NO synthase II in mouse skeletal muscle is associated with caveolin 3

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The inducible-type NO synthase (NOS II; iNOS) is constitutively expressed in slow-twitch skeletal muscle fibres of guinea-pigs [Gath, Closs, Gödtel-Armbrust, Schmitt, Nakane, Wessler and Förstermann (1996) *FASEB J.* **10**, 1614–1620]. Here we studied the expression of NOS II in skeletal muscle of wild-type and NOS II-deficient mice and investigated the molecular basis for the membrane association of this NOS in muscle. A basal expression of NOS II mRNA and protein was detected in skeletal muscle from untreated wild-type mice; expression increased when mice were treated with bacterial lipopolysaccharide (LPS). No NOS II was found in any tissue of untreated or LPS-treated NOS II-deficient mice. Immunoprecipitation experiments were performed with homogenates of gastrocnemius muscle from untreated or LPS-treated wild-type mice. A NOS II-

specific antibody precipitated caveolin 3 in all homogenates investigated, the effect being most pronounced in skeletal muscle from LPS-treated animals. Conversely, an antibody against caveolin 3 co-precipitated NOS II in muscle homogenates. Similarly, a weak co-precipitation of NOS II and caveolin 3 was seen in homogenates of untreated murine C2C12 myotubes; co-precipitation was markedly enhanced in cells stimulated with LPS/interferon γ . The association of NOS II with caveolin 3 might have implications for the regulation of contraction of, and/or glucose uptake by, slow-twitch muscle fibres.

Key words: immunoprecipitation, membrane association, NO synthase II, NO synthase II-deficient mice, RNase protection assay.

INTRODUCTION

Caveolae are plasma membrane invaginations that gather and organize many classes of receptors and signalling molecules, thereby acting as signal transduction centres [1]. The typical structural and regulatory component of caveolae is a 21–24 kDa integral membrane protein termed caveolin; the interaction of caveolins with proteins containing one of two related caveolin-binding motifs is mediated by a so-called scaffolding domain at their N-terminus [1]. As a consequence of this direct binding to caveolin, the signalling proteins are sequestered in caveolae [1]. Three isoforms of caveolin, caveolins 1–3, have been identified and cloned [1]. Whereas caveolin 1 and caveolin 2 have distinct but overlapping cell and tissue distributions [1], the expression of caveolin 3 has been reported to be muscle-specific [2,3].

Caveolins have been shown to contribute to the subcellular localization and activity of endothelial-type NO synthase III (NOS III; eNOS) and neuronal-type NOS I (nNOS). The doubly acylated NOS III [4,5] associates with caveolin 1 in endothelial cells and with caveolin 3 in cardiomyocytes [6]. The inhibitory NOS III–caveolin heteromeric complex has been shown to be sensitive to dynamic regulation by Ca^{2+} –calmodulin [7]. Agonist-stimulated increases in intracellular Ca^{2+} lead to a disruption of the complex, a transient dissociation of NOS III from caveolin and an activation of the NOS [8]. In skeletal muscle, NOS I is found almost exclusively in the membrane fraction [9,10]. Sarcolemmal association of NOS I has been attributed to PDZ (PSD-95/discs large/ZO-1 homology) domain-mediated interactions with α_1 -syntrophin, a member of the dystrophin–glycoprotein complex [11]. Recently, association with caveolin 3 has been described as an additional membrane anchor for NOS I in skeletal muscle [12]. Inducible-type NOS (NOS II; iNOS) in slow-twitch fibres of guinea-pig skeletal muscle is associated with

intracellular membranes [10]. Similarly, in macrophages, a significant portion of NOS II is found in the particulate fraction [13] but the molecular nature of this membrane association has not been clarified [14]. NOS II, like the other isoforms of NOS, contains a putative caveolin-binding motif that confers an inhibition of NOS II activity, as demonstrated with recombinant peptides derived from the scaffolding domains of caveolins 1 and 3 [15].

The present study confirms our finding of constitutive expression of NOS II in skeletal muscle of mice. It demonstrates the lack of expression in skeletal muscle of NOS II-deficient mice, and indicates that the particulate nature of NOS II in muscle is likely to be due to its association with the muscle-specific caveolin 3.

