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Functional impact of neuronal nitric oxide synthase (nNOS) on angiogenesis and the molecular characterization of its isoform expression pattern in skeletal muscle

PhD Thesis submitted by

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Abstract

In rodents and humans skeletal muscle neuronal nitric oxide synthase (nNOS) is present at the sarcolemma of skeletal muscle fibres. nNOS produces high concentrations of the signalling molecule nitric oxide (NO), which could play an important role in the molecular integration of muscle metabolism and the microvascular supply in skeletal muscle.

The association of nNOS expression with the capillary supply in human skeletal muscle fibres was assessed in response to endurance exercise (**Project I**).
Vastus lateralis (VL) muscle biopsies from ten sedentary male subjects before and after moderate training were investigated. nNOS is mainly expressed as an alpha isoform with the additional mu exon and to a lesser extent without the mu exon as analysed by RT-PCR. Only five of the ten subjects exhibited significant ($P \leq 0.05$) elevations in the C/F ratio (+25%) and capillary density (+21%), indicative of angiogenesis. Therefore, the subjects were divided into angiogenesis responder (AR) and non-angiogenesis responder (NR) groups. The mRNA levels of nNOS tended to be elevated after training (+34%; $P > 0.05$) in the VL biopsies of all subjects analysed by real-time PCR. However, when the AR group was analysed separately, the nNOS mRNA levels were significantly up-regulated (+128%; $P \leq 0.05$) in response to exercise. Moreover, these mRNA levels correlated positively ($r = 0.08$; $P \leq 0.01$) with exercise-induced differences in the C/F ratio. The quantification of the nNOS-specific immunoblots showed that the nNOS protein expression was significantly increased (+82%; $P \leq 0.05$), but only in the VL biopsies of the subjects of the AR group.
These data might represent an additional evidence for the functional impact of up-regulated nNOS on exercise-induced angiogenesis in skeletal muscles.

Various isoforms of nNOS have been characterized at protein level, but the primary structure of these variants has not yet been elucidated. Therefore, the expression and structure of nNOS isoforms in skeletal muscles of mice were characterized in more detail (**Project II**).

The nNOS protein expression was analysed in skeletal muscles of C57Bl/6 mice by immunoblotting. Two bands at 165 and 160 kDa represented the nNOS alpha isoform, with and without the additional mu exon. The nNOS beta isoform with and without exon mu was detected at 145 and 140 kDa. Immunofluorescence histochemistry localized the nNOS alpha and beta isoforms at the sarcolemma of skeletal muscle fibres. Performing RT-PCR in skeletal muscle of C57Bl/6 mice, three cDNA variants (1500 bp, 650 bp and 300 bp) were detected using the primer pair specific for exon 1c and exon 5. The remaining nNOS sequence was covered using seven different primer pairs. One cDNA variant was amplified between exons 2-15 and exons 17-28. Using the primer pair specific for exon 14 and exon 18, a double band was amplified which represented isoforms with and without the alternative spliced mu exon (102 bp). Nucleotide sequencing revealed that in C57Bl/6 mice the nNOS alpha isoform (1500 bp) had the following exon sequence: 1c-pi-rho-sigma-tau-4 to 29. The shorter nNOS beta isoform (650 bp) consisted of: 1c-pi-rho-4 to 29. Each of the isoforms existed with and without exon mu. Unexpectedly, two cDNA variants (450 bp and 300 bp) were detected using the primer pair specific for exon 1c and exon 5 in skeletal muscles of nNOS-knockout mice. The expression pattern of the remaining nNOS sequence was similar to the pattern in C57Bl/6 mice with the exception that no cDNA band was detected using the primer pair specific for exon 2 (containing the initiation codon) and exon 6. In nNOS-knockout mice, none of the cDNA variants were translated.

Taken together, in mice skeletal muscle, four nNOS cDNA variants were translated: the nNOS alpha isoform, with and without the mu exon, and the nNOS beta isoform, with and without the mu exon.

The functional impact of the nNOS alpha isoform on angiogenesis should be further characterized in mice skeletal muscle by gene electrotransfer (**Project III**).

Therefore, the full-coding nucleotide sequence (4676 bp) of nNOS was isolated, cloned into the pIRES2-ZsGreen1 vector and over-expressed in hind limb muscles from mice by gene electrotransfer. The pIRES2-ZsGreen1-nNOS plasmid and the control pIRES2-ZsGreen1 plasmid were injected into the tibialis anterior (TA) muscles. After the gene electrotransfer, the mice were separated into one sedentary and one exercise group. The successful gene electrotransfer of both plasmids was confirmed by detection of the ZsGreen1 fluorescence in muscle fibres. However, no clear nNOS over-expression was detected in muscle fibres transfected with the pIRES2-ZsGreen1-nNOS plasmid by immunofluorescence. The real-time PCR analysis showed that the nNOS mRNA expression was not significantly up-regulated in the TA muscles with the pIRES2-ZsGreen1-nNOS plasmid compared to the TA muscles with the control plasmid of the sedentary as well as exercise group.

Therefore, further characterization of the function of the nNOS alpha isoform with this approach was not possible so far.

Abbreviations

3’ UTR	3’ Untranslated region
5’ UTR	5’ Untranslated region
AMPK	Adenosine monophosphate-activated protein kinase
Ang-1/-2	Angiopoetin-1/-2
AR	Angiogenesis responder
BH4	Tetrahydrobiopterin
BSA	Bovine serum albumin
C/F	Capillary-to-fibre
CaMK	Calcium/calmodulin-dependent protein kinase
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
EDL	Extensor digitorum longus
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ERR-alpha	Estrogen-related receptor alpha
EST	Expressed sequence tag
FAD	Flavin adenine dinucleotide
FGF	Fibroblast growth factor
FMN	Flavin mononucleotide
GLUT4	Glucose transporter 4
HIF-1alpha	Hypoxia inducible factor-1alpha
HSP	Heat shock protein
iNOS	Inducible nitric oxide synthase
LGMD	Limb-girdle muscular dystrophies
L-NAME	L-N-arginine methyl ester
L-NMMA	L-N-monomethylarginine
MAPK	Mitogen-activated protein kinase
MCSFA	Mean cross-sectional fibre area

MHC	Myosin heavy chain
MLC	Myosin light chain
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NADP/H	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Non-angiogenesis responder
NRF-1/-2	Nuclear respiratory factor-1/-2
PBS	Phosphate buffered saline
PDGF-B	Platelet-derived growth factor B
PGC-1alpha	Peroxisome proliferator-activated gamma coactivator 1 alpha
PIN	Protein inhibitor of nNOS
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SMTC	S-methyl-L-thiocitrulline
TA	Tibialis anterior
TSP-1	Trombospondin-1
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2
VL	Vastus lateralis
VO_2max	Maximal oxygen uptake

1 Introduction

1.1 Skeletal muscle

Skeletal muscle is one of the major muscle types besides cardiac and smooth muscle and is also known as striated muscle under control of the central nervous system. A fibrous fascia, also called the epimysium, surrounds the whole muscle and further divides it into several fasciculi. Each fasciculus is surrounded by the perimysium and contains bundles of muscle fibres which build a specific mosaic in every mammalian skeletal muscle. Each muscle fibre is surrounded by more connective tissue called endomysium. The endomysium consists of collagen filaments and proteoglycans in which capillaries and nerve fibres are embedded. Each single muscle fibre is surrounded by a membrane called the sarcolemma and consists of myofibrils which are surrounded by the sarcoplasm (figure 1) (Billeter et al. 1994; Baechle et al. 2000; Schünke et al. 2005).

