Endothelial nitric oxide synthase is central to skeletal muscle metabolic regulation and enzymatic signaling during exercise in vivo

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Lee-Young RS, Ayala JE, Hunley CF, James FD, Bracy DP, Kang L, Wasserman DH. Endothelial nitric oxide synthase is central to skeletal muscle metabolic regulation and enzymatic signaling during exercise in vivo. Am J Physiol Regul Integr Comp Physiol 298: R1399–R1408, 2010. First published March 3, 2010; doi:10.1152/ajpregu.00004.2010.—Endothelial nitric oxide synthase (eNOS) is associated with a number of physiological functions involved in the regulation of metabolism; however, the functional role of eNOS is poorly understood. We tested the hypothesis that eNOS is critical to muscle cell signaling and fuel usage during exercise in vivo, using 16-wk-old catheterized (carotid artery and jugular vein) C57BL/6J mice with wild-type (WT), partial (+/-), or no expression (-/-) of eNOS. Quantitative reductions in eNOS expression (~40%) elicited many of the phenotypic effects observed in enos/-/- mice under fasted, sedentary conditions, with expression of oxidative phosphorylation complexes I to V and ATP levels being decreased, and total NOS activity and Ca2+/CaM kinase II Thr286 phosphorylation being increased in skeletal muscle. Despite these alterations, exercise tolerance was markedly impaired in enos/-/- mice during an acute 30-min bout of exercise. An eNOS-dependent effect was observed with regard to AMP-activated protein kinase signaling and muscle perfusion. Muscle glucose and long-chain fatty acid uptake, and hepatic and skeletal muscle glycogenolysis during the exercise bout was markedly accelerated in enos/-/- mice compared with enos+/+ and WT mice. Correspondingly, enos/-/- mice exhibited hypoglycemia during exercise. Thus, the ablation of eNOS alters a number of physiological processes that result in impaired exercise capacity in vivo. The finding that a partial reduction in eNOS expression is sufficient to induce many of the changes associated with ablation of eNOS has implications for chronic metabolic diseases, such as obesity and insulin resistance, which are associated with reduced eNOS expression.

ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) is one of three NOS isozymes, which catalyze the reaction of l-arginine to l-citrulline and nitric oxide (NO) (35). eNOS is expressed in a number of different tissues (28), and on the basis of studies using genetically modified mice, the production of NO via eNOS is thought to play a role in a number of physiological functions ranging from, but not limited to, the regulation of vascular tone (13, 20, 25, 32), cardiovascular function (4), and skeletal muscle mitochondrial function and biogenesis (26, 47). A partial impairment in eNOS expression in skeletal muscle also correlates with the pathogenesis of chronic metabolic disease states such as obesity and insulin resistance (11, 18, 47). However, the degree to which the diverse biochemical and morphological changes associated with the reduction of eNOS translate into functional metabolic effects is poorly understood.

In skeletal muscle, moderate-intensity exercise increases total NOS activity (i.e., the sum of eNOS, neuronal (n) NOS and inducible (i) NOS, as well as AMP-activated protein kinase (AMPK) activity (27, 40)). It has been suggested that the activation of AMPK is, in part, regulated by endogenous NO in a positive feedback mechanism, such that an increase in NO activates AMPK, which further augments NOS activity and NO production (8, 29, 50). Thus, it could be hypothesized that impaired skeletal muscle NOS activity and NO production would suppress AMPK activity. In line with this positive feedback paradigm, mice expressing a catalytically inactive AMPKα2 subunit in skeletal muscle are unable to increase total NOS activity during moderate-intensity exercise (27). These mice are exercise intolerant (16, 27, 37), as are mice with a full deletion of eNOS (34, 39).

In humans and rodents, the nonspecific NOS inhibitors Nω-monomethyl-l-arginine and Nω-nitro-l-arginine methyl ester have been shown to impair skeletal muscle glucose uptake (MGU) and GLUT-4 translocation during exercise in vivo (5, 21, 41), and attenuate MGU in response to contraction in situ (42); however, other rodent studies utilizing contraction in vitro (14, 19, 46) and exercise in vivo (43) show no effect of NOS inhibition on MGU. A caveat to the use of these NOS inhibitors is that they affect all NOS isoforms, and thus, the role of specific isoforms in the physiological, metabolic, and enzymatic response to exercise is unclear. Here, we studied mice with heterozygous (enos+/-) and homozygous (enos-/-) eNOS deletion, and wild-type littermates (WT). This allowed us to study the specific eNOS isoform, as well as quantifiable eNOS dose-response effects. Physical exercise in vivo was used as a means to unmask phenotypes associated with eNOS action. We tested the hypotheses that 1) exercise capacity, 2) skeletal muscle enzymatic signaling, and 3) the uptake of metabolic fuels by skeletal muscle during exercise in vivo is critically dependent on the extent to which eNOS is expressed.

