The phenotype of capillaries in skeletal muscle of neuronal nitric oxide synthase (nNOS)-knockout mice

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Running title: Capillary phenotype in skeletal muscle of nNOS-KO mice

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Abstract
Because neuronal nitric oxide synthase (nNOS) has a well-known impact on arteriolar blood flow in skeletal muscle, we compared the ultrastructure and the hemodynamics of/in the ensuing capillaries in the extensor digitorum longus (EDL) muscle of male nNOS-KO mice and wild-type (WT) littermates.
The capillary-to-fiber (C/F)-ratio (-9.1%) was lower \( (P \leq 0.05) \) in the nNOS-KO mice than in the WT mice, while the mean cross-sectional fiber area (-7.8%) and the capillary density (-3.1%) varied only non-significantly \( (P > 0.05) \). Morphometrical estimation of the area occupied by the capillaries, the volume and surface densities of the subcellular compartments differed non-significantly \( (P > 0.05) \) between the two strains. Intravital microscopy revealed neither the capillary diameter (+3% in nNOS-KO mice versus WT mice) nor the mean velocity of red blood cells in EDL muscle (+25% in nNOS-KO mice versus WT mice) to significantly vary \( (P > 0.05) \) between the two strains. The calculated shear stress in the capillaries was likewise non-significantly different (3.8 ± 2.2 dyne/cm² in nNOS-KO mice and 2.1 ± 2.2 dyne/cm² in WT mice; \( P > 0.05 \)). The mRNA levels of VEGF-A were lower in the EDL muscle of nNOS-KO mice than in the WT littermates (-37%; \( P \leq 0.05 \)), whereas mRNA levels of KDR/VEGF-R2 (-11%), HIF-1alpha (+9%), FGF-2 (-14%) and thrombospondin-1 (-10%) differed non-significantly \( (P > 0.05) \). Our findings support the contention that VEGF-A mRNA expression and C/F-ratio but not the ultrastructure or the hemodynamics of/in capillaries in skeletal muscle at basal conditions depend on the expression of nNOS.
Introduction

Skeletal muscle can adapt to diverse external stimuli, as it e.g. atrophies to immobilization (19), hypertrophies after overload (17) and undergoes mitochondriogenesis after endurance exercise (2). Although most studies dealing with the plasticity of skeletal muscle have focused on the adaptive capacity of the fibers per se, there is evidence suggesting that the capillaries also respond to pathological situations or changes in the microenvironment, e.g. by a thickening of their basement membrane (4) and by undergoing either regression after heart failure (37), inflation during ischemia (16) or angiogenesis in response to training (20). However, the molecular signals that orchestrate this interplay between the skeletal muscle fibers and the endothelial cells (EC) of their surrounding capillaries are largely unknown (21).

Nitric oxide (NO), which is generated by the catalytic activity of intracellular NO synthases (NOS), might diffuse from cells into the surrounding milieu, thereby activating signaling cascades in neighboring cells (26). As such, NO would be an ideal molecule to coordinate the paracrine interaction between skeletal muscle fibers and the capillaries.

Skeletal muscle contains high concentrations of neuronal NOS (nNOS) anchored at the sarcolemma (29). In rodents, it is preferentially associated with the fast twitch, oxidative (type IIa) fibers (43) whereas in humans, no fiber type-specific localization of nNOS has been identified (18, 23). As autocrine signaling molecule, NO generated by the catalytic action of nNOS influences the metabolism and the contractile activity of skeletal muscle fibers (8). Furthermore, several studies suggest that nNOS-generated NO also acts as a paracrine signaling molecule on the vascular system in skeletal muscle. Using specific chemical inhibitors, nNOS has been shown to promote the vasodilation of arteries and arterioles in skeletal muscle.
of both rats (10) and humans (7). The impact of nNOS-derived NO on vasodilation could be a direct or an indirect consequence of the interaction with adenosine (38). Using nNOS-knockout (KO) mice, nNOS has been shown to control the cGMP-dependent relaxation of smooth muscle cells and thus to contribute to the arteriolar blood flow regulation in contracting skeletal muscle (31). However, it has not yet been ascertained whether nNOS-generated NO influences the structure or function of capillaries in skeletal muscle.