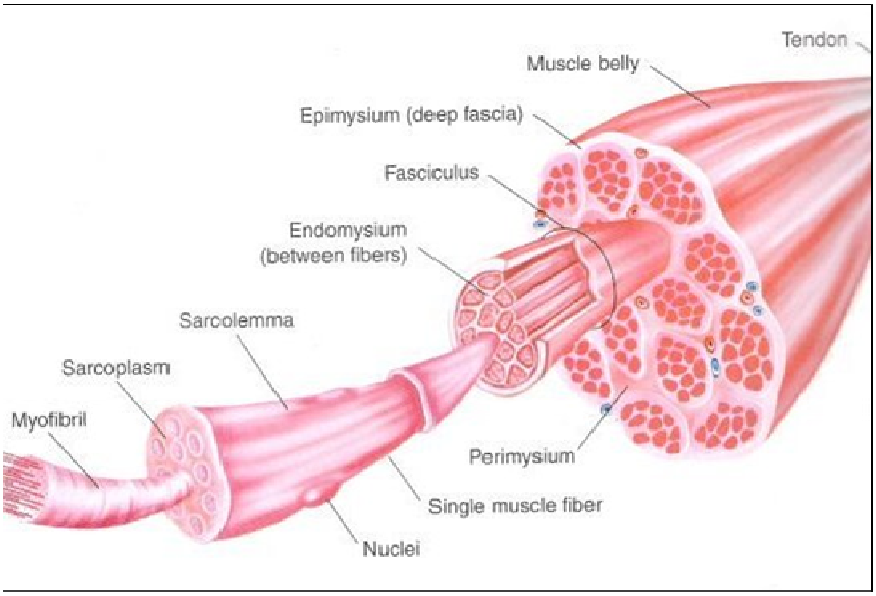

Figure 1: Detailed structure of a skeletal muscle (Baechle et al. 2000).

Skeletal muscles have an abundant supply of blood vessels and nerves which is related to the primary function of skeletal muscles, mechanical contraction. Contraction of various muscle fibres is controlled by a single motor-neuron. Depolarization of the membrane during contraction leads to an action potential which innervates a group of fibres at the same time (Monti et al. 2001).
Skeletal muscle fibres can be attributed to different metabolic and contractile phenotypes, which differ in their oxidation and glycosylation potential, as well as their velocities of contraction. Myosin, the motor of contraction, exists in different isoforms in skeletal muscle fibres. Although isoforms of both myosin heavy chain (MHC) and myosin light chain (MLC) portions of the molecules exist, research is mainly focused on the expression of MHC isoforms (Canepari et al. 2010). Different skeletal muscle fibres can be identified by their MHC composition (Schiaffino et al. 1996). In humans three different fibre types are determined, while in rodents four fibre types are known. In both species, type 1 slow-twitch, oxidative fibres are slow in force generation and have an oxidative profile whereas type 2A fibres are fast in force generation and have a similar oxidative profile as type 1 fibres. In humans type 2X fibres are fast in force generation and have a glycolytic profile. In contrast, rodent type 2X fibres are fast, oxidative, glycolytic and type 2B fibres are glycolytic and have an even faster-twitch profile (Schiaffino 2010).

The functional heterogeneity of skeletal muscles can be explained by the existence of mixed fibre types having different contractile and energetic properties (Canepari et al. 2010). The differentiation of skeletal muscles into fast and slow also corresponds to a morphological difference. Fast muscles appear white and slow muscles red. The redness is due to high amounts of myoglobin and a high capillary density (Gödecke et al. 1999; Scott et al. 2001). The fibre type composition of different skeletal muscles depends on their function. Muscles used for long, endurance work consist mainly of slow, type 1 fibres (e.g. soleus) in contrast to muscles used for short and intense work which consist of fast, type 2 fibres (e.g. vastus lateralis) (figure 2). Other muscles contain a rather balanced fibre type distribution (e.g. gastrocnemius). For this reason long distance runners have rather slow twitch fibres whereas sprinters have more fast twitch fibres (Saltin et al. 1977; Hoppeler 1983).

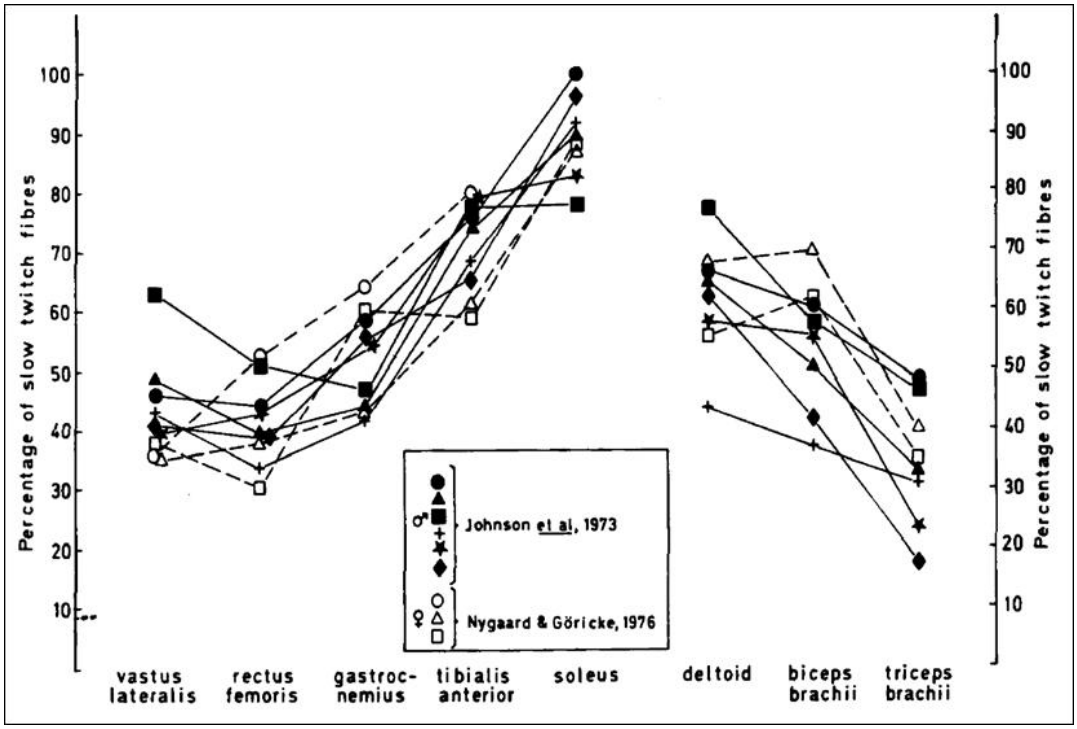

Figure 2: Percentage of slow twitch fibres in different human skeletal muscles (Saltin et al. 1977).

