Skeletal muscle glucose uptake during treadmill exercise in neuronal nitric oxide synthase-μ knockout mice

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Skeletal muscle glucose uptake during treadmill exercise has previously been determined in eNOS−/− mice, which were found to have higher glucose uptake than wild-type controls (28). This was postulated to be due to the exercise-induced hypoxia in contracting muscle which, in turn, might have stimulated a greater muscle glucose uptake (28), since hypoxia is a potent stimulator of skeletal muscle glucose uptake (6). In addition, NO production during ex vivo contraction was not different between eNOS−/− and eNOS−/−− muscles (13), suggesting that eNOS may not be directly involved in NO-mediated intramuscular signaling. Given that neuronal nitric oxide synthase-μ (nNOSμ) is the major NOS isoform activated during contraction (27), it was surprising to find that nNOSμ knockout muscles did not have attenuated muscle glucose uptake during ex vivo contraction (16). Nevertheless, NO inhibition of isolated nNOSμ knockout (nNOSμ−/−) and wild-type (nNOSμ+/+) muscles still attenuated the increase in muscle glucose uptake (16), suggesting that NO was still playing a role in muscle glucose uptake during contraction. It should be considered that ex vivo contraction lacks the complex integrated interactions underlying in vivo exercise conditions such as neural input, blood flow, and hormonal changes. Highly relevant to this context is that nNOS has been shown to mediate arterial relaxation in contracting skeletal muscle (27). Thus, in vivo studies are essential to define the role of nNOSμ in muscle glucose uptake during exercise.

In this study, nNOSμ−/− and nNOSμ−/− mice were used to investigate the effect of nNOSμ on skeletal muscle glucose uptake in conscious and unrestrained chronically catheterized mice running on a treadmill. This allowed examination of the role of nNOSμ in skeletal muscle glucose uptake in a physiological unstressed condition with intact hemodynamic and intramuscular signaling responses. We hypothesized that the increase in muscle glucose uptake during treadmill running...
would be attenuated in nNOS<sup>−/−</sup> mice because nNOS is the major NOS isoform activated during contraction (27).

MATERIALS AND METHODS

Animals. All procedures were approved by The Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee, and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th Edition). nNOS<sup>−/−</sup> and nNOS<sup>+/−</sup> littermates were generated by mating C57Bl/6 nNOS<sup>−/−</sup> mice originally obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping was performed using tail samples obtained at day 21 of age by a commercial vendor (Transnetyx, Cordova, TN). Mice were housed in standard cages and maintained under constant temperature of 21 ± 1°C with a 12:12-h light-dark cycle in the AMREP Animal Facility. Animals had access to standard rodent chow and water ad libitum. Both male and female mice were used for experiments at 16 wk of age.

Exercise stress test. Mice were subjected to an incremental exercise stress test as previously described (29) to determine their maximum exercise capacity. Briefly, 2 days following a familiarization test (10 m/min for 10 min) mice commenced running at a speed of 10 m/min on a 0% incline treadmill. Running speed was increased by 4 m/min every 3 min until mice were exhausted, which was defined as the point whereby mice continuously remained at the back of the treadmill for more than five s despite tail prodding. Treadmill electrical stimulation was not used in any of the tests.

Surgery and experimental procedures. Surgery procedures were performed as previously described (2), except that only jugular vein cannulation was performed due to an observed intolerance of nNOS<sup>−/−</sup> mice to chronic carotid cannulation. Briefly, mice were anesthetized with 5% isoflurane in oxygen and maintained with 2% isoflurane in oxygen throughout the cannulation procedure. Carprofen was given subcutaneously for pain relief prior to the skin incision. The right jugular vein was cannulated with a Silastic catheter. The free end of the catheter was tunneled under the skin to the back of the neck, where it was exteriorized. The catheter was kept patent with saline containing 200 U/ml heparin and 5 mg/ml ampicillin and sealed with stainless steel plugs. Mice were housed individually after surgery, and body weight was monitored. Mice were used for experiments at least 3 days postsurgery when they had fully recovered as indicated by normal activity, healthy appearance, and weight regained after surgery.