The targeting of nNOS to the sarcolemma is pivotal for its proper paracrine signaling function. Its down-regulation and redistribution in skeletal muscles of patients with Duchenne’s muscular dystrophy is associated with the force loss (32) and the excessive fatigue often observed in these patients after mild exercise (28). Accordingly, it has been shown using mouse models that the redistribution of nNOS decreases cGMP-mediated vasodilation of the supplying arteries during contraction and accounts causally for the exaggerated exercise-induced fatigue associated with dystrophies (28). In addition, a correct anchorage of nNOS in the sarcolemma is a prerequisite for the antagonistic inhibition of sympathetic vasoconstriction in rodents (9) and healthy humans (30).

Given the well-known impact of nNOS on arteriolar blood flow in skeletal muscles and its close expression in proximity to the microcirculation, we hypothesized that nNOS-generated NO influences the phenotype of the capillaries in skeletal muscle. To test this hypothesis, we compared the (i) vascularity, (ii) the ultrastructure and (iii) the hemodynamic environment of the capillaries and as well as (iv) the mRNA expression patterns of genes governing for the communication between muscle fibers and the microvasculature in the EDL muscle of nNOS-KO mice and wild-type (WT) mice.
Materials and Methods

Animals
The nNOS-knockout (KO) strain of mice utilized in this study was originally generated by the recombinant replacement of exon-2 of the nNOS-gene with a neomycin cassette (22). Breeding pairs with this genotype were purchased from Jackson Laboratories (Bar Harbor, Maine, ME, USA). These mice were crossed into the C57BL/6J wild-type (WT) background for eight generations. All mice were bred in our animal-unit under standard conditions, as previously described (5). By inbreeding of heterozygous (WT x nNOS-KO) mice (F1-offsprings), seven litters were obtained (F2-offsprings) which were subjected to genotyping using DNA from biopsies of the tails tips. All homozygous male mice of these litters (9 nNOS-KO mice and 8 WT mice) were euthanized at the age of 16-weeks to collect the skeletal muscles for molecular and structural analyses. Intravital microscopy was performed on 16-20-week-old male WT and homozygous nNOS-KO mice. The mice were euthanized in accordance with the regulations of the Free University of Berlin and the University of Bern, as well as with those of the state authorities for animal welfare in Berlin (LAGETSI) and Bern (Cantonal committee on animal welfare (51/08)).

DNA and RNA isolation
DNA was extracted from biopsies of the tips from the tail tips of the mice using the DNeasy Blood and Tissue Kit according to the protocol of the supplier (Qiagen, Hilden, Germany).
RNA was extracted from 100 of 25-µm-thick cryosections using the RNeasy Mini Kit (Qiagen), as previously reported (23).

**Genomic PCR and RT-PCR**

Genotyping of the mice was conducted by genomic PCR applying the protocol published by the Jackson Laboratories, Bar Harbor, ME, USA (http://jaxmice.jax.org/strain/002986.html#genotype) with specific primer pairs. For reverse transcription (RT)-PCR, the GoTaq Hot Start Polymerase (Promega, Dübendorf, Switzerland) was used. Specific primer pairs were designed using Primer3 software and purchased from Microsynth (Balgach, Switzerland). PCR fragments were visualized by 1% agarose gel electrophoresis supplemented with 0.5 µg/ml ethidium bromide.

**Real-time PCR**

Real-time PCR was performed using the ABI Prism 7900 HT sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) and SYBR Green PCR Master Mix for quantitative PCR (Applied Biosystems), as previously reported (23). Primers were designed using the software Primer Express (Applied Biosystems). For normalisation, 18S rRNA levels were measured. Data were evaluated by the relative quantification method ($2^{\Delta \text{CT}}$).

**Immunoblotting**

Immunoblotting was performed using 50 µg of protein from skeletal muscle, as previously described (12). A polyclonal antibody (N-7280, Sigma), which specifically identifies amino acids 1409-1429 in the C-terminal region of nNOS, was incubated in a 1:10,000 dilution in washing buffer (0.1% (w/v) Tween 20 in PBS, pH 7.4)
overnight at 4°C. The immunoblots were developed by enhanced chemiluminescence (GE Healthcare Life Sciences, Glattbrugg, Switzerland). Additional immunoblotting was performed with antibodies against eNOS (610296, BD Biosciences; Allschwil, Switzerland, in a 1:250 dilution) and iNOS (610332, BD Biosciences, in a 1:500 dilution). Ponceau Red staining of the blot matrices was conducted for the normalization of the loading required for the densitometric quantification (12).