MATERIALS AND METHODS

Animal maintenance. All procedures were approved by the Vanderbilt University Animal Care and Use Committee. WT, enos+/-, and enos-/- littermate mice were generated by mating C57BL/6J enos+/- mice (45) purchased from Jackson Laboratories (Bar Harbor, ME). Twenty-one days after birth, littermates were separated by sex and maintained in microisolator cages until they were 16 wk of age. Genotyping was performed using standard PCR techniques on genomic DNA isolated from tail biopsies. Following separation, mice were fed a standard chow diet (5.5% fat by weight; 5001 Laboratory Rodent Diet; Purina, Richmond, IN) and had access to water ad libitum. Studies were conducted on 16-wk-old mice.

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Body composition, feeding, and indirect calorimetry. At 16 wk of age, body composition was determined using a mq10 NMR analyzer ( Bruker Optics, The Woodlands, TX). For food intake measurements, mice were placed in individual cages with measured amounts of food and bedding. Food was weighed 48 h later, excluding fecal matter and bedding. Oxygen consumption ($V_{\text{O}_2}$) and carbon dioxide production ($V_{\text{CO}_2}$) was measured using an Oxymax indirect calorimetry system (Columbus Instruments, Columbus, OH), as described previously (2). The respiratory exchange ratio (RER) was calculated as $V_{\text{CO}_2}/V_{\text{O}_2}$, and energy expenditure was calculated as described previously (2).

Metabolic experiments. Seven days prior to the experiment, surgical procedures were performed as previously described (3). Briefly, mice were anesthetized with pentobarbital sodium (70 mg/kg body wt ip), the carotid artery and jugular vein were catheterized, and the free ends of the catheters were tunneled under the skin to the back of the neck, where they were attached via stainless-steel connectors to lines made of Micro-Renathane. These lines were exteriorized, sealed with stainless-steel plugs, and kept patent with saline containing 200 U/ml of heparin and 5 mg/ml of ampicillin. Mice were housed individually postsurgery, and body weight was recorded daily. Five days following surgery, all mice were familiarized to treadmill exercise by performing a 10-min bout of exercise at 13 m/min (0% incline). Experiments were performed 2 days later. Male and female mice were utilized for all experiments, and no sex-specific effects were observed.

On the day of the experiment, mice were fasted for 5 h prior to commencing exercise. Approximately 1 h prior to the experiment, Micro-Renathane tubing was connected to the exteriorized catheters, and all mice were placed in the enclosed treadmill to acclimate to the environment. At $t = 0$ min, a baseline arterial blood sample ($\sim 100 \mu l$) was taken to measure arterial levels of glucose, insulin, nonesterified fatty acids (NEFA), lactate, and hematocrit. Mice then remained sedentary or performed treadmill exercise for a maximum of 30 min at 16 m/min (0% incline). Sedentary mice were allowed to move freely in the stationary treadmill for 30 min. In all mice, a bolus containing $2^{14}$C-dioxygenolysine $(2^{14}$CDJG; 13 $\mu$Ci) and [9,10-$^3$H]-(R)-2-bromopalmitate ([$^3$H]-R-BrP; 26 $\mu$Ci) was injected into the jugular vein at $t = 5$ min to provide an index of tissue-specific glucose and long-chain fatty acid (LCFA) uptake and clearance, respectively. At $t = 7, 10, 15$, and 20 min, $\sim 50 \mu l$ of arterial blood was sampled to determine arterial glucose, NEFA, $2^{14}$CDJG, and [$^3$H]-R-BrP. Hematocrit was also measured at $t = 20$ min. At $t = 30$ min or exhaustion (if earlier than 30 min), $\sim 100 \mu l$ of arterial blood was taken for the measurement of arterial glucose, insulin, NEFA, lactate, $2^{14}$CDJG, and [$^3$H]-R-BrP. Following the final arterial sample, 50 $\mu$l of yellow DYE-TRAK Microspheres (15 $\mu$m; Triton Technology, San Diego, CA) were injected into the carotid artery to assess percent cardiac output to the gastrocnemius (QC)g, as well as to the left and right kidney. Mice were then anesthetized with an arterial infusion of pentobarbital sodium (3 mg). Tissues were rapidly excised and frozen in liquid nitrogen and stored at $-70^\circ$C. For microsphere analysis, the left gastrocnemius and left and right kidneys were placed into 15-ml polypropylene tubes and stored at 4°C until analyzed.

Plasma and tissue analyses. Plasma $2^{14}$CDJG radioactivity was assessed following deproteinization, as previously described (3), while plasma [$^3$H]-R-BrP radioactivity was determined directly from the plasma (44). Tissue phosphorylated $2^{14}$CDJG (2-[14$C$]CDJG-P) and [$^3$H]-R-BrP was determined using a modified method of Folch et al. (15), which has been previously described (27). Immunoreactive plasma insulin was assayed with a double antibody method (36), and plasma NEFAs were measured spectrophotometrically using an enzymatic colorimetric assay (Wako NEFA C kit; Wako Chemicals, Richmond, VA). Plasma lactate was determined using the enzymatic method of Lowry and Passoneau (30), using lithium L-lactate (Sigma, St. Louis, MO) as the standard.

Glycogen was determined using the method of Chan and Exton (7). ATP was measured using HPLC (1). %QC was determined as previously described (27).