Changes in the environment like temperature, nutritional conditions and exercise can lead to specific adaptations in skeletal muscle tissue. These structural and functional modifications are reversible and maintained as long as the stimulus exists (Hoppeler et al. 2002). Physical activity can result in nuclear and mitochondrial gene expression, leading to mitochondrial biogenesis and improved endurance performance or myofibre hypertrophy and force development (Hood et al. 2006). The modifications can be regulated by several signalling pathways which are present in the skeletal muscle. These signalling pathways include the Ras / mitogen-activated protein kinase (MAPK), the calcium / calmodulin-dependent protein kinase (CaMK), the peroxisome proliferator-activated gamma coactivator 1 alpha (PGC-1alpha) and the vascular endothelial growth factor (VEGF) (Zierath et al. 2004; Arany et al. 2008). Further details of the signalling pathways are provided in sections 1.3 and 1.4.

1.2 Nitric oxide synthase

1.2.1 Genes

Three different isoforms of the nitric oxide synthase (NOS) are known, named after cells or tissues in which they were originally found and purified (figure 3). The neuronal NOS (NOS1, nNOS) was purified by cDNA cloning of brain tissue, the inducible NOS (NOS2, iNOS) from activated macrophages and the endothelial NOS (NOS3, eNOS) from endothelial cells (Schmidt et al. 1992; Stamler et al. 2001).
The nNOS is prominent in neurons, epithelial cells of the lung, in macula densa cells of the kidney and in skeletal muscle fibres. As signalling molecule, nNOS-derived NO is an endogenous mediator in neural transmission. In skeletal muscle, nNOS has different functions as a paracrine and autocrine signalling molecule (Schmidt et al. 1992; Kobzik et al. 1994; Ortiz et al. 2003). The eNOS is highly expressed in the endothelial tissue of vessels where it increases cGMP levels leading to relaxation of smooth muscle cells and thus influencing blood pressure (Li et al. 2002; Kojda et al. 2005). The iNOS is rapidly upregulated in all cell types following immunological stimuli and serves a nonspecific immune function (Mungrue et al. 2003; Li et al. 2011).

The activity of nNOS, iNOS and eNOS is regulated at transcriptional, translational and posttranslational levels. Typically, iNOS is upregulated by cytokines whereas nNOS expression is increased by muscle activity and aging process. eNOS is specifically upregulated by chronic exercise and in blood vessels by shear stress. All isoforms are transcriptionally regulated by hypoxia (Stamler et al. 2001). The activity of nNOS and eNOS is regulated by calcium-dependent calmodulin binding whereas the activity of iNOS is described as calcium-independent but presumes a tight interaction with calmodulin (Brenman et al. 1995; Mungrue et al. 2003).

Moreover, all NOS isoforms catalyse the reaction of arginine, NADPH and oxygen to nitric oxide (NO), citrulline and NADP. NO and many NO-related molecules (e.g. S-nitrosothiols, peroxynitrite) are generated from the catalytic conversion of L-arginine to L-citrulline. It seems that the availability of cofactors (BH_4, FAD, FMN, haem) as well as subcellular localisation can influence the enzymatic product (Alderton et al. 2001; Stamler et al. 2001; Mungrue et al. 2003).

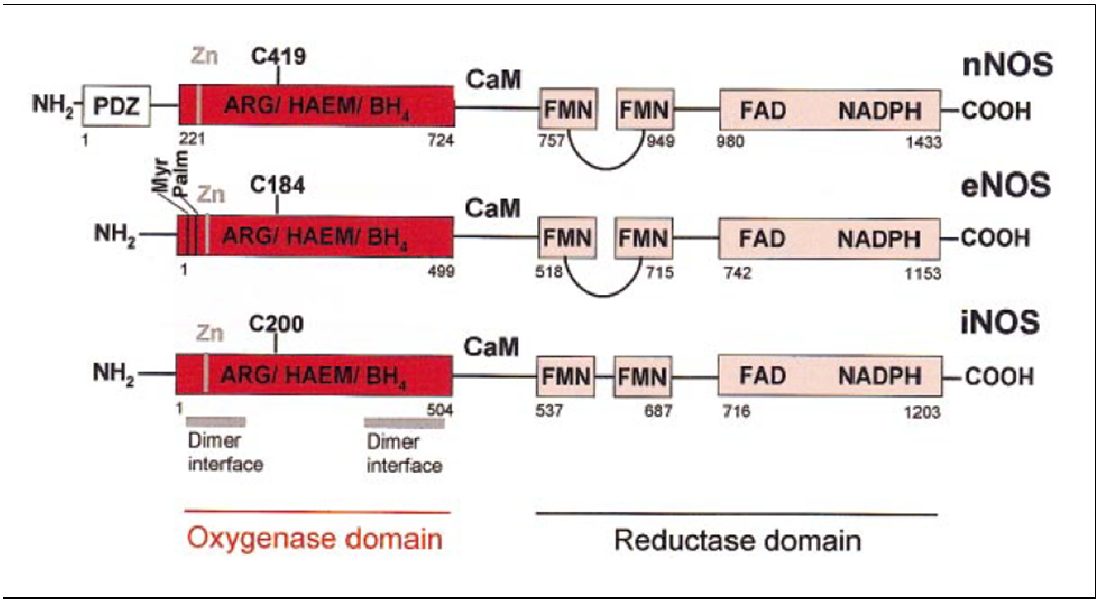

Figure 3: Domain structure of nNOS, eNOS and iNOS with the corresponding binding sites for the cofactors and -substrates (Alderton et al. 2001).

1.2.2 Nitric oxide

Nitric oxide is a diffusible gaseous radical with impact on many physiological and pathological processes. NO has a short half-life which is influenced by the interaction with various proteins (e.g. haemoglobin, myoglobin). As NO is electrically neutral, it diffuses across the plasma membrane and thus has intracellular as well as extracellular effects (Stamler et al. 2001). Until the work of Ignarro et al. (1988) and Furchgott et al. (1989), NO was mainly linked to cell toxicity. But these groups could show that endogenously produced NO is a biological mediator, produced by mammalian cells, and identical to the endothelium-derived relaxing factor (EDRF).

NO as well as EDRF are produced and released by the endothelium and cause smooth muscle cell relaxation by activating the guanylate cyclase (Ignarro et al. 1988; Furchgott et al. 1989; Alderton et al. 2001).