EXPERIMENTAL

Animals and tissue preparation

For studies on the expression of NOS II mRNA and protein, mice with targeted disruption of the NOS II gene (NOS II-deficient) were used. Intercrosses of (129sv $\times$ C57BL/6) F_1 progeny yielded wild-type control mice. NOS II-deficient mice were kindly provided by Dr. C. Nathan and Dr. J. D. MacMicking (Cornell University Medical College, New York, NY, U.S.A.) and Dr. J. S. Mudgett (Merck Research Laboratories, Rahway, NJ, U.S.A.). All mice, kept under specific pathogen-free conditions, were used at 12–16 weeks of age and were matched by age and sex. For immunoprecipitation (IP) experiments, male SPF Balb/c mice purchased from Charles River (Sulzfeld, Germany) were used. Pretreatment of animals with bacterial lipopolysaccharide (LPS) was done with an intraperitoneal injection (7.5 mg/kg LPS, dissolved in PBS). Animals were killed 6 h later by cervical dislocation. After removal of the

Abbreviations used: IFN- γ , interferon γ ; IP, immunoprecipitation; LPS, bacterial lipopolysaccharide; NOS, NO synthase; NOS II, inducible-type NOS (iNOS).

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skin of the hindlimb, the Achilles tendon was separated from the heel, and the lateral and medial parts of the gastrocnemius muscle were dissected out. Diaphragm, cerebellum, liver and small intestine (ileum) were also removed from the animals.

C2C12 cell culture

C2C12 myoblasts, a subclone of the C2 cell line derived from mouse femoral muscle satellite cells [16], were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), and grown under subconfluent conditions (approx. 70% confluency) in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin at 37 °C in a humidified air/CO₂ (9:1) atmosphere. For differentiation into myotubes, myoblasts (up to passage 10) were grown to confluence. Then the growth medium was replaced by low-mitogen medium [Dulbecco's modified Eagle's medium supplemented with 3% (v/v) horse serum (Life Technologies)]. In some experiments, LPS (2 µg/ml) and interferon γ (IFN- γ ; 100 i.u./ml) were added on day 4 of differentiation, when the culture consisted mainly of multinucleated, spontaneously contracting myotubes. Cells were harvested 40 h later.

Cloning of mouse-specific cDNA fragments and generation of anti-sense RNA

Single-stranded cDNA species were generated by reverse transcription of total RNA isolated from induced and non-induced murine RAW 264.7 macrophages (for NOS II and β -actin respectively). The reverse-transcription reaction was performed with the Superscript reverse transcriptase kit (Life Technologies) and oligo(dT) primer (Pharmacia, Uppsala, Sweden) essentially in accordance with the instructions of the manufacturer (Life Technologies) (42 °C for 50 min, 72 °C for 15 min) in a total volume of 20 µl, with the use of 3 µg of total RNA. The resulting cDNA (3 µl of the reverse-transcription product) was amplified by PCR in 50 µl of *Taq* polymerase buffer, consisting of 60 mM Tris/HCl, pH 10.0, 15 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, dNTPs (0.2 mM each), 50 pmol of oligonucleotide primers and 2.5 units of *Taq* polymerase (Pharmacia). Amplification of cDNA for NOS and β -actin was performed in 35 cycles (1 min at 95 °C, 1 min at 60 °C, 2 min at 72 °C) after the initial denaturation step (5 min at 95 °C). The final extension period at 72 °C was for 10 min. The following oligonucleotide primer pairs were used: for NOS II (compare with murine NOS II cDNA [17]), 5'-GACAAGAGGCTGCCCCC-3' (bp 2683–2700, sense), 5'-GCTGGGAGTCATGGAGCCG-3' (bp 3241–3223, anti-sense); for β -actin (compare with bovine actin cDNA, accession number K00622 [18]) 5'-ACCAACTGGGACGACATGGAG-3' (bp 1–21, sense), 5'-CGTGAGGATCTTCATGAGGTAGTC-3' (bp 354–331, anti-sense). The amplification products (NOS II, 559 bp; β -actin, 354 bp) were inserted into the *EcoRV* site of pCR-Script (Stratagene, La Jolla, CA, U.S.A.) with the SureClone ligation kit (Pharmacia). Further restriction of the β -actin cDNA clone with *Bst*EII and *Hind*III generated a clone with an insert of 108 bp. The resulting cDNA clones were sequenced by the dideoxy termination method with the T7 sequencing kit (Pharmacia); plasmids were termed pmNOS II and pmactin. To generate anti-sense RNA (cRNA) probes for ribonuclease protection analyses, plasmids were restricted either with *Nco*I (pmNOS II) or *Asp*718 (pmactin) for transcription *in vitro* with T3 RNA polymerase (Pharmacia). The linearized plasmids were then extracted with phenol/chloroform, precipitated with ethanol and resuspended in diethyl pyrocarbonate-treated water.