Quantification of NO levels in muscle homogenates

Tibialis anterior (TA) muscle was homogenized in reaction buffer (final concentration: 1 mM BH₄, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride in PBS-HCl, pH 7.4) at 4°C. Subsequently, 2 mg protein of the homogenates were supplemented with NADPH (1 mg/ml), L-arginine (2 mM) and calmodulin (10.000 U; Sigma, Deisenhofen, Switzerland), incubated for 30 min at 37°C and centrifuged through a 10 kDa cut-off filter (M0411; Sigma). The nitrate and nitrite levels in the filtrates were quantified in triplicate using a fluorometric assay kit (780051) according to the protocol of the manufacturer (Cayman, Ann Arbor; MI, USA). The two-step principle of this assay is based on the catalytic reduction of NO-derived nitrate to nitrite, which converts 2,3-diaminonaphthalene (DAN) to a proportionally fluorescence-emitting compound.

Light microscopy

Specimens from the mid-belly of the extensor digitorum longus (EDL) muscle were chemically fixed for several days at 4°C in 6.5% (v/v) glutaraldehyde diluted in 0.1 M sodium cacodylate buffer, pH 7.4. Tissue blocks of the specimen, with a volume of approximately 0.5 mm³, were prepared, embedded in Epon 812 (Fluka, Buchs,
Switzerland), post-fixed in 1% (w/v) osmium tetroxide and block-contrasted, as previously described (4). One-micrometer-thick semithin sections were cut using a diamond knife and stained with Toluidine Blue.

For the morphometric analysis, transverse or slightly oblique sections through the EDL muscle were cut from two randomly selected Epon blocks. A systematic sampling strategy was implemented to acquire 10 microphotographs of each section at a magnification of x63 in a Leica Leitz DMR light microscope. Using these light photographs, the number of capillaries and that of muscle fibers were estimated taking into account the forbidden line rule (50). These microphotographs were overlain with a quadratic grid consisting of 10 x 10 test lines separated by a distance of 10 µm. For an estimate of the mean cross-sectional fiber area (MCSFA), the number of test points falling on fibers divided by the total number of test points and multiplied by the total imaging area was divided by the number of fibers that fell within the confines of the grid.

Transmission electron microscopy and morphometry

For transmission electron microscopy (TEM) in a Philips EM 400, ultrathin sections were prepared from two randomly selected Epon blocks of each EDL muscle, as previously described (4).

On each section, 10 randomly emerging capillaries were photographed with a MORADA digital camera (OSIS, Münster, Germany) at a magnification of x14.000. The microphotographs were overlain with a quadratic grid consisting of 12 x 12 test lines separated by a distance of 0.44 µm. The 144 points of intersection together represent an imaging area 0.1936 µm².

For an unbiased determination of the mean volume density (Vv) of the various estimators (capillary lumen, endothelial cells (EC), pericytes (PC) and peri-capillary
basement membrane (BM)), the number of test points falling on each was set in relation to the total number of reference points. The mean cross-sectional areas of the capillaries and the various compartments were calculated by multiplying the number of test points with the imaged area.

The number of test line intersections of the counting grid with (i) the luminal side of the EC, (ii) the abluminal side of the EC, and (iii) the border between the BM and the endomysium, was set according to the number of test points that is permitted for an approximation of surface density using the formula $S_v = I / L$, where with ‘$I$’ is the number of intersections and ‘$L$’ being the sum of the length of the test lines on the counting grid (50).

The PC coverage of capillaries was estimated by dividing the number of test lines that simultaneously crossed the EC/BM border and the PC surface by the number of test lines that crossed the EC/BM border. Accordingly, the proportion of intraluminal EC protrusions was estimated by dividing the number of test lines that crossed these filopodia with those by the total number of test lines that crossed the luminal side of the EC.

**Intravital microscopy**

The 16- to 20-week-old male nNOS-KO and WT mice were anesthetized with an intraperitoneal injection of a mixture of xylazine (10 µg/g of body mass), ketamine (100 µg/g body mass) and atropine (0.1 µg/g of body mass).