Physiologically NO plays a role in many processes like regulation of vascular tone, insulin secretion, angiogenesis, mitochondrial oxygen metabolism and development of the nervous system. Furthermore, pathologically it plays a role in immune response, septic shock and host defence against pathogens and tumours (Grozdanovic 2001). NO is involved in mitochondrial biogenesis at several levels. It directly regulates the supply of oxygen to mitochondria via binding and release from haemoglobin. Moreover, NO indirectly regulates the supply of respiratory substrates to mitochondria via blood flow regulation. Mitochondrial activity has a critical role in various processes in skeletal muscles like fibre type switching and muscle regeneration. NO can induce the change in energy metabolism in these processes (Clementi et al. 2005).

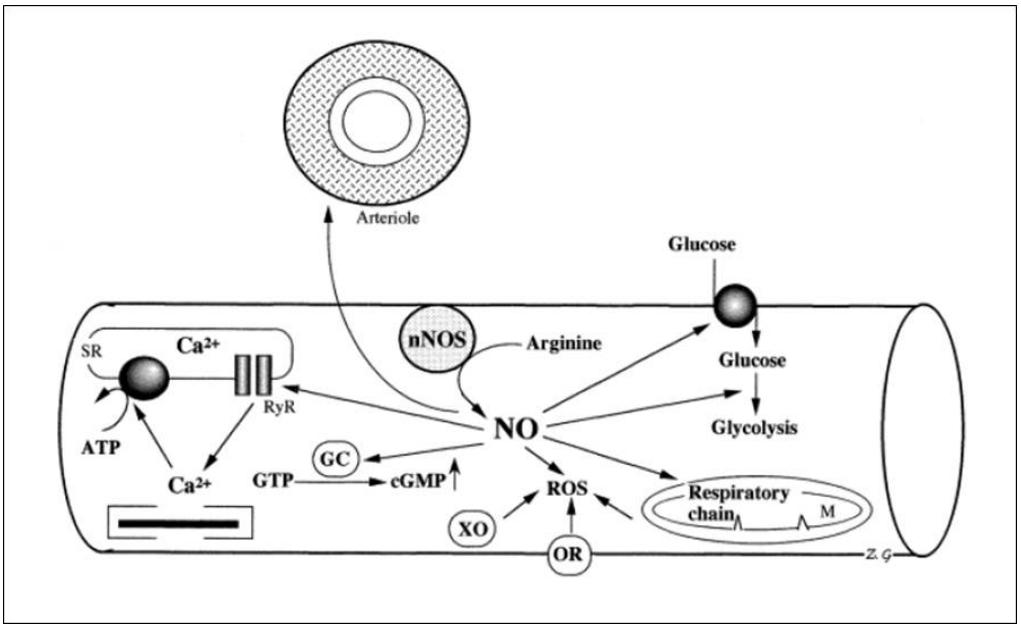

Figure 4: Schematic overview of the main target sites of NO in skeletal muscle. ROS: reactive oxygen species; XO: xanthine oxidase; OR: oxidoreductase; GC: guanylate cyclase (Grozdanovic 2001).

1.2.3 Neuronal nitric oxide synthase

Structure

The nNOS gene is more than 4500 bp long and consists of 29 exons. The initiation codon is located in exon 2 and the termination codon in exon 29. The nNOS protein is expressed as a dimer with an oxidase domain at the N-terminal and a reductase domain at the C-terminal end. Several binding domains for cofactors and -substrates (e.g. L-arginine, haem, BH_4, FAD, FMN and NADPH) are distributed along the sequence (Nathan et al. 1994; Stamler et al. 2001; Newton et al. 2003).
Most nNOS isoforms contain a characteristic PDZ/GLGF motif of ~100 amino acids at the N-terminal end which is linked to the dystrophin glycoprotein complex located at the sarcolemma. This connection is due to the direct binding of nNOS to α-syntrophin via PDZ-PDZ interactions (Kobzik et al. 1994; Brenman et al. 1995; Brenman et al. 1996). A further binding of the β-hairpin finger loop, located between residues 101-130 of nNOS, to the peptide binding groove in the PDZ domain of α-syntrophin is involved in the localisation of nNOS to the sarcolemma (Abdelmoity et al. 2000). If dystrophin is absent in the muscle, like in human Duchenne muscular dystrophy (DMD) patients and in its' animal model *mdx* mice, nNOS is dislocated from the sarcolemma (Brenman et al. 1995).

In skeletal muscle, different nNOS isoforms are known (figure 5). The nNOS alpha isoform consists of all 29 exons and produces a 160 kDa protein. The alpha isoform uniquely manifests a 237 amino acid long stretch, which is encoded by exon 2. This exon contains the characteristic PDZ domain that assures sarcolemmal anchorage. The nNOS beta isoform expresses a truncated N-terminus with a deletion of 236 amino acids, which is initiated at the boundary between exon 1 and exon 3. This deletion is owing to a shift in the frame-reading that is caused by an alternative splicing event. The beta isoform lacks the PDZ domain which leads to a delocalization from the sarcolemma but retains full enzymatic activity. It produces a 136 kDa protein. The nNOS gamma isoform is similar to the nNOS beta isoform but with a deletion of the first 335 amino acids of the nNOS alpha isoform. As exon 2 with the initiation codon and the PDZ domain is missing, the initiation codon is dislocated in exon 5. The nNOS gamma isoform produces a 125 kDa protein. The nNOS mu

isoform, especially known in skeletal muscle, has an alternatively spliced 102 bp (34 amino acid residues, 5 kDa) long stretch between exon 16 and 17 (Brenman et al. 1996; Silvagno et al. 1996; Brenman et al. 1997; Alderton et al. 2001; Stamler et al. 2001).

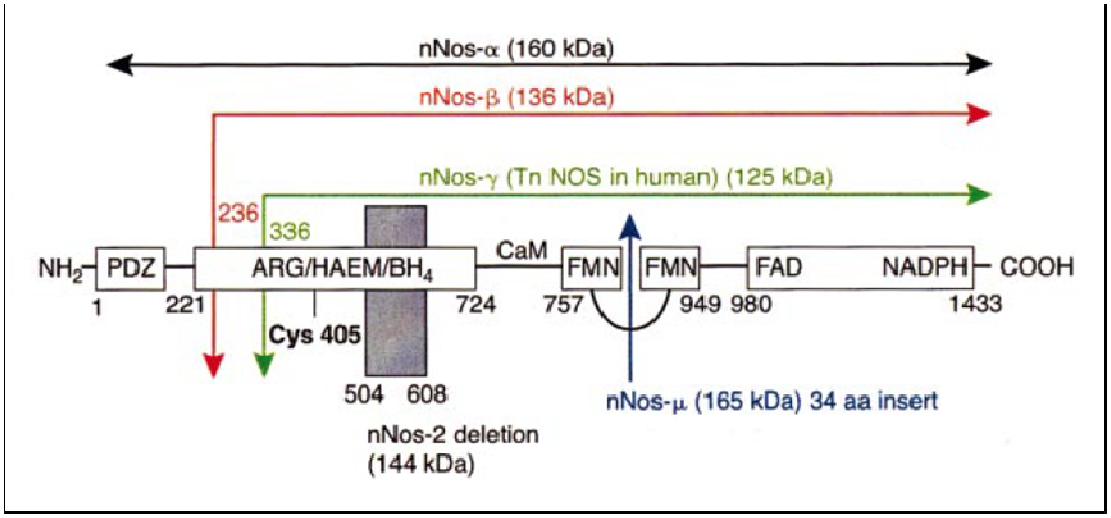

Figure 5: Schematic diagram of the nNOS splice variants: nNOS alpha (160 kDa), nNOS beta (136 kDa), nNOS gamma (125 kDa) and nNOS mu (165 kDa) (Alderton et al. 2001).