Muscle samples were homogenized in lysis buffer containing (final concentrations) 50 mM Tris HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 $\mu$m/l trypsin inhibitor, 5 $\mu$l/ml protease inhibitor cocktail, 50 mM NaF, and 5 mM Na pyrophosphate, as previously described (27). Protein content in the supernatant was determined using the Bradford method. Protein expression of eNOS, nNOS, iNOS, GLUT-4, hexokinase (HK) II, AMPK $\alpha$, acetyl-CoA carboxylase (ACC) $\beta$, Ca$^{2+}$/calmodulin kinase (CaMKII), as well as as AMPK$\alpha$ Thr172 phosphorylation, ACC$\beta$ Ser211 phosphorylation, and CaMKII Thr286 phosphorylation in the gastrocnemius muscle was determined from 100 $\mu$g of protein. Proteins were resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene fluoride membranes. Blots were probed with anti-eNOS rabbit polyclonal (Abcam, Cambridge, MA), anti-nNOS and anti-iNOS mouse monoclonal (BD Biosciences, San Jose, CA), anti-GLUT-4 and anti-HK II rabbit polyclonal (Abcam), anti-AMPK$\alpha$ pan and anti-ACC$\beta$ rabbit polyclonal (Cell Signaling, Beverly, MA), as well as anti-AMPK$\alpha$ Thr172, anti-ACC$\beta$ Ser79 (equivalent to Ser211 in skeletal muscle), and anti-CaMKII Thr286 rabbit polyclonal (Cell Signaling) antibodies. Oxidative phosphorylation (OXPHOS) complexes I to V of the electron transport chain were determined from 25 $\mu$g of protein, and Mitoprotein Total OXPHOS Rodent WB Antibody Cocktail, according to manufacturer instructions (MitoSciences, Eugene, OR). Protein expression was normalized to GAPDH (Abcam). OXPHOS complexes were normalized to voltage-dependent anion channel (VDAC) expression, a known mitochondrial marker (12). AMPK$\alpha$ Thr172, ACC$\beta$ Ser211, and CaMKII Thr286 phosphorylation was normalized to AMPK$\alpha$, ACC$\beta$, and CaMKII protein levels, respectively. Antibody binding was detected with either IRDye 800-conjugated anti-rabbit IgG or IRDye 800-labeled streptavidin (Rockland Immunochemicals, Gilbertsville, PA). ACC$\beta$ expression was detected using IRDye 800-labeled streptavidin (Rockland Immunochemicals).

AMPK$\alpha$2 and NOS activities were measured using 200 $\mu$g and $\sim 70$ $\mu$g of protein, respectively, as previously described (27). Briefly, AMPK$\alpha$2 was immunoprecipitated using Recombin protein A beads (Pierce, Rockford, IL), and activity was measured for 24 min at 30°C in the presence of 200 $\mu$M AMP and 100 $\mu$M SAMS peptide (Genway Biotech, San Diego, CA) and was calculated as picomoles of phosphate incorporated into the SAMS peptide per minute per milligram of protein. NOS activity was measured on whole cell lysates, and it is the difference between samples incubated with or without $N^\omega$-nitro-L-arginine methyl ester. NOS activity was calculated as the amount of l-[3$^3$H]-arginine converted to l-[3$^3$H]-citrulline (in disintegrations/min) per minute per milligram of protein.

Calculations. The tissue-specific clearance of $2^{14}$CDJG and [$^3$H]-R-BrP ($K_g$ and $K_r$, respectively), and the metabolic index for glucose and LCFA ($R_g$ and $R_l$) were calculated as previously described (23, 27). $K_g$ and $K_r$ are used as concentration-independent indices of muscle glucose and LCFA uptake, respectively.

Percent cardiac output to the tissue was calculated from fluorescent intensity as previously described (27) and is expressed as the percentage of microspheres in the gastrocnemius muscle relative to the total amount infused. Adequacy of microsphere mixing was assumed if microsphere content in the left and right kidney was within 10%. On average, the difference between %Q to the left and right kidney was $6 \pm 3$% across genotypes.

Statistical analyses. Data are presented as means $\pm$ SE. Statistical analysis was performed using either a Student’s $t$-test, one-way ANOVA, or two-way repeated-measures ANOVA where appropriate with the statistical software package SigmaStat. If the ANOVA were significant ($P < 0.05$), specific differences were located using Fisher’s least significant difference (LSD) test.