Ribonuclease protection analyses with mouse-specific cRNA probes

Ribonuclease (RNase) protection analyses were performed as described previously [10,19]. In brief, radiolabelled cRNA probes were generated by transcription of 0.5 µg of plasmid DNA *in vitro* with T3 RNA polymerase (Pharmacia) and 90 µCi of [α -³²P]UTP per reaction (incubation for 1 h). Thereafter, template DNA was degraded by incubation with DNase I for 45 min and labelled cRNA probes were purified on NucTrap probe purification columns (Stratagene). For each hybridization, 20 µg of total RNA was incubated overnight at 51 °C with 200000 c.p.m. of NOS II cRNA probe and 30000 c.p.m. of β -actin cRNA probe. Then unhybridized probe was digested by treatment with a mixture of RNase A and RNase T1 (Boehringer Mannheim). RNase activity was stopped by the addition of proteinase K in a buffer containing 10% (w/v) SDS. Protected fragments were extracted with phenol/chloroform, precipitated with ethanol and separated electrophoretically in a denaturing (containing 8 M urea) 6% (w/v) polyacrylamide gel. DNA fragments derived from pUC19 restricted with *Sau*3A were labelled with [γ -³²P]ATP and served as size markers. Densitometric analyses of gels were performed with a Molecular Imager (Bio-Rad) and results were quantified by comparison with the hybridization signal of a β -actin cRNA probe.

Preparation of extracts with CHAPS and IP

Cells and tissues were homogenized on ice by sonication or with a Polytron homogenizer respectively, as described [20]. Homogenates were solubilized with the detergent CHAPS (20 mM final concentration) overnight at 4 °C in a rotating shaker. The supernatants of the subsequent centrifugation at 160000 g (1 h, 4 °C) were defined as CHAPS extracts. These extracts contained the combined soluble and membrane-associated NOS protein [21]. As indicated, in some experiments CHAPS extracts were further enriched for NOS by 2',5'-ADP-Sepharose chromatography [20].

For IP, aliquots of CHAPS extracts were precleared by incubation (10 min on a rotating shaker) with 10 µl of normal rabbit serum and 20 µl of Protein A-agarose in a total volume of 1 ml of IP buffer [50 mM Tris/HCl (pH 7.5)/0.5 mM EDTA/0.5 mM EGTA/10 µg/ml pepstatin/10 µg/ml aprotinin/10 µg/ml leupeptin/0.2 mM PMSF] containing 150 mM NaCl and 8 mM CHAPS. Supernatants of the subsequent centrifugation (1000 g, 5 min) were cleared from antibodies in two incubations with 20 µl of Protein A-agarose (10 min on a rotating shaker, 5 min at 1000 g) before incubation with 4 µg/ml primary antibody for 2 h at 4 °C on a rotating shaker. Primary antibodies used were polyclonal antibodies against NOS II (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and against caveolin 3 (Transduction Laboratories, Lexington, KY, U.S.A.). After the removal of complexed material by centrifugation (10000 g, 10 min), the resulting supernatants were incubated with 20 µl of Protein A-agarose for 90 min on a rotating shaker. Immunoprecipitates were collected by centrifugation at 1000 g for 5 min, washed four times with IP buffer containing 150 mM NaCl and 8 mM CHAPS (1000 g, 5 min), once with IP buffer containing 150 mM NaCl, and twice with IP buffer without additions. After the final wash, immunoprecipitates were pelleted by centrifugation at 14000 g for 5 min. Pellets were resuspended in 20 µl of 2× sample buffer, boiled for 5 min and centrifuged (14000 g for 5 min) to pellet the agarose beads. Supernatants (total immunoprecipitated proteins) were analysed by SDS/PAGE and subsequent Western blotting.