Due to alternative splicing and the alternative usage of multiple promoters, nine independent exon 1 mRNA variants are detectable and designated as exons 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h and 1i in different tissues (Hall et al. 1994; Xie et al. 1995; Brenman et al. 1996; Lee et al. 1997). As these exon 1 mRNA variants are 5' upstream of the coding sequence, they do not encode in different protein variants but affect the translational efficiency of the nNOS gene. Multiple exon 1 variants might be simultaneously expressed in one cell type e.g. exon 1a, 1b and 1c are highly demonstrated in skeletal muscle (Wang et al. 1999; Newton et al. 2003).

nNOS-knockout mice were generated with a targeted disruption of exon 2 which contains the translation initiation codon (Huang et al. 1993). Consequently, the PDZ domain, which is important for the association of the protein to the sarcolemma, is also deleted with the segment. The nNOS-knockout mice do not show any reproductive or survival defects (Huang et al. 1993; Huang 1999). An additional

nNOS-knockout strain was generated by targeted deletion of the haeme-binding region present within exon 6 (Gyurko et al. 2002). These mice completely lack all nNOS activity in comparison to the nNOS-knockout mice of Huang et al. (1993) which still exhibit low levels of nNOS activity (Mungrue et al. 2003).

Localisation

The nNOS is found in several tissues of different species including humans, rats, mice, chicken and hamsters. Nevertheless, it was shown, that nNOS expression is higher in skeletal muscle tissue than in brain tissue where it was first isolated. It is detected in several different skeletal muscles including gastrocnemius, tibialis anterior (TA), quadriceps, soleus and extensor digitorum longus (EDL) (Nakane et al. 1993; Stamler et al. 2001).
The expression of nNOS in the different skeletal muscle fibre types is species specific. An equal distribution of nNOS expression was shown in human muscles enriched in slow-twitch, type 1 fibres (soleus) and fast-twitch, type 2 fibres (EDL, gastrocnemius) (Frandsen et al. 1996; Grozdanovic et al. 1996). Whereas in mice, a higher nNOS activity was only detected in muscles enriched in fast-twitch, type 2 skeletal muscle fibres (Kobzik et al. 1994; Kusner et al. 1996; Kapur et al. 1997; Planitzer et al. 2001).

The nNOS localization was shown along the sarcolemma of skeletal muscle fibres (Kobzik et al. 1994; Frandsen et al. 1996). At the inner surface of the sarcolemma as well as at subsarcolemmal areas near mitochondria, an association of nNOS, α1-syntrophin and dystrophin was detected (Wakayama et al. 1997). Moreover, in skeletal muscle homogenates, the nNOS activity resides mainly in the particulate fraction (Brenman et al. 1995; Chao et al. 1996; Kameya et al. 1999).
The activation of nNOS due to extracellular signals increases the Ca^{2+} concentration and facilitates the interaction with calmodulin. Caveolin-3 inhibits the activity of nNOS and may also inhibit the activities of iNOS and eNOS in skeletal muscle (Venema et al. 1997). At neuromuscular junctions, nNOS is enriched and colocalizes with the N-methyl-D-aspartate (NMDA) type glutamate receptor (Brenman et al. 1996; Grozdanovic et al. 1997). Denervated muscle fibres have a decreased expression of nNOS with preserved expression of dystrophin but upon reinnervation the enzyme

level is restored (Tews et al. 1997). Urazaev et al. (1995) reported that in rat diaphragm NOS activity is stimulated through NMDA-mediated influx of calcium and regulates the resting potential (Urazaev et al. 1995).

Function

Skeletal muscle continuously produces NO and reactive oxygen species (ROS) which are involved in various complex processes. As one of the main NO producer, nNOS has various functions as autocrine and paracrine signalling molecule in skeletal muscle (reviewed in (Stamler et al. 2001)). nNOS-derived NO lowers the contraction force, hinders the depletion of energy stores and supports the uptake of glucose. Furthermore, nNOS generated NO controls the cGMP-dependent relaxation of vascular smooth muscle cells, thereby contributing to vasodilation (Lau et al. 2000; Stamler et al. 2001; Da Silva-Azevedo et al. 2009). The submaximal contraction force in mammalian skeletal muscle was increased by several NOS inhibitors (e.g. L-N-arginine methyl ester (L-NAME) or L-N-monomethylarginine (L-NMMA)) and haemoglobin (Kobzik et al. 1994). Thus, NO has an effect on skeletal muscle force production by shifting the force-frequency curve rightward. Contraction force is also increased if cGMP signalling is decreased. This indicates that the NO / cGMP signalling inhibits contraction (Kobzik et al. 1994; Huang 1999; Reid 2001). In patients with DMD and in mdx mice, the nNOS is dislocated from the sarcolemma and the expression is down-regulated. The loss of nNOS leads to muscle damage due to degeneration of muscle fibres and increased fatigability. These observations lead to the assumption that nNOS plays a key role in the pathogenesis of this muscle illness (Brenman et al. 1995; Tidball et al. 2007; Lai et al. 2009).

Furthermore, NO participates in scavenging of ROS (e.g. superoxide) as this reaction is faster than the dismutation of ROS (Nathan et al. 1994). The scavenging of ROS prevents the damage of proteins and lipids inside the skeletal muscle fibres, in particular by the reaction of superoxide to peroxynitrite (Smith et al. 2006; Jackson 2009). ROS increases calcium transients and thereby force in contracting muscle. As antioxidant, NO prevents the effect of ROS and decreases the contraction force indirectly (Reid 2001; Jackson et al. 2007). Indeed, the ROS metabolism has been

shown to be up-regulated in the skeletal muscles of nNOS-knockout mice (Da Silva-Azevedo et al. 2009).

1.3 Angiogenesis

All muscle fibres in skeletal muscles are surrounded by a characteristic number of capillaries that provide the tissue with oxygen and nutrition. The microvascular bed can be enlarged beginning with the growth of capillaries, with larger arterioles being formed from the capillary network (Hudlicka et al. 1992). The process underlying the growth of the microvascular system is called angiogenesis. The capillary supply can be increased due to different signals like exercise (Hudlicka 1998). Two different mechanisms of angiogenesis are known: sprouting and intussusception. Sprouting angiogenesis, the most common type, forms new vessels whereas intussusception angiogenesis splits one existing vessel into two new ones (Djonov et al. 2003). In both mechanisms, signalling molecules like VEGF, fibroblast growth factor (FGF), and others are activated (Hudlicka et al. 2009).