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Table 1. Body weight, body composition, and indirect calorimetry measurements of 16 wk of chow-fed C57BL/6J mice with wild type, partial, or no expression of endothelial NOS

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>enos+/−</th>
<th>enos−/−</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>25.5 ± 0.8</td>
<td>24.8 ± 0.8</td>
<td>22.3 ± 0.8</td>
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<tr>
<td>Lean mass, g</td>
<td>21.4 ± 0.7</td>
<td>19.1 ± 0.5</td>
<td>17.5 ± 0.6</td>
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<tr>
<td>Lean mass, %</td>
<td>84.1 ± 1.4</td>
<td>77.0 ± 0.7</td>
<td>78.2 ± 0.6</td>
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<tr>
<td>Fat mass, g</td>
<td>1.5 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>1.7 ± 0.1</td>
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<tr>
<td>Fat mass, %</td>
<td>6.2 ± 1.0</td>
<td>9.1 ± 0.7</td>
<td>7.6 ± 0.5</td>
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<tr>
<td>Light cycle (0600–1800)</td>
<td></td>
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<tr>
<td>VO₂, ml·kg⁻¹·min⁻¹</td>
<td></td>
<td></td>
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<tr>
<td>RER</td>
<td>0.92 ± 0.02</td>
<td>0.97 ± 0.01</td>
<td>0.95 ± 0.01</td>
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<tr>
<td>CHOox, µmol·kg⁻¹·min⁻¹</td>
<td>50.1 ± 4.1</td>
<td>62.9 ± 4.5</td>
<td>59.0 ± 3.0</td>
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<tr>
<td>Fatox, µmol·kg⁻¹·min⁻¹</td>
<td>7.1 ± 1.4</td>
<td>2.8 ± 1.1</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>24-h food consumption, g</td>
<td>7.3 ± 0.5</td>
<td>6.7 ± 0.6</td>
<td>6.6 ± 0.6</td>
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</table>

Data are expressed as means ± SE for n = 6–8 per group. *P < 0.05 vs. WT; †P < 0.05 vs. wild type (WT) and enos+/−; **P = 0.05 vs. WT. eNOS, endothelial nitric oxide synthase; WT, wild type; enos+, partial expression of eNOS; enos−/−, no expression of eNOS; RER, respiratory exchange ratio; VO₂, oxygen consumption; CHOox, carbohydrate and fat oxidation, respectively.

RESULTS

Baseline characteristics. At 16 wk of age, body weight of WT and enos+/− mice was significantly greater than enos−/− mice (Table 1). enos+/− and enos−/− mice had less muscle mass than WT (Table 1). In absolute terms, fat mass was elevated in enos+/− mice vs. WT (Table 1). Under sedentary conditions, no genotype effects were observed for arterial levels of glucose (11 ± 1 vs. 10 ± 1 vs. 9 ± 1 mM for WT, enos+/− and enos−/−, respectively) or NEFA (1.7 ± 0.1 vs. 1.8 ± 0.1 vs. 1.8 ± 0.2 mM, respectively). eNOS protein expression was reduced ~40% in skeletal muscle of enos+/− mice, and was undetectable in enos−/− mice (Fig. 1A). Neuronal nitric oxide synthase mu (nNOSμ) expression did not significantly differ between genotypes in skeletal muscle (Table 2), while iNOS expression was undetectable. GLUT-4 and HKII protein levels were also similar between genotypes (Table 2). Basal ATP levels in skeletal muscle differed between genotypes with WT > enos+/− > enos−/− (55 ± 1 vs. 47 ± 2 vs. 41 ± 1 µmol·100g⁻¹, respectively; P < 0.05).

A partial reduction of eNOS was sufficient to impair OXPHOS complexes I through V in skeletal muscle (Fig. 1, B and C). A further reduction in complex II and V expression was seen in enos−/− mice when compared with enos+/− mice. The changes in OXPHOS complex expression between genotypes was not due to changes in mitochondrial content, as assessed via expression of mitochondrial VDAC (2.4 ± 0.3 vs. 2.2 ± 0.2 vs. 2.0 ± 0.3 arbitrary units for WT, enos+/− and enos−/−, respectively).

We next assessed whether changes in eNOS protein expression altered enzymatic signaling under basal conditions. Partial and full deletion of eNOS resulted in a paradoxical increase in total NOS activity in skeletal muscle (Fig. 1D). Basal AMPK activity was similar between genotypes (Fig. 1E), as was total AMPKα protein expression (Table 2) and total AMPKα Thr72 phosphorylation. Expression of the upstream AMPK kinase LKB1 was similar between genotypes, as was ACCβ, a downstream target of AMPK.

![Fig. 1. Baseline characteristics of 16-wk-old chow-fed C57BL/6J mice with wild type (WT), partial (+/−) or no expression (−/−) of endothelial nitric oxide synthase (eNOS). Protein expression of eNOS (A), oxidative phosphorylation (OXPHOS) complexes I to V of the electron transport chain (B and C), OXPHOS activity (D), AMP-activated protein kinase (AMPK) α2 activity (E), and Ca²⁺/calmodulin protein kinase (CaM-K) II Thr²⁵⁶ phosphorylation (F) was determined in gastrocnemius muscle of sedentary mice that had been fasted for 5 h. Data are expressed as means ± SE for n = 5–7 per group. *P < 0.05 vs. WT; †P < 0.05 vs. WT and enos+/−; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; +ve, positive control (rat brain mitochondria).](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00169.2010)
Table 2. Protein expression of the skeletal muscle isoform of neuronal nitric oxide synthase, GLUT-4, hexokinase II, AMP-activated protein kinase β, LKB1, and Ca²⁺/calmodulin protein kinase II in gastrocnemius muscle of 16-wk-old Chow-fed C57BL/6J mice with WT, +/-, or --/-- of eNOS