The carotid artery was exposed, and a transducer (PDCR 75, Natec Schultheiss, Garching, Germany) was connected to it for the continuous monitoring of the arterial blood pressure (BMT Medical Technology) and heart rate (Mino PFM 2 Hugo Sachs Elektronik, March-Hugstetten, Germany).
The EDL muscle was surgically exposed by careful displacement of the tibialis anterior (TA) muscle. To maintain body temperature at 37°C, the animals were placed on a heating pad, which was positioned for intravital observation in a Leitz microscope (Wetzlar, Germany). The muscle was superfused at a rate of 3 ml/min with Tyrode's solution (131.6 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 3 mM NaHCO₃, pH 7.35–7.4), which was equilibrated with a gaseous mixture of 5% carbon dioxide and 95% nitrogen.

Capillaries in the EDL muscle were observed using a water-immersion objective (x25/N0.6). Images were digitally recorded on video tapes (DVCAM DSR-25, Sony) using an optical system that permitted epi-illumination of the muscle surface. Capillaries were photographed for 10 min in 3-6 fields, each field containing 2-6 capillaries. At the end of the period of observation, the mice were euthanized with a lethal dose of pentobarbital (160 mg/ml).

The velocities of the red blood cells (RBC) and the diameters of the capillaries were measured off-line using a computer-assisted image analysis-system, which complied with the spatial-correlation principle (34). Using the video recordings, the light intensity pattern along the centerline of any given capillary was monitored over a length of about 80-120 µm on each of 200 consecutive frames. Experimental data from five nNOS-KO mice and five C57BL/6J mice were included in the analysis.

Shear stress in the capillaries was calculated using the formula \( \tau = \eta \times \frac{8 \times V_{RBC}}{d} \), where ‘\( \tau \)’ is the shear stress (in dyne/cm²), ‘\( \eta \)’ the viscosity (= 0.02 Pa) (44), ‘\( V_{RBC} \)’ the velocity of the RBC (in m/s) and ‘\( d \)’ the capillary diameter (in µm), as previously described (25).
Statistics

All numerical data are expressed as mean values together with the standard deviation. Parameters pertaining to the structural, physiological and molecular analyses were compared using a paired Student’s t-test. The significance level was set at $P \leq 0.05$. 
Results

To confirm that the skeletal muscles of nNOS-KO mice used in this study did not express the deleted nNOS, genomic PCR, RT-PCR and immunoblotting analyses were undertaken.

Genotyping revealed the DNA of nNOS-KO mice to lack exon-2 of the nNOS gene but to contain that for neomycin resistance with which it was substituted. RT-PCR analysis was conducted with skeletal muscle mRNA of nNOS-KO mice. An exon-5 reverse primer was combined with forward primers that were derived from the four mRNA variants of the 5'-UTR (exons 1a, 1c, 1f and 1g) that are generated by alternative splicing of the murine nNOS gene (Fig. 1a). When exon-1c was used as the forward primer, two cDNA bands (consisting of 450 and 300 base-pairs, respectively) were detected. Nucleotide sequencing confirmed the 1c-3-4-5 and the 1c-4-5 exon compositions of these PCR-amplificates (data not shown). When any of the other three exon-variants (1a, 1f or 1g) were used as forward primer, no cDNA was amplified.

Further RT-PCR analysis was performed using seven different pairings of exon-specific primers, which covered the complete coding sequence of the nNOS alpha-isoform downstream of the initial AUG codon in exon-2. When RT-PCR was performed using skeletal muscle mRNA of nNOS-KO mice with a primer pair specific for the nucleotide sequence between exon-2 and exon-6, no cDNA was amplified (Fig. 1b). Somewhat surprisingly, one cDNA band was amplified when RT-PCR was conducted using the overlapping primer pairings: exon-5/exon-11, exon-10/exon-15, exon-17/exon-22, exon-21/exon-26 and exon-25/exon-28. A cDNA double band was obtained using the combination of primer pairings exon-14/exon-18 that represent PCR-amplificates with and without exon-mu. Nucleotide sequencing confirmed the identity of all PCR-amplificates (data not shown). Similar results were obtained using
extracts of the EDL muscle (Fig. 1a,b), the tibialis anterior muscle (TA) and the rectus femoris muscle (data not shown).

The immunoblotting analysis confirmed the absence of nNOS-immunoreactive bands in TA muscle extracts of nNOS-KO mice as well as the presence of the nNOS alpha- and beta-isoforms in those of the WT counterparts (Fig. 1c). Similar results were obtained using extracts of the EDL muscle (data not shown). Levels of eNOS were significantly higher (23%; \( P \leq 0.05 \)) in TA muscle extracts of nNOS-KO mice compared to those in WT mice (Fig. 1d). We were not able to detect iNOS in skeletal muscle extracts of the two strains (data not shown). The concentration of NO-derived nitrate/nitrite was significantly lower (-68%; \( P \leq 0.05 \)) in TA homogenates of nNOS-KO mice than WT mice (Fig 1e).