On the day of the experiment, the exteriorized jugular catheter was connected, via a stainless steel connector, with Micro-Renathane tubing ~1 h prior to the experiment. Mice were then placed on a single-lane treadmill to acclimate to the environment. During the experiment, mice remained sedentary or began a single bout of exercise (t = 0 min). Exercise started at 15 m/min (0% incline) for 3 min and then increased to 17 m/min throughout the rest of the experiment until t = 30 min (28, 45). Sedentary mice were allowed to move freely on the stationary treadmill for 30 min. In all mice, a bolus of 13 μCi of 2-deoxy-<sup>1,2</sup>H<sub>2</sub>glucose (1'<sup>14</sup>H<sub>2</sub>-DG) was injected into the jugular vein at t = 5 min for evaluation of tissue-specific glucose uptake. At the end of the experiment, mice were anesthetized with a jugular vein injection of pentobarbital sodium (3 mg). A tail blood sample was immediately obtained for determination of blood glucose levels. The brain and gastrocnemius and superficial vastus lateralis muscles from each limb were rapidly excised, frozen with liquid nitrogen-cooled tongs, and stored at −80°C. A blood sample was collected via cardiac puncture after exercise and used for plasma insulin and lactate determination.

Muscle glucose uptake determination. The determination of muscle glucose uptake was performed as previously described (8). Muscle samples and brain tissue (~30 mg) were homogenized with 1.5 ml of MilliQ water. Phosphorylated [1'<sup>14</sup>H<sub>2</sub>-DG ([1'<sup>14</sup>H<sub>2</sub>-DG-6-P) was extracted from an aliquot of centrifuged homogenates (6,000 rpm for 10 min at 4°C) using an anion exchange resin column (AG1-X8, Bio-Rad). Radioactivity of the samples was determined using a β-counter (Tri-Carb 2800TR, PerkinElmer, Chicago, IL). Glucose uptake for each muscle was expressed as an index of [1'<sup>14</sup>H<sub>2</sub>-DG-6-P accumulation in the muscle normalized to [1'<sup>14</sup>H<sub>2</sub>-DG-6-P in the brain of that mouse, as done previously (8, 11). Brain glucose uptake was used as a control for the integrated plasma [1'<sup>14</sup>H<sub>2</sub>-DG concentration differences over the duration of the experiments (8), as glucose uptake into the brain occurs via facilitated diffusion depending on glucose concentration gradients between the blood and brain tissue (31). In addition, intracellular glucose phosphorylation under normoglycemic conditions has no impact on brain glucose uptake (14). Importantly, [1'<sup>14</sup>H<sub>2</sub>-DG-6-P in the brain was not different between genotypes.

Blood and plasma biochemistry. Plasma insulin concentrations were determined using an enzyme-linked immunosorbent assay (Merckodia, Uppsala, Sweden) as per the manufacturer’s instructions. Plasma lactate concentrations were analyzed with the enzymatic method of Lowry and Passonneau (30). Blood glucose levels were determined directly from the tail blood by using an ACCU-CHEK Advantage monitor (Roche Diagnostics, Indianapolis, IN).

Immunoblotting. Immunoblotting was performed using ground frozen gastrocnemius muscle homogenized with 200× volume of solubilizing buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT, and 0.01% bromophenol blue) as described previously (15, 38). Five μg of total protein from whole homogenates was separated on SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA), which was then wet-transferred onto polyvinylidine difluoride (PVDF) membranes. Following membrane blocking with 5% skim milk in TBS solution, they were probed with the following primary antibodies overnight: phospho-AMPKα Thr<sup>172</sup> (1:1,000), phospho-TBC1D1 Ser<sup>665</sup> (1:1,000), AMPKα (1:1,000), TBC1D1 (1:500), and α-tubulin (1:1,000) (Cell Signaling Technology, Danvers, MA); nNOS (1:10,000), eNOS (1:10,000), and iNOS (1:2,000) (BD Biosciences, San Jose, CA); GLUT4 (1:8,000; Thermo Scientific, Rockford, IL); and actin (1:40,000; Sigma-Aldrich, St. Louis, MO). Chemiluminescent signal was developed with ECL substrate (SuperSignal West Femto, Pierce, MA) and was captured with a charge-coupled device (CCD) camera using Quantity One software (Bio-Rad). Prestained molecular weight markers were immediately imaged under a white light source without changing the membrane position. To quantify both phosphorylated and total protein abundance, phosphorylation-specific primary antibody signal was first determined and then stripped (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 0.8% β-mercaptoethanol), reblocked, and reprobed with primary antibody against the total protein. Loading control proteins were always probed on nonstripped membranes, and actin was used for all proteins except GLUT4. Actin and GLUT4 have similar molecular weights, and it was not possible to probe both of these proteins without undertaking the stripping process; therefore, α-tubulin was used as a loading control for GLUT4 abundance.