VEGF-A and the VEGF receptor-2 (VEGFR-2) as well as the angiopoetins, angiopoetin-1 (Ang-1) and angiopoetin-2 (Ang-2), are essential factors in the angiogenic process in skeletal muscle (Gustafsson et al. 2007). Ang-1 and Ang-2 act as antagonists as the capillary stability or instability is promoted by the ratio of their concentrations (Gavin 2009). Beside the positive effect of VEGF on angiogenesis, the remodelling of the extracellular matrix of the capillaries must also be regulated. This process of degradation is controlled by matrix metalloproteinases (MMPs) which can be increased after a single bout of exercise (Bloor 2005; Gavin 2009). One important negative angiogenic regulator is trombospondin-1 (TSP-1). This matrix glycoprotein inhibits angiogenesis for example by inhibition of endothelial cell proliferation, diminution of cell spreading and inhibition of proteolytic enzymes. TSP-1 influences angiogenesis in skeletal muscle by direct or indirect effects on VEGF (Malek et al. 2009; Olfert et al. 2011).

NO has several functions in the vasculature such as suppression of smooth muscle cell proliferation, modulation of leukocyte activation and adhesion as well as stimulation of VEGF expression (Huang 1999; Egginton 2009). Several studies demonstrated that the availability of NO is a critical factor for the implementation of angiogenesis in rodent skeletal muscle. Down-regulation of NOS activity reduced the growth of the capillary system in skeletal muscles of rodents after chronic electrical stimulation or muscle overload as well as in response to prazosin administration (Hudlická et al. 2000; Williams et al. 2006). But the clear impact of NO / nNOS on angiogenesis in skeletal muscle still needs to be more precisely determined.

1.4 Exercise

Skeletal muscle adapts to endurance exercise, like prolonged running, with characteristic and coordinated changes in the expression levels of many genes, which subsequently lead to multifaceted alterations in muscular ultrastructure and function. There are several established hallmarks of endurance exercise (Coffey et al. 2007; Hoppeler et al. 2007). The most common adaptations of skeletal muscle to exercise are fibre type transformation, mitochondrial biogenesis and angiogenesis (Fig. 6) (Yan et al. 2011).

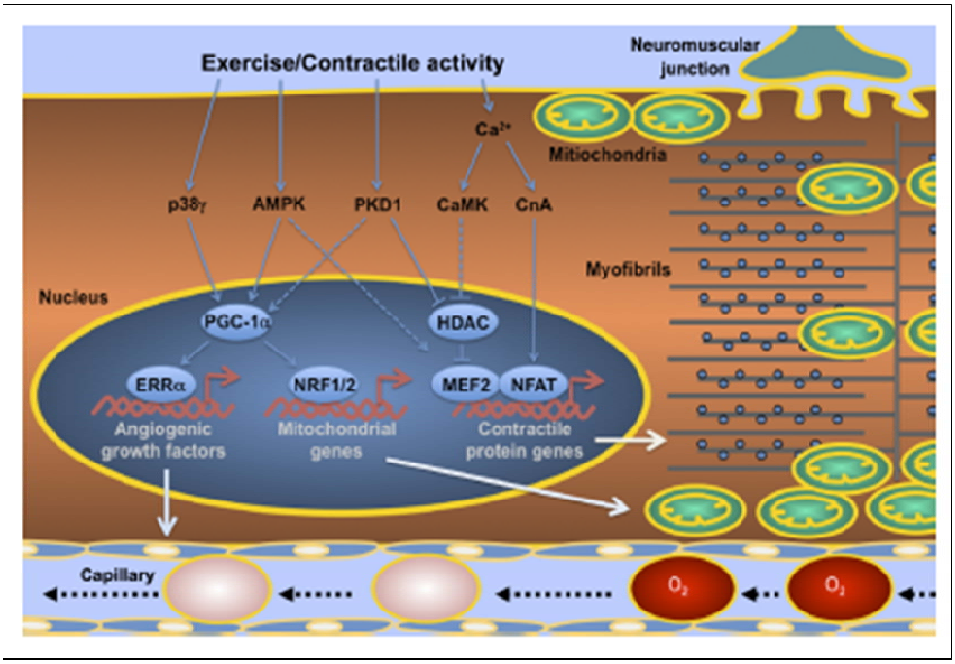

Figure 6: Schematic presentation of the signalling and molecular mechanisms underlying exercise-induced adaptations in skeletal muscle (Yan et al. 2011).

An adaptation of skeletal muscle to endurance exercise is fibre type shifting whose phenotypic ability correlates with physical performance. As mentioned before not every skeletal muscle has the same functional demand and therefore has a different fibre type distribution. The most common fibre type adaptation in skeletal muscle in response to exercise is a reduction in fast-twitch glycolytic fibres (type 2B / 2X) and an accumulation of fast-twitch oxidative fibres (type 2A) (Waters et al. 2004). Especially due to endurance exercise, another fibre type transformation is from fast-

glycolytic (type 2B / 2X) to slow-oxidative (type 1) fibres (Howald et al. 1985; Schmutz et al. 2006). Fibre type transformation due to exercise is influenced by different factors as AMP-activated protein kinase (AMPK), PGC-1alpha and peroxisome proliferator-activated receptor beta / delta (PPAR-beta / -delta) (Yan et al. 2011).

Mitochondrial biogenesis (up-regulation of mitochondrial volume density and function) is important in exercise-induced improvement of muscle function and whole body metabolic homeostasis (Lanza et al. 2008). Both, PGC-1alpha and estrogen-related receptor alpha (ERR-alpha) are major regulators of mitochondrial function in response to exercise (Arany et al. 2008; Chinsomboon et al. 2009). The expression of PGC-1alpha is up-regulated due to exercise which results in a rapid increase in the expression levels of nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2). These increases represent key regulatory components of the stimulation of mitochondrial biogenesis by exercise.
Additionally, PGC-1alpha mediates up-regulation of the mRNA content of glucose transporter 4 (GLUT4), mitochondrial respiratory chain proteins (like cytochrome oxidase (COXII and COXIV) and F_1F_0-ATP synthase (Baar et al. 2002; Olesen et al. 2010). Skeletal muscles release increased amounts of NO and superoxide during contractile activity or passive stretching by activating their generating enzymes, like nNOS and plasma membrane oxidoreductases. NO can induce mitochondrial biogenesis by up-regulating PGC-1alpha and interacting with AMPK (Lira et al. 2010). Skeletal muscles adapt to exercise to protect against muscle damage after a second exercise period. Therefore, contractile activity also increases the amount of heat shock proteins (HSPs, like HSP70) and antioxidant enzymes (McArdle et al. 2000; Jackson 2005).