The body weights of the male nNOS-KO mice and WT mice were monitored from the 8th to the 16th week (Fig. 1f). At all junctures, the nNOS-KO mice were permanently weighing less than their WT littermates (7.2-9.6%), even though the consumption of food and water did not differ significantly between the two strains (data not shown). The mass of the skeletal muscles in the two strains differed significantly (\( P \leq 0.05 \)) when expressed in absolute terms but not when related to body weight (data not shown).

The vascularity of the EDL muscle was evaluated light-microscopically using semithin sections (Fig. 2). The capillary-to-fiber (C/F)-ratio was significantly lower in the EDL muscle of nNOS-KO mice than in the WT littermates (-9.1%; \( P \leq 0.05 \)). In contrast, differences in the MCSFA (-7.8%) and capillary density (-3.1%) did not attain statistical significance (\( P > 0.05 \)).

The ultrastructural characteristics of the capillaries in the EDL muscle of the two strains of mice were evaluated in the transmission electron microscope by morphometrically estimating the area occupied by the capillaries as well as their
volume and surface densities (Tab. 1). The PC coverage and the percentage of the capillary lumen that was occupied by EC-protrusions (filopodia) were likewise assessed (Tab. 1). In no case differences between the two strains of mice were observed, thereby indicating that the lack of nNOS expression has no impact on the ultrastructure of capillaries in the EDL muscle.

Continuous monitoring of the arterial blood pressure during intravital microscopy revealed similar mean values for the two strains of mice (95 ± 3 mmHg in nNOS-KO mice versus 90 ± 6 mmHg in WT mice; \( P > 0.05 \)), as shown in Fig. 3a.

The hemodynamics of the microvasculature in the EDL muscle of the two strains of mice was likewise evaluated during intravital microscopy (Fig. 3b-d). Neither the mean capillary diameter (6.5 ± 1.0 µm in nNOS-KO mice and 6.3 ± 0.9 µm in WT mice; Fig. 3b) nor the mean velocity of red blood cells (91 ± 51 µm/s in nNOS-KO mice and 73 ± 47 µm/s in WT mice; Fig. 3c) differed significantly \( (P > 0.05) \) between the two strains of mice. Consistent with these findings, the calculated wall shear stress in the capillaries was not significantly different in the two strains (3.8 ± 2.2 dyne/cm² in nNOS-KO mice and 2.1 ± 2.2 dyne/cm² in WT mice; \( P > 0.05 \); Fig. 3d).

The mRNA levels of five growth factors involved in microvascular remodeling were monitored in the EDL muscle of the two strains of mice by Realtime-PCR (Fig. 4). VEGF-A were significantly lower in the EDL muscle of nNOS-KO mice than in that of WT littermates (-37%; \( P \leq 0.05 \)), whereas mRNA levels of KDR/VEGF-R2 (-11% in nNOS-KO mice), HIF-1alpha (+9% in nNOS-KO mice), FGF-2 (-14% in nNOS-KO mice) and thrombospondin-1 (-10% in nNOS-KO mice) did not attain statistical significance \( (P > 0.05) \).
Discussion

The deletion of the nNOS exon-2 in the genome of the nNOS-KO mice used in this study was confirmed by a genomic PCR analysis. As a result, skeletal muscles lack the expression of nNOS mRNA-species with the initial AUG codon that is required for the translation of the alpha-isoform. RT-PCR analysis combined with nucleotide sequencing of the PCR fragments revealed the absence also of the beta-isoform and other splice variants of nNOS with alternative initial codons. Consistent with these data, no immunoreactivity for nNOS proteins was detected in skeletal muscles of nNOS-KO mice by immunoblotting, which confirms our findings of a previous immunohistochemical analysis (5). Hence, the expression of nNOS protein is indeed completely abolished in skeletal muscles of nNOS-KO mice. In a previous investigation using mice with the same genetic background as those of this study, the expression of nNOS beta- and gamma-isoforms in skeletal muscle was reported which are to be generated by unusual alternative splicing of exon-1 in the 5’-UTR (8). The reason for the contradictory findings is unclear.