NOS activity assay. NOS activity was determined as described previously (29), using radiolabeled L-[1<sup>14</sup>C]arginine. NOS activity was expressed as picomoles of L-[1<sup>14</sup>C]citrulline formed per minute per milligram of protein. It was determined based on the difference between samples incubated with and without L-NAME.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed using the SPSS statistical package using one-factor ANOVA (genotype) or two-factor ANOVA (genotype and exercise). If there was a significant interaction, specific differences between mean values were identified using Fisher’s least significance test. The significance level was set at P < 0.05. No sex-specific differences were observed in muscle glucose uptake during exercise (male vs. female: nNOS<sup>−/−</sup>: 1.72 ± 0.23 vs. 1.50 ± 0.14, P > 0.05; nNOS<sup>+/−</sup>: 1.72 ± 0.10 vs. 2.10 ± 0.17, P > 0.05); therefore, data from male and female mice were pooled and analyzed together.

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Table 1. Body weight and exercise capacity of nNOS\(^{+/+}\) and nNOS\(^{-/-}\) mice

<table>
<thead>
<tr>
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<th>nNOS(^{+/+})</th>
<th>nNOS(^{-/-})</th>
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<tbody>
<tr>
<td>Male/female, n</td>
<td>15:15</td>
<td>6:8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.0 ± 0.8</td>
<td>23.6 ± 1.0</td>
</tr>
<tr>
<td>Max running speed, m/min</td>
<td>31.5 ± 0.9</td>
<td>29.4 ± 1.2</td>
</tr>
<tr>
<td>Max running time, min</td>
<td>17.7 ± 0.6</td>
<td>15.9 ± 0.9</td>
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Values are means ± SE; \(n = 30\) and 14 for neuronal NO synthase-\(\mu\) wild-type (nNOS\(^{+/+}\)) and deficient (nNOS\(^{-/-}\)) mice, respectively. *\(P < 0.05\); Fig. 1 A and C.

**RESULTS**

Body weight and exercise capacity of nNOS\(^{+/+}\) and nNOS\(^{-/-}\) mice. At 16 wk of age, the body weight of nNOS\(^{-/-}\) mice was significantly (\(P < 0.05\)) lower than that of nNOS\(^{+/+}\) littermates (Table 1). The ratio of male to female mice was not significantly different in either genotype (Table 1). The maximum running speed achieved during the exercise stress test was similar between genotypes (Table 1). The maximum running speed of nNOS\(^{-/-}\) mice tended (\(P = 0.10\)) to run for a shorter time than nNOS\(^{+/+}\) littermates (Table 1).

Blood glucose level. At the end of the experiment, blood glucose concentration from the sedentary mice was not significantly different between genotypes (7.9 ± 0.5 vs. 7.3 ± 0.8 mmol/l for nNOS\(^{+/+}\) and nNOS\(^{-/-}\), respectively, \(P > 0.05\)). Exercise had no effect on the blood glucose concentration compared with the sedentary state and remained similar between genotypes (8.7 ± 1.0 vs. 7.1 ± 0.3 mmol/l for nNOS\(^{+/+}\) and nNOS\(^{-/-}\), respectively, \(P > 0.05\)).

Skeletal muscle glucose uptake. Gastrocnemius muscle glucose uptake at rest (sedentary state) was not different between genotypes (Fig. 1A). Exercise significantly increased glucose uptake in gastrocnemius muscle (5- to ~7-fold), and the exercise-induced glucose uptake (fold increase) was significantly higher in nNOS\(^{-/-}\) than in nNOS\(^{+/+}\) mice (\(P < 0.05\); Fig. 1B). A similar muscle glucose uptake pattern was observed in the superficial vastus lateralis (SVL) muscle (Fig. 1 C and D).

Plasma insulin and lactate levels. At the end of the exercise, plasma insulin was not different between genotypes (1.00 ± 0.16 vs. 0.89 ± 0.17 \(\mu\)g/l for nNOS\(^{+/+}\) and nNOS\(^{-/-}\), respectively, \(P > 0.05\)). Plasma lactate was significantly elevated following exercise compared with the sedentary state (main effect, \(P < 0.05\)), and the increases following exercise were similar across genotypes (6.0 ± 0.5 vs. 5.4 ±
0.7 mmol/l for nNOS$^{+/+}$ and nNOS$^{-/-}$, respectively, $P > 0.05$).