Figure 1 RNase protection analysis of NOS II mRNA and β -actin mRNA (used for standardization)

Total RNA was prepared from cerebellum, liver, gastrocnemius muscle and small intestine of untreated (—) and LPS-treated (+) mice. Wild-type (wt) and NOS II-deficient (knock-out, ko) animals were studied. RNA (20 μ g) was used for each hybridization with anti-sense RNA probes. Protected fragments (184 nt for NOS II, 108 nt for β -actin) were separated on a 6% (w/v) denaturing polyacrylamide gel. Molecular size markers (sizes indicated at the left) and a tRNA control (20 μ g) are also shown. NOS II mRNA was detected in skeletal muscle and small intestine of untreated wild-type mice, and was induced in these tissues after treatment with LPS. In addition, in the liver and cerebellum of LPS-treated mice, but not untreated wild-type mice, significant NOS II expression was detected. No NOS II mRNA was found in any tissue from untreated or LPS-treated NOS II-deficient mice. The results shown are representative of four separate experiments.

SDS/PAGE and Western blotting

Proteins were separated by denaturing discontinuous PAGE in 7.5% (w/v) (for detection of NOS II) or 12.5% (w/v) (for detection of caveolin 3) resolving gels, and blotted on to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.) in a semi-dry electrophoretic transfer cell (Trans-Blot; Bio-Rad). Blots were blocked for 60 min in TBS buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl], containing 3% (w/v) dried milk powder, 10% (v/v) normal goat serum and 0.05% (w/v) Tween 20. After three wash steps (5 min each) in TBS containing 1% (w/v) BSA and 0.1% (w/v) Tween 20, blots were incubated in this solution for 60 min (at room temperature) with the primary antibodies (polyclonal antibodies against NOS II and caveolin 3; see above). Thereafter, blots were washed three times for 5 min each in a solution of 3% dried milk powder in TBS containing 0.05% Tween 20. They were then incubated for 60 min at room temperature with the secondary antibody conjugated to alkaline phosphatase (Sigma). Finally, blots were washed in TBS containing 0.05% Tween 20; immunoreactive proteins were revealed with Nitro Blue Tetrazolium Chloride/5-bromo-4-chloro-3-indolyl phosphate.

Assay of NOS activity

The NO-forming capacity of differentiated murine C2C12 myotubes and 2',5'-ADP-Sepharose-purified CHAPS extracts from the particulate fraction of skeletal muscle was determined by bioassay with RFL-6 reporter cells, essentially as described previously [21,22]. In brief, myotubes either untreated or treated for 40 h with a combination of LPS (2 μ g/ml) and IFN- γ (100 i.u./ml) were preincubated in Locke's solution containing 1 mM L-arginine and 20 units/ml superoxide dismutase for 20 min.

Then myotubes were washed twice with two changes of Ca^{2+} -free Locke's solution containing 1 mM L-arginine, 0.3 mM 3-isobutyl-1-methylxanthine and 20 units/ml superoxide dismutase. After a 2 min incubation at 37 °C in a fresh 1 ml of this Locke's solution, supernatants of myotubes were transferred to RFL-6 cells and incubated again for 2 min at 37 °C. The reaction was terminated by removal of the supernatants, the addition of ice-cold 50 mM sodium acetate buffer, pH 4.0, and the immediate freezing of the RFL-6 cells with liquid nitrogen. CHAPS extracts of skeletal muscle from untreated animals or animals treated with LPS (7.5 mg/kg for 6 h) were incubated for 3 min on RFL-6 cells in Ca^{2+} -free Locke's solution containing 100 μ M L-arginine, 100 μ M NADPH, 3 μ M tetrahydrobiopterin, 300 nM FAD, 250 μ M glutathione and 500 units/ml calmodulin [19]. The accumulation of cGMP in the RFL-6 reporter cells was measured by radioimmunoassay, as described [21,22].

RESULTS

NOS activity in skeletal muscle

Significant basal NOS activity was found in skeletal muscle from untreated animals (stimulating the formation of 11.3 ± 1.1 pmol of cGMP/2 min per 10^6 RFL-6 cells in the reporter cell assay). NOS II activity was markedly enhanced 6 h after treatment with LPS *in vivo* (stimulating the formation of 24.3 ± 1.9 pmol of cGMP/2 min per 10^6 RFL-6 cells). In addition, in murine C2C12 myotubes, a basal, Ca^{2+} -independent NOS activity was detected (stimulating the formation of 0.35 ± 0.08 pmol of cGMP/2 min per 10^6 RFL-6 cells), which increased markedly after treatment with LPS/IFN- γ (stimulating the formation of 4.4 ± 0.5 pmol of cGMP/2 min per 10^6 RFL-6 cells). Results are given as the means $\pm$ S.E.M.