Previous studies have revealed that nNOS-KO mice have lower body weights than WT mice (41). In addition, the tibialis anterior (TA) muscle is not only less massive and mechanically weaker, but also characterized by a poorer microtubular organization (42). Furthermore, it has been shown that the fiber type composition and the mean cross-sectional fiber area (MCSFA) in the TA muscle are also significantly different between the two strains of mice (9). We have used the TA muscle to ascertain the expression patterns of the nNOS beta-isoform in skeletal muscle fibers (6).

The mass of the skeletal muscles was significantly lower in the nNOS-KO mice than the WT mice only when expressed in absolute terms but not when related to the body weight. It still remains to be clarified whether this difference in muscle mass
between the two strains of mice goes back to a reduction in the number of muscle fibers or a decrease in MCSFA, which actually tended to be lower (−7.8%; *P* = 0.11) in EDL of nNOS-KO mice than in that of WT mice.

Although the eNOS protein levels were 23% higher, the concentration of NO-derived nitrate/nitrite was 68% lower in TA muscle homogenates of nNOS-KO mice than in those of WT mice. These data corroborate that the genetic lack of nNOS is not entirely compensated by the increase of the catalytic activity of another NOS form in skeletal muscles at basal conditions.

In the present study, the EDL muscle rather than the TA muscle was analyzed. In WT (C57BL/6) mice, both skeletal muscles contain a high proportion of type IIa fibers (2). In rodents, nNOS expression is predominantly expressed in these fibers (43). However, the different fiber types are more homogenously distributed in the EDL muscle than in the TA muscle. The latter is subdivided into distinct deep, mid and superficial regions, each of which is characterized by a different composition of fiber types and capillaries (14). Consequently, the chances of introducing a sampling bias into the analysis of the capillary network (e.g. during intravital microscopy) are lower for the EDL muscle than for the TA muscle.

In accordance with data published by Lidington et al. (33), the mean arterial blood pressure (MABP) monitored during intravital microscopy did not differ significantly between the two strains of mice. On the other hand, Thomas et al. (9) have reported the MABP to be higher in WT (C57BL/10) mice than in nNOS-KO mice (103 mmHg versus 82 mmHg). Although we currently cannot account for these discrepant findings, differences in either the genetic background of the nNOS-KO mice, the age of the animals or the experimental set-up could be contributory factors.

Shear stress has a profound influence on the characteristics of the capillary network in skeletal muscles (15, 24). Acting tangentially on the luminal surface of the
endothelium, it is sensed by the EC to induce signaling cascades important for vascular remodeling (13). Since in the capillaries neither the viscosity of the blood nor their caliber is subject to much variation, shear stress in the microcirculation depends mainly on the velocity of the RBC (25). Because arteriolar vasodilation is impaired and, hence, blood flow is reduced in skeletal muscle of nNOS-KO mice (31, 6), we suspected that the shear stress in capillaries would be lower in the EDL muscle of nNOS-KO mice than in that of WT mice. However, the intravital microscopy analysis revealed no differences in the RBC velocity and the capillary diameter – and hence in the shear stress acting on the capillary wall - between the two strains of mice.

These findings indicate that the blood flow in the capillary network of the EDL muscle of the nNOS-KO mice was adjusted to abolish the effects of impaired vasodilation. This tenet is supported by the morphometric findings, which revealed no differences in the ultrastructure of the capillaries in EDL muscle between nNOS-KO mice and WT mice.

The functional integrity of the microcirculation in skeletal muscles depends upon the coordinated expression of crucial angiogenic and anti-angiogenic growth factors, several of which have been identified (39). The 165 KDa-isoform is the major splicing variant of VEGF-A, which is expressed in skeletal muscle fibers (20) and EC of skeletal muscle capillaries (11). By binding to KDR - its functionally relevant receptor - VEGF-A initiates a signaling cascade in target EC. Muscle-specific VEGF-A-KO mice have a lower capillary density in their skeletal muscles suggesting that this factor is pivotal for the maintenance of capillaries at basal conditions (40). Likewise, knockout mice that lack the gene for either thrombospondin-1 (35) or HIF-1alpha (36) exhibit a lower numerical density of capillaries in their skeletal muscles than their WT counterparts, thereby confirming the importance of these proteins in
the microvascular remodeling at basal conditions. FGF-2 is also believed to play a role in this process, since its administration has been shown to enhance the numerical density of skeletal muscle capillaries (1).