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>eNOS +/-</th>
<th>eNOS --/--</th>
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<tbody>
<tr>
<td>nNOS µ</td>
<td>0.16 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.15 ± 0.02</td>
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<tr>
<td>GLUT-4</td>
<td>1.09 ± 0.14</td>
<td>1.09 ± 0.21</td>
<td>1.10 ± 0.14</td>
</tr>
<tr>
<td>HK II</td>
<td>0.36 ± 0.05</td>
<td>0.29 ± 0.09</td>
<td>0.39 ± 0.09</td>
</tr>
<tr>
<td>AMPKα</td>
<td>0.41 ± 0.04</td>
<td>0.41 ± 0.02</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>ACCβ</td>
<td>0.55 ± 0.07</td>
<td>0.45 ± 0.04</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>LKB1</td>
<td>0.62 ± 0.04</td>
<td>0.56 ± 0.03</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>CaMKII</td>
<td>1.25 ± 0.08</td>
<td>1.30 ± 0.14</td>
<td>1.26 ± 0.15</td>
</tr>
</tbody>
</table>

Data are expressed as arbitrary units normalized to glyceraldehyde-3-phosphate dehydrogenase and are means ± SE for n = 6–8 per group.

WT mice, RER increased from the light to dark cycle, the dark cycle, RER was reduced in WT mice and became hypoglycemic at the end of exercise. A main effect for genotype (P < 0.05) and time (P < 0.05) was observed for arterial NEFA (Fig. 3B) and insulin (Fig. 3C). Arterial lactate increased during exercise in all genotypes (Fig. 3D); however, the increase in eNOS --/-- mice (11.3 ± 1.4-fold) was significantly greater than in both eNOS +/- (3.9 ± 0.6-fold) and WT mice (2.9 ± 0.4 fold). Thus, exercise in vivo elicited an eNOS-dependent phenotype characterized by reductions in exercise capacity and arterial glucose, altered arterial NEFA and insulin, and increased arterial lactate levels.

Percent cardiac output to skeletal muscle during exercise in vivo is highly dependent on eNOS. In the sedentary state, %QG did not differ between genotypes (Fig. 4A). In response to exercise, %QG was proportional to eNOS expression, increasing ~6-, 3-, and 2-fold in WT, eNOS +/-, and eNOS --/-- mice, respectively (Fig. 4A). Regression analysis revealed a correlation between eNOS expression and the increase in %QG in response to exercise (r = 0.73, P < 0.01; Fig. 4B).

On the basis of the equation of the line, 20 ± 5% of the increase in %QG during exercise in WT mice was eNOS independent.

Ablation of eNOS markedly accelerates indices of muscle substrate uptake in vivo. Compared with WT, sedentary K₆ (Fig. 5A) and R₆ (Fig. 5B) in gastrocnemius muscle was elevated in eNOS +/- mice. Exercise significantly increased gastrocnemius K₆ and R₆ in WT and eNOS --/-- mice; however, this increase was considerably higher (~5-fold) in eNOS --/-- mice (Fig. 5, A and B). To determine whether this phenomenon occurred in other tissues, we assessed K₆ and R₆ in superficial vastus lateralis (SVL) (Fig. 5, C and D) and cardiac muscle (Fig. 5, E and F). In these tissues, K₆ and R₆ during exercise were also markedly elevated in eNOS --/-- mice.

No genotype differences in gastrocnemius Kt (Fig. 6A) or R₆ (Fig. 6B) were observed in the sedentary state. Kt increased during exercise in eNOS +/- and eNOS --/-- mice, whereas R₆ only significantly increased in eNOS --/-- mice. As with K₆, gastrocnemius Kt increased to the greatest extent in eNOS --/-- mice during exercise. Similar findings were observed in SVL muscle.
In cardiac muscle of enos+/H11001/H11002 and enos+/H11002/H11002 mice, basal $K_f$ (Fig. 6E) and $R_f$ (Fig. 6F) were similar between genotypes. Exercise increased $K_f$ in enos+/H11002/H11002 mice, whilst $R_f$ was not altered in any genotype.

Ablation of eNOS accelerates net muscle and liver glycogenolysis in vivo. Gastrocnemius glycogen levels were similar between WT, enos+/H11001/H11002 and enos+/H11002/H11002 mice under sedentary conditions, and exercise caused a similar decrement in glycogen (Table 3). Liver glycogen was also similar at rest and decreased to a similar extent in response to exercise (Ex) following a 5-h fast in WT, enos+/H11001/H11002, and enos+/H11002/H11002 mice (Table 3). However, the average running time for enos−/− mice was ~30% less than for WT and enos+/− mice. Thus, the rate of glycogenolysis was ~3-fold greater in enos−/− mice in the gastrocnemius and liver (Table 3).

**DISCUSSION**

Here, we examined the role of eNOS in the regulation of enzymatic signaling and fuel metabolism in skeletal muscle in vivo. Novel findings were that a partial reduction in eNOS was sufficient to induce many of the phenotypic effects observed in enos−/− mice under fasting, sedentary conditions. Likewise, physical exercise was able to reverse physiological deficits, which were dependent upon eNOS expression, and precipitate others that were unique to enos−/− mice. Collectively, our
findings show that eNOS plays a multifaceted role in the interaction of physiological responses both at rest and during exercise in vivo. These findings emphasize the exquisite sensitivity of skeletal muscle to deficits in eNOS expression.