Figure 2 Western blots of ADP-Sepharose-purified CHAPS extracts from skeletal muscle (gastrocnemius and diaphragm), small intestine, liver and cerebellum of untreated and LPS-treated wild-type and NOS II-deficient mice (A), and of ADP-Sepharose-purified CHAPS extracts from untreated and LPS/IFN- γ -treated C2C12 myotubes (B)

Electrophoreses were performed in 7.5% (w/v) polyacrylamide resolving gels and blots were immunostained with anti-(NOS II) antibody. Samples were applied at 20–50 μ g of protein per lane (A), identical loads for all samples of a given tissue or 20 μ g of protein per lane (B). CHAPS extracts from LPS/IFN- γ -induced RAW 264.7 macrophages were used as positive controls. Results are representative of three (A) or four (B) independent experiments. The positions of molecular mass markers [lane MW in (B)] are indicated at the left.

Characterization of NOS II expression in tissues from wild-type and NOS II-deficient mice and in C2C12 myotubes

RNAse protection analyses with total RNA from tissues of untreated wild-type mice with the use of a NOS II-specific cRNA probe revealed a protected fragment of the expected molecular size in gastrocnemius muscle and small intestine (used as control [23]) (Figure 1). In these tissues the NOS II signal was enhanced when the animals were pretreated with LPS. Induction of NOS II by LPS treatment was also observed in liver and cerebellum of wild-type mice. In contrast, in NOS II-deficient mice no signal for NOS II mRNA was detected (whether in tissues from untreated mice or in tissues from animals pretreated with LPS) (Figure 1).

In Western blot studies with partly purified CHAPS extracts from tissues of wild-type and NOS II-deficient mice, a basal expression of NOS II protein was detected in skeletal muscle (gastrocnemius and diaphragm) and small intestine of wild-type mice. This expression was markedly enhanced by treatment of the animals with LPS (Figure 2A). Expression of NOS II protein was also induced in liver and cerebellum of wild-type mice. No

Figure 3 IP of NOS II with caveolin 3

CHAPS extracts (3 mg) of gastrocnemius muscle from untreated and LPS-treated mice were subjected to IP with either an antibody against NOS II (A) or an antibody against caveolin 3 (B). Similar experiments were performed with CHAPS extracts (3 mg) from untreated C2C12 myotubes, and with myotubes treated with a combination of LPS and IFN- γ . Precipitated proteins were separated on 12.5% (w/v) (A) or 7.5% (w/v) (B) polyacrylamide resolving gels. Precipitates obtained with the anti-(NOS II) antibody were probed with an anti-(caveolin 3) antibody (A) and vice versa (B). CHAPS extracts of murine gastrocnemius muscle (100 μ g of protein per lane) and LPS/IFN- γ -induced RAW 264.7 macrophages (20 μ g of protein per lane) were used as positive controls for the detection of caveolin 3 (cav-3) and NOS II respectively. IgG_H indicates the immunoglobulin heavy chain of the precipitating antibody. Results are representative of four independent experiments with identical results. The positions of molecular mass markers are indicated at the left.

staining for NOS II protein was observed in any tissue from NOS II-deficient mice (Figure 2A). In extracts from C2C12 myotubes, basal expression of NOS II was hardly detectable with Western blotting (Figure 2B). However, expression of the enzyme was markedly increased after treatment of the cells with LPS/IFN- γ (Figure 2B).

Protein–protein interaction between NOS II and caveolin 3 in skeletal muscle

In IP studies with a NOS II-specific antibody, caveolin 3 was co-precipitated in CHAPS extracts from gastrocnemius muscle of untreated mice and from untreated murine C2C12 myotubes (Figure 3A). The intensity of the signal for caveolin 3 was strongly increased in precipitates from gastrocnemius muscle of

LPS-treated mice and in LPS/IFN- γ -treated C2C12 myotubes (Figure 3A). Conversely, an antibody against caveolin 3 co-precipitated NOS II in homogenates from gastrocnemius muscle of untreated and LPS-treated mice. The same was true for untreated and LPS/IFN- γ -treated C2C12 myotubes (Figure 3B). NOS II protein expression in skeletal muscle was relatively low and could be detected on Western blots only after NOS enrichment on ADP-Sepharose (see Figure 2). Co-immunoprecipitation of the NOS II with an anti-(caveolin 3) antibody resulted in a clearly detectable signal in subsequent Western blots with an anti-(NOS II) antibody (Figure 3B). This indicates that a large portion of the total NOS II protein is bound to caveolin 3. With Western blotting, no NOS II protein was detectable in supernatants after IP with an antibody against caveolin 3 ($n = 4$).