The expression of mitochondrial genes and oxidative enzymes is up-regulated, whereas glycolytic enzymes are down-regulated in the skeletal muscle fibres. This shift in metabolism is accompanied by a distinct increase in the density of capillaries surrounding the muscle fibres due to angiogenesis. The increase of capillaries is attributed to an increase in capillary red blood cell velocity and often to an elevated shear stress (Egginton 2009; Hudlicka et al. 2009). The increased blood flow is

required to provide additional supply of nutrients and oxygen which cannot be gained by increased cardiac output (Andersen et al. 1985).

Important factors for exercise induced skeletal muscle angiogenesis are for example myocyte VEGF-A, VEGFR-2 and PGC-1alpha (Gustafsson et al. 2007; Olfert et al. 2010). One important negative angiogenic factor in skeletal muscle is TSP-1. It was shown, that after three days of exercise TSP-1 mRNA is degraded. There might be an altered balance between VEGF and TSP-1 in response to exercise which regulates the onset of angiogenesis (Olfert et al. 2006). Besides, the transcriptional coactivator PGC-1alpha regulates angiogenesis in response to exercise. PGC-1alpha activates ERR-alpha which mediates the increase of VEGF expression.

The molecular mechanisms and regulations underlying the exercise-induced changes in skeletal muscle phenotype, particularly of the microvascular system, are only incompletely understood.

2 Methods and Results

2.1 Project I

Exercise-induced angiogenesis correlates with the up-regulated expression of neuronal nitric oxide synthase (nNOS) in human skeletal muscle

The first project of my PhD thesis focuses on the hypothesis that the expression of nNOS is associated with the capillary density in human skeletal muscle in response to moderate endurance exercise.

Biopsies of the vastus lateralis muscle had been derived from 10 sedentary male subjects before and after moderate training (four 30 minutes weekly jogging sessions for six months, with a heart-rate corresponding to 75% VO_2max).

Summarizing the results of this project, the expression of nNOS at mRNA and protein levels was up-regulated in response to endurance exercise, especially in those subjects who responded to exercise with an increase in the capillary-to-fibre ratio and the numerical density of capillaries. Additionally, the nNOS up-regulation was significant positive correlated to the capillarity after endurance exercise in human VL biopsies. These data suggest that nNOS is involved in the angiogenic response to exercise in human skeletal muscle.

The data are presented in the following manuscript, which has been accepted for publication in European Journal of Applied Physiology (Huber-Abel et al. in press).

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ORIGINAL ARTICLE

Exercise-induced angiogenesis correlates with the up-regulated expression of neuronal nitric oxide synthase (nNOS) in human skeletal muscle

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Abstract The contribution of neuronal nitric oxide synthase (nNOS) to angiogenesis in human skeletal muscle after endurance exercise is controversially discussed. We therefore ascertained whether the expression of nNOS is associated with the capillary density in biopsies of the vastus lateralis (VL) muscle that had been derived from 10 sedentary male subjects before and after moderate training (four 30-min weekly jogging sessions for 6 months, with a heart-rate corresponding to 75% VO_2max). In these biopsies, nNOS was predominantly expressed as alpha-isoform with exon-mu and to a lesser extent without exon-mu, as determined by RT-PCR. The mRNA levels of nNOS were quantified by real-time PCR and related to the capillary-to-fibre ratio and the numerical density of capillaries specified by light microscopy. If the VL biopsies of all subjects were co-analysed, mRNA levels of nNOS were non-significantly elevated after training (+34%; $P > 0.05$). However, only five of the ten subjects exhibited significant ($P \leq 0.05$) elevations in the capillary-to-fibre ratio (+25%) and the numerical density of capillaries (+21%) and were thus undergoing angiogenesis. If the VL biopsies of these five subjects alone were evaluated, the mRNA levels of nNOS were significantly up-regulated (+128%; $P \leq 0.05$) and correlated positively ($r = 0.8$; $P \leq 0.01$) to angiogenesis. Accordingly, nNOS protein expression in VL biopsies quantified by immunoblotting was significantly increased (+82%; $P \leq 0.05$) only in those subjects that underwent angiogenesis. In conclusion, the expression of nNOS at mRNA and protein levels was statistically linked to capillarity after exercise suggesting that nNOS is involved in the angiogenic response to training in human skeletal muscle.

Keywords Angiogenesis · Human exercise physiology · Neuronal nitric oxide synthase · Skeletal muscle

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Introduction

Skeletal muscles adapt to endurance exercise with characteristic and coordinated changes in the expression levels of many genes, which subsequently lead to multifaceted alterations in muscular ultrastructure and function. There are several established hallmarks of endurance exercise (reviewed in Coffey and Hawley 2007; Hoppeler et al. 2007). The expression of mitochondrial genes and oxidative enzymes is up-regulated, whereas glycolytic enzymes are downregulated in the skeletal muscle fibres. This shift in metabolism is accompanied by a distinct increase in density of capillaries surrounding the muscle fibres due to angiogenesis (reviewed in Egginton 2009; Hudlicka and Brown 2009).

The molecular mechanisms underlying the exercise-induced changes in skeletal muscle phenotype, particularly of the microvascular system, are only incompletely understood. Conceptually, information relating to the higher rates of muscle contractility is translated into an up-stream signalling event, which is subsequently transduced into the extracellular environment surrounding the muscle fibres.

Neuronal NO synthase (nNOS) is expressed in high concentrations at the sarcolemma of the muscle fibres, where it generates nitric oxide (NO), a diffusible gaseous radical with impact on many processes in skeletal muscle (reviewed in Stamler and Meissner 2001). As an autocrine

signalling molecule, nNOS-derived NO promotes the relaxation of skeletal muscle fibres to counteract their contraction (Kobzik et al. 1994) and participates in the regulation of the redox state in skeletal muscle fibres (Da Silva-Azevedo et al. 2009; Jackson et al. 2007). As a paracrine signalling molecule, nNOS-derived NO controls the cGMP-dependent relaxation of vascular smooth muscle cells in skeletal muscle, thereby contributing to vasodilation (Lau et al. 2000; Melikian et al. 2009).

Several studies demonstrated that the availability of NO is a critical factor for the implementation of angiogenesis in skeletal muscle of rodents. Down-regulation of NOS activity by chemical inhibition reduced the growth of the capillary system in skeletal muscles of rats after chronic electric stimulation (Hudlicka et al. 2000). Furthermore, sprouting angiogenesis was abolished in nNOS-knockout-mice in response to muscle overload induced by extirpation of a functional agonist (Williams et al. 2006). In accordance with this role of nNOS as modulator of angiogenesis, nNOS protein levels and activity revealed to be increased in skeletal muscles of rodents after chronic exercise (Vassilakopoulos et al. 2003). In contrast, the regulation and functional impact of nNOS to angiogenesis in response to endurance exercise in skeletal muscle of humans has been controversially discussed (Frandsen et al. 2000; McConell et al. 2007).