In the present study, the mRNA levels of VEGF-A were significantly higher in the EDL muscle of nNOS-KO mice than in their WT littermates, whereas those of KDR/VEGFR-2, HIF-1alpha and FGF-2 and thrombospondin-1 did not differ significantly between the two strains. These findings indicate that in vivo the expression of VEGF-A is modulated up-stream by the activity of nNOS at basal conditions. This interpretation agrees with other findings that NO impairs the expression of VEGF-A in skeletal muscles (7). In contrast to the findings of this study with nNOS-KO mice, the levels of VEGF-A expression in extracts of the TA muscle and those of endothelial cells were higher in eNOS-KO mice than in the corresponding WT mice (3). Hence, the influence of NO on expression of VEGF might depend upon the site of NO at which it is generated, as previously suggested (15, 1).

Studies using nNOS-KO mice (12) and nNOS-overexpressing transgenic mice (45) have previously shown that an alteration of the nNOS expression levels in skeletal muscle is accompanied by a change in the availability of superoxide and, subsequently, hydrogen peroxide (SOD-1 dependent) and/or peroxynitrite. These reactive oxygen species (ROS) exert important cell signaling functions (27). It thus is possible that the lower levels in VEGF-A mRNA and the lower C/F-ratio in EDL muscle of nNOS-KO described in this study are caused directly by the decrease in NO availability and/or indirectly by changes in the ROS metabolism.

**Perspectives and Significance**
In the present study, we hypothesized that nNOS-generated NO influences the phenotype of the capillaries in skeletal muscle and thus expected to find differences in structure and function of the microvasculature within the EDL muscle of nNOS-KO mice compared to their WT littermates. However, neither the MCSFA and the hemodynamics nor the ultrastructure of the capillaries in the EDL muscle differed significantly between the two strains of mice. The expression levels of four growth factors that are involved in microvascular remodeling (KDR/VEGF-R, HIF-1alpha, FGF-2 and thrombospondin-1) were not significantly altered by the nNOS deficiency. In contrast, the C/F-ratio and the mRNA-levels of VEGF-A were significantly lower in the EDL muscle of the nNOS-KO mice than in that of their WT counterparts. However, our experimental data do not permit to ascertain whether the capillary density is a cause or a consequence of the altered VEGF-A expression in EDL muscle of nNOS-KO mice, or whether the relationship between these parameters is coincidental. It furthermore remains to be elucidated whether the changes in capillarity are caused directly by nNOS/NO-mediated signaling or indirectly as a consequence of changes in the ROS metabolism. By addressing these questions, our understanding of the adaptive establishment of the capillary phenotype in skeletal muscle and, thus, of the physiological response to exercise or the pathogenesis of dystrophic diseases might be improved.

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Conflicts of interest

There is no conflict of interest.

Author contributions
References


Figure Legends

Fig. 1: Comparison of the phenotypes in male nNOS-KO mice and WT mice.
A, B: Expression of the mRNA for nNOS in the EDL muscle of nNOS KO mice. (A): Representative agarose gels after RT-PCR using nNOS-specific forward primers for the murine exons-1a, -1c, -1f and -1g, and a reverse primer for exon-5. Only when using the forward primer derived from exon-1c, two cDNA bands were detected, namely, two, consisting of 450 and 300 base-pairs (bp), respectively. Nucleotide sequencing revealed the exon-structures of these two mRNA-variants of nNOS to be 1c-3-4-5 and 1c-4-5, respectively, thus encoding mRNA-species without the initial codon in exon-2. (B): Evaluation of the mRNA-expression patterns of nNOS isoforms using overlapping primer-pairings spanning the exon-range 2-28. Note that no cDNA band was amplified, when using the primer pair exon-2/exon-6. Combining the primers derived from exon-14 and 18 yielded a double band, owing to the alternative splicing of exon-mu between exon-16 and 17. The identity of each band was confirmed by nucleotide sequencing.
C: Immunoblotting analysis of detergent extracts of the TA muscle revealed the presence of the alpha- (160 kDa) and the beta- (140 kDa) isoforms of nNOS in WT mice but not in nNOS-KO mice. Protein loading of the gels was evaluated by Ponceau Red staining of the blot matrices.
D: Immunoblotting analysis using detergent extracts of the TA muscle demonstrated significantly 23% higher levels ($P \leq 0.05$) of eNOS in male nNOS-KO mice (n= 4) than in WT littermates (n= 4).
E: Quantification of the concentration of NO-derived nitrate/nitrite in TA muscle homogenates of male nNOS-KO mice (n= 4) and WT littermates (n= 4). Values represent means ± standard deviation. Significance levels: ** = $P \leq 0.01$. 
F: Temporal course of changes in the body weights of male nNOS-KO mice (n= 9) and WT littermates (n= 8) between weeks 8-16. Values represent means ± standard deviation. Significance levels: * = $P \leq 0.05$ and ** = $P \leq 0.01$.