DISCUSSION

Expression of NOS II can be induced in many cell types on exposure to inflammatory cytokines, LPS and a number of other agents [24]. In skeletal muscle, the induction of NOS II by LPS has been linked to contractile dysfunction in sepsis [10,25]. An up-regulation of NOS II expression has also been shown in skeletal muscle of patients with chronic heart failure. This has been attributed to elevated levels of circulating tumour necrosis factor α [26]. Indeed, tumour necrosis factor α has been described as the primary factor in the development of skeletal muscle atrophy and weakness caused by a variety of chronic diseases, including sepsis and congestive heart failure [27]. Moreover, in a murine model of muscle wasting, tumour necrosis factor α has been shown to induce NOS II; an inhibition of NOS activity significantly ameliorated the cachectic condition in these mice [28].

In a previous study we found constitutive expression of NOS II (mRNA, protein and activity) in the skeletal muscle of guinea pigs [10]. In that study, NOS II protein was shown to be associated with slow-twitch (type I) fibres and to be further increased in these fibres by LPS [10]. Interestingly, Hussain et al. [29] also found constitutive as well as LPS-induced expression of Ca^{2+} /calmodulin-independent NOS activity in rat skeletal muscle, which was higher in muscle types with a high content of slow-twitch fibres, and low in muscles with mainly fast-twitch fibres. The constitutive expression of NOS II mRNA and protein in human skeletal muscle has also been reported [26,30]. Murine C2C12 cells are known to express proteins specific for slow-twitch skeletal muscle [31,32]. In agreement with this, in the present study we detected a weak basal Ca^{2+} -independent (NOS II-like) activity, which was markedly stimulated by LPS/IFN- γ . Similarly, in Western blots, NOS II protein was hardly detectable in untreated C2C12 cells but significant protein expression was seen after induction with LPS/IFN- γ (Figure 2B). In addition, Williams et al. [33] detected unstimulated NOS activity in C2C12 cells, but did not investigate its Ca^{2+} dependence.

Here we show that the mouse also expresses NOS II constitutively in skeletal muscle (Figures 1 and 2). The absence of its expression in NOS II-deficient mice (Figures 1 and 2) confirms its identity as NOS II. In agreement with previous reports [23], we also found high levels of constitutive NOS II in small intestine but not in liver and cerebellum of wild-type mice (Figures 1 and 2). However, NOS II could be induced with LPS in the latter tissues. In contrast, NOS II was not expressed in any tissue of untreated or LPS-treated NOS II-deficient mice (Figures 1 and 2).

In murine macrophages, NOS II is mainly soluble. However, 30–40% is found in the particulate fraction [13]. The molecular

basis of this membrane association is not clear [14]. In skeletal muscle, NOS II is almost exclusively membrane-bound [10,29]. The present study suggests that a protein–protein interaction with caveolin 3 can contribute to the particulate nature of NOS II in skeletal muscle (Figure 3). The location of NOS II in intracellular membranes [10] matches the distribution of caveolin 3 in skeletal muscle [34]. In differentiated C2C12 myotubes, caveolin 3 was co-localized with developing T-tubules [2].

The function of NOS II in skeletal muscle is still unclear. NO has been found to modulate the activity of the ryanodine receptor Ca^{2+} release channel [35] and to decrease actomyosin ATPase activity in skeletal muscle [36]. NO has also been reported to interfere with the intracellular signalling of insulin in rat soleus muscle [37]. In rat L6 myotubes, NOS II-derived NO generated resistance to insulin, possibly caused by an impairment of the insulin-stimulated translocation of glucose transporters [38]. Resistance of peripheral tissues to insulin can also be observed in sepsis, when NOS II is up-regulated [39]. In contrast, non-insulin-dependent glucose uptake is increased in sepsis [40]. Similar effects have been observed in skeletal muscle with high concentrations of exogenous or endogenously stimulated NO [37,38,41]. Interestingly, slow-twitch fibres, expressing NOS II constitutively, possess the highest capacity for glucose uptake [42,43].

In conclusion, our study demonstrates that, similarly to NOS I, NOS II is also bound to caveolin 3 in skeletal muscle. This might contribute to the membrane association of NOS II in this tissue.

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