The finding that a partial deletion of eNOS is sufficient to induce a phenotypic effect under basal conditions that is similar to that of enos−/− mice has important implications for metabolic disease states. Obese individuals have a partial reduction in eNOS protein expression in skeletal muscle (18); high-fat fed enos−/−/− mice exhibit exaggerated insulin resistance during an insulin clamp (11); genetic and environmental induction of obesity attenuates eNOS protein expression and lowers ATP levels in skeletal muscle of rodents (47). Findings from the present and aforementioned studies suggest that a reduction in eNOS expression does not play a causative role with regard to obesity per se. Indeed, enos−/− and enos+/− mice do not weigh more than WT mice; however, enos++/− mice do have slightly elevated fat mass, while fat oxidation is reduced in the light cycle. It is likely that obesity impairs eNOS protein expression, potentially via elevated levels of tumor necrosis factor α (47), and this downregulation of eNOS exerts subsequent effects on ATP levels and OXPHOS complexes in skeletal muscle, as seen in the present study.

Here, we show that partial and full deletion of eNOS decreases OXPHOS complexes in skeletal muscle without altering the mitochondrial marker VDAC. It has previously been shown that enos−/− mice have impaired skeletal muscle mitochondrial β-oxidation, although this occurred in parallel with reduced mitochondrial content (26). Presently, the role of eNOS with regard to mitochondrial content and biogenesis is unclear, with some (26, 38), but not all studies (34, 48), showing a regulatory role for eNOS in these processes. Furthermore, differences have been observed in mice generated from the same group (45), with Wadley et al. (48) finding no differences in mitochondrial content of enos−/− and WT mice, and Le Gouill et al. (26) showing decreased mitochondrial content in enos−/− mice. Similar to Wadley et al. (48), we found no differences in mitochondrial content between geno-

**Fig. 3.** Arterial blood glucose (A), plasma nonesterified fatty acids (NEFAs; B), plasma insulin (C), and plasma lactate (D) at rest and during exercise in 16-wk-old chow fed C57BL/6j mice with wild-type (WT), partial (+/−), or no expression (−/−) of eNOS. Following a 5-h fast, chronically catheterized mice performed a maximum of 30 min of running on a motorized treadmill, and arterial blood was sampled at times shown. Data are expressed as means ± SE for n = 7 (WT), n = 15 (enos+/−), and n = 10 (enos−/−). ‡P < 0.05 vs. corresponding rest value: ∗P < 0.001 vs. WT; †P < 0.001 vs. WT and enos−/−; †Main effect for genotype, P < 0.05; §Main effect for time, P < 0.05.

**Fig. 4.** Exercise-induced increase in percent cardiac output to gastrocnemius muscle (%QG) in 16-wk-old chow fed C57BL/6j mice with wild-type (WT), partial (+/−), or no expression (−/−) of eNOS. Following a 5-h fast, mice either remained sedentary or performed a maximum of 30 min of running on a motorized treadmill. At the end of exercise, 50 μl of yellow DYE-TRAK Microspheres were injected into the carotid artery to determine %QG (see MATERIALS AND METHODS). A: %QG in sedentary mice and exercised mice. B: exercise-induced increase in %QG. Data are expressed as means ± SE; n = 5 per group. ∗P < 0.01 vs. WT; †P < 0.05 vs. corresponding rest value.
in 12-mo-old enos−/− mice. Indeed, basal $\dot{V}O_2$ is not increased in enos−/− mice. This is in line with previous observations showing that NOS activity in these mice likely reflect an increase in nNOS activity. However, our finding of a similar elevation in NOS activity is unclear, as other studies in enos−/− mice have observed no change (24) or a partial reduction (26) in NOS activity. It should be noted that heart rate is reduced by 5–10% under basal conditions in enos−/− mice. It is possible that the elevated level of NOS activity in enos−/− mice relates to alterations in $Ca^2+$ or CaM, which both serve as cofactors for the activation of eNOS and nNOS (6, 9). Indeed, CaMKII Thr$^{286}$ phosphorylation was also elevated in enos+/− and enos−/− mice, and it has been well established that phosphorylation of CaMKII at the Thr$^{286}$ residue occurs following CaM binding.

An unexpected finding from the present study was that basal NOS activity was elevated in enos+/− and enos−/− mice when compared with WT mice. As shown in the present study and by others (34), iNOS expression and activity are not present in enos+/− and enos−/− mice. As such, the elevated rates of basal NOS activity in these mice likely reflect an increase in nNOSµ activity. This is in line with previous observations showing that nNOSµ activity predominates over eNOS activity under basal conditions (40). Presently, the role of eNOS in the regulation of NOS activity is unclear, as other studies in enos−/− mice have observed no change (24) or a partial reduction (26) in NOS activity. However, our finding of a similar elevation in NOS activity in enos+/− mice corroborates our findings in enos−/− mice. It is possible that the elevated level of NOS activity in enos+/− and enos−/− mice relates to alterations in $Ca^2+$ or CaM, which both serve as cofactors for the activation of eNOS and nNOS (6, 9). Indeed, CaMKII Thr$^{286}$ phosphorylation was also elevated in enos+/− and enos−/− mice, and it has been well established that phosphorylation of CaMKII at the Thr$^{286}$ residue occurs following CaM binding.

