binding activity. In contrast, exercise was without effect on skeletal muscle ZEB-binding activity. ZEB serves as a negative regulator of skeletal muscle differentiation (37). In adult skeletal muscle, MEF2 is essential for the expression of GLUT4 (49). Given the increase in ZEB activity in response to high-fat diet, we hypothesized that activated ZEB enhanced and coordinated Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit and PLM gene expression in skeletal muscle of high-fat-fed sedentary rats. Thus, in skeletal muscle of high-fat-fed exercise-trained rats, activation of MEF2 may increase MEF2:ZEB binding, thus competitively inhibiting ZEB binding to its consensus sequence in \(\alpha_1\)-subunit and PLM gene promoters.

In conclusion, lifestyle factors such as diet and exercise regulate Na\(^+\)-K\(^+\)-ATPase. High-fat diet modulates DNA-binding activity of candidate transcription factors in skeletal muscle and coordinatively regulates Na\(^+\)-K\(^+\)-ATPase subunit expression. Short-term exercise training restores isoform expression to levels observed in chow-fed sedentary rats. Changes in skeletal muscle Na\(^+\)-K\(^+\)-ATPase expression, in particular the decrease in \(\alpha_2\)-subunit expression in response to high-fat diet, occur prior to the development of insulin resistance. Moreover, diet-induced changes in cell surface isoform expression are restricted to the \(\alpha_2\)-subunit of Na\(^+\)-K\(^+\)-ATPase. In obese insulin-resistant humans, insulin action on K\(^+\) fluxes is impaired (13), concomitant with an increased Na\(^+\)/K\(^+\) ratio in skeletal muscle (31). In obese type 2 diabetic patients, skeletal muscle Na\(^+\)-K\(^+\)-ATPase content is decreased; this effect is not genetically determined (15). Collectively, these findings implicate Na\(^+\)-K\(^+\)-ATPase expression as playing a role in the development of impaired K\(^+\) fluxes associated with obesity or insulin resistance.

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REFERENCES