**Protein expression and phosphorylation.** The expressions of actin and $\alpha$-tubulin proteins were not different between genotypes, and they were used as loading controls. Total AMPK$\alpha$ expression in gastrocnemius muscle was not different between genotypes (Fig. 2, A and B). For sedentary muscles, AMPK$\alpha$ Thr$^{172}$ phosphorylation relative to AMPK$\alpha$ abundance was also not different between genotypes. Exercise significantly increased skeletal muscle AMPK$\alpha$ Thr$^{172}$ phosphorylation relative to AMPK$\alpha$ abundance of both nNOS$^{-/-}$ and nNOS$^{+/+}$ mice compared with their respective sedentary group (Fig. 2C). The increase in AMPK$\alpha$ Thr$^{172}$ phosphorylation was significantly greater in nNOS$^{-/-}$ mice than in nNOS$^{+/+}$ mice (Fig. 2C). Expression of TBC1D1 in gastrocnemius muscle was also similar between genotypes (Fig. 3, A, and B), and there was no difference in sedentary TBC1D1 Ser$^{660}$ phosphorylation relative to TBC1D1 abundance between genotypes (Fig. 3C). Exercise increased TBC1D1 Ser$^{660}$ phosphorylation (Fig. 3C, main effect, $P < 0.05$). There was no nNOS detected in either nNOS$^{+/+}$ or nNOS$^{-/-}$ skeletal muscle. eNOS (Fig. 4A) and GLUT4 (Fig. 4B) protein expressions were not different between genotypes.

**Expression of nNOS$\mu$, nNOS splice variants, and NOS activity.** nNOS$\mu$ and nNOS$\beta$ (which are splice variants of nNOS) were detected in gastrocnemius muscles of nNOS$^{+/+}$ but not nNOS$^{-/-}$ mice (results not shown), as we previously reported in EDL muscles (16). Exercise caused a significant increase in NOS activity in gastrocnemius muscles from nNOS$^{+/+}$ mice ($P < 0.05$). Low levels of NOS activity were detected in gastrocnemius muscle from nNOS$^{-/-}$ mice (Fig. 5), which is in accord with previous data from EDL muscles (16) and brain tissues of nNOS$^{-/-}$ mice (18).

**DISCUSSION**

In this study, we observed that skeletal muscle glucose uptake increased to a significantly greater extent during 30 min of moderate-intensity treadmill running in nNOS$^{-/-}$ mice than in nNOS$^{+/+}$ littermates. The higher muscle glucose uptake in nNOS$^{-/-}$ mice was observed together with a greater increase in skeletal muscle AMPK phosphorylation during exercise in nNOS$^{-/-}$ mice. Given that NO is involved in GLUT4 translocation and nNOS$\mu$ is the main NOS isoform that produces NO during exercise in an integrated fashion, could affect skeletal muscle glucose uptake in an integrated fashion, could affect skeletal muscle glucose uptake. Under physiological in vivo exercise conditions, various factors beyond the signaling events within the muscle, including endocrine, vascular, neural, and internal milieu inputs that work in an integrated fashion, could affect skeletal muscle glucose uptake.

The nNOS$^{-/-}$ mice used in this study were generally comparable with their nNOS$^{+/+}$ littermates in a number of phenotypic features that may directly or indirectly influence muscle glucose uptake. The blood glucose levels at rest (sedentary, AJP-Endocrinol Metab • doi:10.1152/ajpendo.00513.2015 • www.ajpendo.org
...entary) and during exercise were similar in both genotypes, implying that the higher glucose uptake in \( \text{nNOS}^{\mu-/-} \) mice was not due to higher blood glucose levels (17). Similarly, plasma insulin levels after exercise were not different between genotypes, suggesting that the observed higher glucose uptake in \( \text{nNOS}^{\mu-/-} \) mice was not due to a potential additive effect of insulin on contraction-stimulated glucose uptake (56).