Fig. 2: Quantification of capillarity and fibers size in the EDL muscle of nNOS-KO mice and WT mice.
The capillary/fiber (C/F)-ratio (A), the numerical density of capillaries (B) and the mean cross-sectional fiber area (MCSFA; C) were estimated morphometrically on Toluidene Blue-stained semithin sections through the EDL muscle of male nNOS-KO mice (n= 9) and WT littermates (n= 8). Values represent means ± standard deviation from eight mice in each group. * = refer to values of WT mice significantly differing ($P \leq 0.05$) from values of nNOS-KO mice.

Fig. 3: The hemodynamic characteristics of capillaries in the EDL muscle do not significantly differ between nNOS-KO mice and WT mice
A: The mean arterial blood pressure (MABP) as recorded during the intravital microscopy analysis. B-D: The diameter of capillaries (B) and the velocity of the red blood cell (RBC) therein (C) were monitored in EDL muscle of nNOS-KO mice (n= 5; N= 66 capillaries) and WT littermates (n= 5; N= 78 capillaries). These data were used to calculate the shear stress in the capillaries (D). Values represent means ± standard deviation.

Fig. 4: Quantification of the mRNA-expression levels of genes that are involved in the establishment of the microcirculation in skeletal muscle. The mRNA levels of VEGF-A, KDR/VEGF-R2, HIF-1alpha, FGF-2 and thrombospondin-1 were determined in the EDL muscle of nNOS-KO mice (n= 6) and WT littermates.
(n= 6) by Realtime-PCR. Note that mRNA levels of nNOS were also quantified as positive control. Values represent means ± standard deviation. Significance levels (nNOS-KO mice versus WT mice): *: $P \leq 0.05$; *** = $P \leq 0.001$.

**Table 1: Morphometric characterization of the capillary ultrastructure in the EDL muscle of nNOS-KO and WT mice.**

Transmission electron microphotographs of capillaries of male nNOS-KO mice (n= 9) and WT littermates (n= 8) in the EDL muscles were used for a morphometric analysis of the listed parameters. Values represent means ± standard deviation for 10 capillaries in each of the mice. NS: statistically non-significant ($P > 0.05$). A: mean cross-sectional area; Vv: volume density; Sv: mean surface density; cap: capillary; BM: basement membrane; PC: pericyte; EC: endothelial cell.
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Fig. 1
Table 1: Morphometric characterization of the capillary ultrastructure in the EDL muscle of nNOS-KO mice.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=8)</th>
<th>nNOS-KO (n=9)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (cap) with BM and PC µm²</td>
<td>13.2 ± 2.3</td>
<td>12.3 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>A (cap) without BM and PC µm²</td>
<td>10.5 ± 2.1</td>
<td>10.1 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (lumen; cap) %</td>
<td>0.49 ± 0.06</td>
<td>0.50 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (EC; cap) %</td>
<td>0.31 ± 0.03</td>
<td>0.31 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (PC; cap) %</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (BM; cap) %</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit (= Vv (RBC; lumen))%</td>
<td>0.46 ± 0.11</td>
<td>0.51 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Vv (EC nucleus; EC) %</td>
<td>0.09 ± 0.03</td>
<td>0.11 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Sv (lumen, capillary) µm⁻¹</td>
<td>0.55 ± 0.07</td>
<td>0.55 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Sv (EC, capillary) µm⁻¹</td>
<td>0.59 ± 0.05</td>
<td>0.59 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Sv (BM, capillary) µm⁻¹</td>
<td>0.64 ± 0.05</td>
<td>0.65 ± 0.06</td>
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<tr>
<td>Pericyte coverage %</td>
<td>0.25 ± 0.06</td>
<td>0.22 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Intraluminal filopodia propor</td>
<td>0.11 ± 0.06</td>
<td>0.10 ± 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>