In the present study, we used exercise in vivo as a means to unmask phenotypes associated with impaired eNOS expression. The use of mice with a wide range of eNOS expression allowed for a comprehensive assessment of eNOS in the regulation of physiological responses to exercise. It is intriguing that despite similar decreases in OXPHOS complexes and lower ATP levels, and similar levels of total NOS activity and $V\dot{O}_2$ in enos+/− and enos−/− mice, the enos−/− mice were relatively exercise intolerant. This finding suggests that alterations in OXPHOS capacity within skeletal muscle, or the activity of NOS and CaMKII, do not affect exercise capacity within the 30-min exercise period utilized. Impaired substrate delivery arising from reduced muscle perfusion likely accounted for the enos−/− phenotype. Indeed, substrate delivery was also closely tied to eNOS expression during exercise (i.e., %QG in WT > enos+/− > enos−/− mice). It should be noted that heart rate is reduced by 5–10% under basal conditions in enos−/− mice (45). Although heart rate during exercise in these mice is unknown, a reduced heart rate could contribute to the impaired blood flow to contracting muscle seen in these mice. Moreover, the inability of enos−/− mice to significantly increase %QG during exercise will result in impaired oxygen delivery, which would lead to hypoxia. In skeletal muscle, hypoxia is a potent stimulator of glucose uptake, as well as phosphocreatine and glycogen breakdown (17, 22, 51).
increases plasma lactate, hepatic glucose production, and muscle glycogenolysis, these processes are further augmented during exercise performed under hypoxic conditions (22, 51). The fact that muscle metabolic flux, glycogenolysis, and plasma lactate levels were markedly enhanced in enos−/− mice during exercise suggests that hypoxia, arising from impairments in %QG, may have elicited an additive effect on substrate uptake and metabolism. We have previously observed a similar finding in AMPKα2 kinase-dead mice, whereby skeletal muscle complex I and IV activities, muscle perfusion during exercise in vivo, and exercise tolerance were impaired when compared with WT littermates (27).

The finding that the exercise-induced increase in AMPK signaling, and AMPKα2 activity, was ablated in skeletal muscle of enos−/− mice is remarkable. AMPKα2 is the predominant α subunit activated in skeletal muscle during moderate to high-intensity exercise (10, 27). Previous studies have suggested that the activation of AMPK is regulated via endogenous NO in a positive-feedback mechanism (29, 50). Our findings argue against absolute rates of NOS activity per se, and thus absolute NO production, as a regulator of AMPK activity in skeletal muscle (40), our findings suggest that the activation of AMPK is regulated via endogenous NO in a positive-feedback mechanism (29, 50). Our findings argue against absolute rates of NOS activity per se, and thus absolute NO production, as a regulator of AMPK activity in skeletal muscle during exercise despite similar rates of NOS activity in all genotypes. Thus, while exercise in rodents increases eNOS and nNOSα activity in skeletal muscle (40), our findings provide evidence that it is eNOS-derived NO, and thus specifically eNOS activity in skeletal muscle, which interacts with AMPK under these conditions.

To date, contrasting effects have been observed regarding the role of NOS in the direct regulation of substrate uptake during contraction in vivo (5, 21, 43) and in isolated or

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Table 3. Glycogen levels and glycogenolysis in liver and gastrocnemius of 16-wk-old chow-fed C57BL/6J mice with WT, +/−, or −/− of enos

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>enos+/−</th>
<th>enos−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gastrocnemius glycogen, mmol/100 g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedentary</td>
<td>2.1 ± 0.4</td>
<td>2.3 ± 0.2</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>Exercise</td>
<td>1.0 ± 0.3†</td>
<td>1.1 ± 0.4*</td>
<td>0.6 ± 0.2*†</td>
</tr>
<tr>
<td>Glycogenolysis, μmol·100 g−1·min−1</td>
<td>39 ± 9</td>
<td>47 ± 8</td>
<td>100 ± 15†</td>
</tr>
<tr>
<td><strong>Hepatic glycogen, mmol/100 g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedentary</td>
<td>6.1 ± 2.5</td>
<td>6.6 ± 2.4</td>
<td>8.2 ± 2.4</td>
</tr>
<tr>
<td>Exercise</td>
<td>1.4 ± 0.5*</td>
<td>1.1 ± 0.38</td>
<td>0.4 ± 0.1†</td>
</tr>
<tr>
<td>Glycogenolysis, μmol·100 g−1·min−1</td>
<td>157 ± 15</td>
<td>193 ± 19</td>
<td>468 ± 60†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for n = 5–8 per group. Gastrocnemius and liver glycogen levels were assessed from mice that remained sedentary or performed a maximum of 30-min treadmill exercise, and they are measured as described in MATERIALS AND METHODS. *P < 0.05 vs. corresponding sedentary value; †P < 0.05 vs. WT and enos+/−.