Exercise stimulated a greater muscle AMPK phosphorylation in \( \text{nNOS}^{\mu-/-} \) mice than in \( \text{nNOS}^{\mu+/+} \) littermates. AMPK is a metabolic fuel sensor that can be activated following metabolic stress/perturbations in which the degradation of ATP and the consequent accumulation of ADP and AMP increase the ADP/ATP and AMP/ATP ratio, which leads to an increase in phosphorylation of AMPK (50). The higher AMPK phosphorylation in \( \text{nNOS}^{\mu-/-} \) mice suggests that they may have endured a higher metabolic stress. However, both groups of mice had similar maximum exercise capacity (maximal running speed and time), which suggests that the metabolic stress levels may have been similar. Although not statistically significant, it is possible that the 10% longer running time in the control mice compared with the \( \text{nNOS}^{\mu-/-} \) mice could be due to the increased AMPK phosphorylation, we have no direct evidence to prove a causal relationship between these parameters in \( \text{nNOS}^{\mu-/-} \) mice, as we have not investigated glucose uptake during exercise in these mice while preventing the increase of AMPK activation. It may be worthwhile to compare skeletal muscle glucose uptake during exercise in both genotypes in vivo contraction in \( \text{nNOS}^{\mu-/-} \) muscles that are crossed with an AMPK dominant-negative mouse strain.

TBC1D1 has been implicated in the regulation of muscle glucose uptake during contraction/exercise, and glucose uptake is decreased in muscle overexpressing TBC1D1 mutated on several predicted AMPK phosphorylation sites (52). TBC1D1 Ser\(^{660}\) phosphorylation is one of the downstream effectors of AMPK (52) that is stimulated during contraction in mice (52) and during exercise in humans (22). The increase in TBC1D1 Ser\(^{660}\) phosphorylation with exercise in \( \text{nNOS}^{\mu-/-} \) mice suggests that an AMPK-TBC1D1 mechanism may potentially be involved in stimulating the higher glucose uptake in these mice, which, however, remained to be investigated. AMPK can also phosphorylate other downstream mediators such as AS160 to stimulate muscle glucose uptake (26), although there is evidence that AMPK-mediated AS160 phosphorylation in \( \text{nNOS}^{\mu-/-} \) mice with lower exercise-induced increases in blood flow to the contracting muscle and a likely greater hypoxic state in the muscles have no greater increase in AMPK phosphorylation during exercise (28). Indeed, we have shown previously that there is little effect of hypoxia on glucose uptake during exercise in humans (55). Therefore, hypoxia-induced increases in AMPK phosphorylation in \( \text{nNOS}^{\mu-/-} \) mice during exercise appear to be an unlikely stimulus for the greater increase in AMPK phosphorylation during exercise, and thus the reasons for this finding remain unclear.

Fig. 3. Representative blots for TBC1D1, TBC1D1 Ser\(^{660}\) phosphorylation, and actin (A), gastrocnemius muscle TBC1D1 abundance in sedentary muscles (B), and gastrocnemius muscle TBC1D1 Ser\(^{660}\) phosphorylation relative to TBC1D1 abundance (C). Data are means ± SE; \( n = 9 \) and 4 for sedentary \( \text{nNOS}^{\mu+/+} \) and \( \text{nNOS}^{\mu-/-} \), respectively, and 9 and 5 for exercise \( \text{nNOS}^{\mu+/+} \) and \( \text{nNOS}^{\mu-/-} \), respectively. \( \dagger P < 0.05 \) main effect for exercise.

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Glucose uptake does not have a role in muscle glucose uptake during contraction (51).

A caveat to the interpretation of the data using genetically modified mice needs to be considered. The loss of a protein of interest during development that spans the entire lifespan could possibly induce secondary adaptations including compensatory overexpression of closely related proteins (33). These changes could mask the effects elicited by the loss of the protein of interest. In this study, no compensatory increase in iNOS, eNOS, nNOS splice variants, or GLUT4, all of which could directly or indirectly affect muscle glucose uptake, were detected in nNOS<sup>−/−</sup> mice. Likewise, there was no difference in total AMPK or TBC1D1 expression between genotypes.

These data suggest that nNOS<sub>µ</sub>, similarly to ex vivo contraction (16), may not play a role in muscle glucose uptake during in vivo exercise, because total loss of nNOS<sub>µ</sub> did not attenuate glucose uptake or elicit a compensatory response in the proteins examined. It should be considered, however, that there may have been compensatory increases in the other potential proteins that may regulate skeletal muscle glucose uptake, including Ca<sup>2+</sup>/calmodulin-dependent protein kinase (CaM-KII) (57), protein kinase C (20), and Rac1/PAK1 (48).

In addition, an exacerbated ROS accumulation during exercise in nNOS<sub>µ</sub><sup>−/−</sup> mice may have contributed to the higher muscle glucose uptake. Muscle contraction/exercise increases ROS production in the heart and skeletal muscles (40, 46), and ROS increases muscle glucose uptake during ex vivo contraction (36, 46). Following acute exercise, there is significantly higher accumulation of ROS in the myocytes from mice lacking nNOS compared with controls (43). If a similar effect is conferred by nNOS in skeletal muscle during exercise as in the myocytes, it is plausible that muscle glucose uptake in nNOS<sub>µ</sub><sup>−/−</sup> mice could be increased as a result of ROS-induced glucose uptake. Nevertheless, some studies have shown that ROS has no stimulatory effect on muscle glucose uptake during in vivo conditions in rats (34) and humans (37).