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Fig. 6. Tissue-specific indexes of long-chain fatty acid (LCFA) clearance (Kg) and LCFA uptake (Rg) in gastrocnemius (Gastroc; A, B), superficial vastus lateralis (SVL; C, D), and cardiac muscle (E, F) at rest and during exercise in 16-wk-old chow fed C57BL/6J mice with wild-type (WT), partial (+/−) or no expression (−/−) of NOS. Mice were chronically catheterized and allowed to recover for 7 days. Following a 5-h fast, mice either remained sedentary (Sed) or performed a maximum of 30 min of treadmill exercise (Ex). At 5 min, mice were given a bolus injection containing 26 μCi of [9,10-3H]-R(-)-2-bromopalmitate. Kg and Rg were calculated as described previously (see MATERIALS AND METHODS). In Sed, data are expressed as means ± SE for n = 7 (WT), n = 9 (enos+/−) and n = 4 (enos−/−). For Ex, n = 7, n = 12, and n = 7, respectively. †P < 0.05 vs. WT and enos−/−; ‡P < 0.05 vs. corresponding sedentary value.
perfused hindlimb muscle (14, 19, 33, 41–43). This study is the first to test the role of eNOS, and further the dose-response effect of eNOS, in these processes in vivo. Despite similar total NOS activity in all genotypes in response to exercise, MGU increased to the greatest extent in enos−/− mice, indicating that the absolute level of NOS activity does not regulate MGU during exercise in vivo. It has been suggested that the relative increase in NOS activity during contraction, as opposed to absolute rates, may more accurately reflect the role of NOS in contraction-mediated MGU (33); however, again our findings argue against this as WT mice had the greatest relative increase in NOS activity from rest to exercise, yet MGU was substantially lower than that for enos−/− mice who had no relative increase in NOS activity.

An interesting observation from the present study was that enos+/− mice responded differently in a number of parameters to WT and/or enos−/− mice. Indeed, upon initial inspection, these mice do not differ from WT mice in terms of total body weight; however, this is due to an increase in fat mass, which compensates for a reduction in lean mass. This increase in fat mass may be, at least, partly due to reduced fat oxidation in the light cycle. Despite a ~30% reduction in expression of OXPHOS complexes when compared with WT mice, enos+/− mice are able to adequately perform 30 min of treadmill exercise, suggesting that mitochondrial dysfunction in the basal state does not transfer to gross alterations during exercise, at least under the conditions used in the present study. Furthermore, enos+/− mice do not increase MGU during exercise in vivo, an effect that was observed across all tissues examined. This was at least partially due to elevated basal MGU in these mice, meaning that absolute levels of MGU in response to exercise were similar to WT.

Despite a ~3-fold increase in hepatic glycogen breakdown in enos−/− mice, marked hypoglycemia occurred, further emphasizing the magnitude of the increase in glucose disposal with exercise in these mice. The elevated indices of MGU in enos−/− mice were not due to increased total GLUT-4 or HKII protein in these mice, suggesting that other factors are involved. Likewise, Kf and Rf were also increased to the greatest extent in gastrocnemius of enos−/− mice during exercise, although this finding is in line with previous observations showing that enos−/− mice have elevated levels of intramyocellular lipid (26). Therefore, our findings suggest that neither expression of eNOS nor an increase in total NOS activity is essential for increases in MGU during stressful exercise. This is also in agreement with previous findings in AMPKα2 kinase-dead mice, as the extraction of glucose by skeletal muscle (27) and GLUT-4 translocation (31) is normal during exercise despite the absence of an increase in total NOS activity (27). Nevertheless, it cannot be discounted that at least part of the reason for differences in MGU relates to exercise intensity. Had the WT and enos−/− mice also been run to exhaustion, it is possible that MGU would have increased to rates observed in enos−/− mice.

In conclusion, through the use of WT, enos+/− and enos−/− littermate mice, we have shown for the first time an eNOS-dependent phenotype under basal conditions, characterized by reductions in OXPHOS complex expression, impaired ATP levels, and increased total NOS activity and CaMKII Thr286 phosphorylation in skeletal muscle. Thus, a partial reduction in eNOS protein expression is sufficient to induce many of the effects observed in enos−/− mice, demonstrating that even a quantitative reduction in eNOS protein expression can result in metabolic dysregulation in vivo. A 30-min bout of acute exercise in vivo also elicits a number of physiological processes that are dependent upon eNOS expression, with the exercise-induced increase in AMPKα Thr172 phosphorylation, ACCβ Ser21 phosphorlation, and %QG all being reduced in parallel with reduced eNOS expression. Ablation of eNOS also results in impaired exercise capacity, hypoglycemia, and increased plasma lactate levels. The alterations associated with partial impairment of eNOS have important implications for chronic metabolic disease states such as obesity and insulin resistance, conditions that are characterized by reduced eNOS protein expression in skeletal muscle.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