The relative roles of nNOS<sub>µ</sub> could also be affected by the exercise intensity. Given that it has been shown that nNOS is expressed at higher levels in fast-twitch muscles than in slow-twitch muscles (24, 36) it would be expected that nNOS would have a greater contribution to glucose uptake during exercise in fast-twitch muscles and/or at higher exercise intensities. In fact, we have shown that NOS inhibition significantly attenuates the increase in glucose uptake during ex vivo contraction in EDL (mainly fast-twitch) but not in soleus (mainly slow-twitch) muscles (36). However, the fiber type effects on muscle glucose uptake during in vivo exercise are unclear. It is possible that there was no effect of a lack of nNOS<sub>µ</sub> on glucose uptake during exercise because the intensity of exercise was insufficient to substantially activate nNOS<sub>µ</sub>. How-

Fig. 5. Gastrocnemius muscle NOS activity at rest (sedentary) and during exercise. Data are means ± SE; n = 7 for nNOS<sub>µ</sub><sup>+/+</sup> and nNOS<sub>µ</sub><sup>−/−</sup>, respectively; and 7 and 5 for exercise nNOS<sub>µ</sub><sup>+/+</sup> and nNOS<sub>µ</sub><sup>−/−</sup>, respectively. *P < 0.05 vs. sedentary of the same genotype; †P < 0.05 vs. nNOS<sub>µ</sub><sup>−/−</sup> of the same condition.

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Fig. 4. Gastrocnemius muscle endothelial (e)NOS (A) and GLUT4 (B) protein expressions in sedentary state relative to actin and tubulin abundance, respectively. Data are means ± SE; n = 9 for nNOS<sub>µ</sub><sup>+/+</sup> and 4 for nNOS<sub>µ</sub><sup>−/−</sup>. For GLUT4 protein expression, bands at 45 and 40 kDa represent glycosylated and deglycosylated GLUT4, respectively. Both bands were used for data analysis.

Fig. 5. Gastrocnemius muscle NOS activity at rest (sedentary) and during exercise. Data are means ± SE; n = 7 and 3 for sedentary nNOS<sub>µ</sub><sup>+/+</sup> and nNOS<sub>µ</sub><sup>−/−</sup>, respectively; and 7 and 5 for exercise nNOS<sub>µ</sub><sup>+/+</sup> and nNOS<sub>µ</sub><sup>−/−</sup>, respectively. *P < 0.05 vs. sedentary of the same genotype; †P < 0.05 vs. nNOS<sub>µ</sub><sup>−/−</sup> of the same condition.
ever, the observed increase in NOS activity during exercise suggests that nNOSmia was indeed activated. Further studies should examine the effects of nNOSmia on glucose uptake during exercise at different intensities.

In this study, we observed very low levels of NOS activity in nNOSmia/mice, whereas eNOS abundance was not different between the genotypes. Together with the previous finding that NOS activity is normal or increased in eNOS+/− and eNOS−/− mice, respectively (28), these data indicate that nNOSmia is the predominant NOS isoform responsible for NOS activity in skeletal muscle. This finding is in agreement with a study showing that nNOS is the predominant NOS isoform that activates NO downstream signaling via cGMP during ex vivo contraction (27). Interestingly, eNOS abundance in skeletal muscle was not different between nNOSmia/mice and their wild-type littermate control mice in this study, as opposed to our previous study that found a compensatory increase of eNOS expression in nNOS−/− muscles (54). However, in that study the control mice were C57Bl/6 mice rather than littermate controls (54). Others have also found no compensation of eNOS expression in myocytes and uteri of mice lacking nNOS compared with their wild-type littersmates (19, 39). This highlights the importance of using littermate controls as a proper experimental control.

In summary, nNOSmia is not essential for skeletal muscle glucose uptake during in vivo exercise. The greater muscle glucose uptake observed in nNOS−/− mice than in nNOS+/− mice during moderate-intensity treadmill exercise may be due to the observed greater increase in AMPK activation during exercise.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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GLUCOSE UPTAKE DURING EXERCISE IN nNOS−/− KO MICE