Our group has previously conducted a study with unexercised male subjects undergoing a moderate training enrolled in a 6-month jogging program (Suter et al. 1995). Analysing biopsies of the vastus lateralis (VL) muscle by means of morphometry, this training regime evoked a heterogenous structural response. While the mitochondrial density was higher in all subjects after exercise, only some study participants showed an alteration in the muscular intracellular lipid content and capillarity (Suter et al. 1995). To ascertain whether the expression of neuronal nitric oxide synthase (nNOS) is associated with exercise-induced angiogenesis in human skeletal muscle, we now quantified the nNOS expression at the mRNA and protein levels in these VL muscle biopsies. Statistical evaluation of the sets of data revealed the mRNA and protein levels of nNOS to be up-regulated in response to endurance exercise and to be correlated with the degree of angiogenesis. These findings suggest that nNOS is involved in the angiogenic response to training in skeletal muscle of humans.

Materials and methods

Subjects and training protocol

Fine-needle biopsies of the vastus lateralis (VL) muscle were derived from 10 male subjects in the context of an earlier study (Suter et al. 1995). Previous to the study, the participants had not been engaged in endurance activities. The moderate, home-based training regime consisted of four 30-min jogging sessions per week for 6 months, with a heart-rate corresponding to a maximal oxygen uptake (VO_2max) of 75%. The heart rate of the subjects participating in this study was continuously recorded during the training with a portable heart rate monitor as previously reported (Suter et al. 1995). VO_2max was determined in a continuous incremental exercise test on a bicycle ergometer (start at 75 W, followed by increments of 30 W every 2 min) to voluntary exhaustion. The study conformed with the guidelines delineated by the Bernese Ethical Commission, and was conducted with its approval.

The male subjects had a mean age of 36.3 (±6.3) years, a mean height of 181.0 (±7.1) cm and a mean weight of 85.6 (±16.0) kg and a mean body-mass index of 26.2 (±4.0) kg/cm^2. The mean VO_2max increased from a pre-training value of 39.6 (±5.5) ml/kg min to a post-training one of 41.3 (±6.3) ml/kg min, but the difference was not significant ($P > 0.05$). VL biopsies, 50–100 mg in weight, were obtained using a Bergström fine needle 48 h after the last training session, as previously described (Suter et al. 1995).

Morphometric analysis

1-mm^3 pieces of the VL biopsies were chemically fixed for several days at 4°C in an aqueous solution containing 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde, which was buffered with 0.1 M sodium cacodylate (pH 7.4) as previously described (Hoppeler et al. 1985; Suter et al. 1995). 1-μm thick sections for light microscopy were prepared and stained with toluidine blue to determine the capillary-to-fibre (C/F) ratio and the numerical density of capillaries on light micrographs (Baum et al. 2004). This approach represents the gold standard to quantitatively assess the capillarity due to the direct visualization of the microvasculature.

Transmission electron microscopy and morphometry to quantify the mitochondrial volume density and the mean fibre cross-sectional area was performed as previously described (Hoppeler et al. 1985).

RNA extraction and reverse transcription

For each biopsy, RNA was extracted from 100 25-μm thick cryo-sections using an RNeasy Mini kit (Qiagen, Hilden, Germany). The concentration of RNA was spectrophotometrically determined (absorbance wavelength: 260 nm) using a NanoDrop (Thermo Scientific, Wilmington, DE, USA).

First-strand DNA was synthesized from the RNA using a Superscript VILO cDNA synthesis kit (Invitrogen, Basel,

Switzerland) in accordance with the manufacturer's instructions.

RT-PCR

For the determination of the mRNA expression profiles of nNOS in human VL by reverse transcription (RT)-PCR, the GoTaq Hot Start Polymerase (Promega, Dübendorf, Switzerland) was used. This analysis was performed on six randomly selected biopsies taken prior to the exercise. nNOS exon-specific primer pairs were designed using Primer3 software and purchased from Microsynth (Balgach, Switzerland): exon 1a: 5′-CAG ATG GCA GCA GAC AGG TA-3′; exon 1c: 5′-CTG ACT GCC CTT GTC TCT CC-3′; exon 1f: 5′-CCC ACA CCC ATA AAC CAG TC-3′; exon 1 g: 5′-CGG GAG GAA GAG GAG GAG TA-3′, exon 5 reverse: 5′-GGA GCC CATG CAG ATG TAC T-3′; exon 4–11: 5′-ACG CTT CCT CAA GGT CAA GA-3′ and 5′-ACC GCG ATA TTG ATC TCC AC-3′; exon 10–15: 5′-CCC TTC AGT GGC TGG TAC AT-3′ and 5′-TCA TGT TCC AGG TGC ACA AT-3′; exon 14–18: 5′-TAT GGC CAA GAG GGT GAA AG-3′ and 5′-CGA GGC CAA AAA CTG AGA AC-3′; exon 17–22: 5′-ACA AGG TCC GAT TCA ACA GC-3′ and 5′-CAG ACG CTG CTT CTC CTT CT-3′; exon 21–26: 5′-TGG TGA AAG TGG AAC TGC TG-3′ and 5′-TGA AGA CCC CCT TGT TCT TG-3′; exon 25–28: 5′-CGG CAA TTT GAT ATC CAA CAC-3′ and 5′-CTC AGA TCT AAG GCG GTT GG-3′. The total reaction volume of 25 µl consisted of 5× Green GoTaq Buffer, dNTP mix (10 mM), $MgCl_2$ solution (1 mM), primers (100 µM), GoTaq Hot Start Polymerase (1.25 U), cDNA template (50 ng) and double-distilled water. The following PCR cycling protocol was applied: initial denaturation step at 94°C for 2 min, then 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 60 s, elongation at 72°C for 90 s and a final elongation step at 72°C for 10 min. 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). Primers were designed using the software Primer Express (Applied Biosystems) and qPrimerDepot: nNOS primer pair: 5′-CAG CCC AAT GTC ATT TCT GTT-3′ and 5′-GAT CAC GGG CGG CTT ACT-3′. For normalization, 18S rRNA levels were measured using the following primer pair: 5′-GCT TAA TTT GAC TCA ACA CGG GA-3′ and 5′-AGC TAT CAA TCT GTC AAT CCT GTC-3′. The total reaction volume of 15 µl consisted of 2× SYBR Green PCR Master Mix, primers (500 nM), cDNA (50 ng) and double-distilled water. Following a 15-min denaturation step at 95°C, specific cDNAs were amplified through 40 cycles at 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. For recording of the dissociation curves the following protocol was used: 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. For analysis of the mRNA expression, cycling reports and melting curves were evaluated after setting of a baseline cycle threshold. Data were evaluated by the relative quantification method ($2^{-\Delta CT}$).

Immunoblotting

Immunoblotting was performed using 50 µg of protein, as previously described (Da Silva-Azevedo et al. 2009). 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.

Statistics

All numerical data are expressed as mean values (±SEM). Parameters pertaining to the anthropometric and molecular analyses were compared using a paired Students *t*-test. Significance values relating to the real-time PCR data were calculated by a two-way ANOVA (repeated measures). The significance level was set at $P \leq 0.05$.

Results

mRNA expression profiles of nNOS isoforms in human VL biopsies

The mRNA expression profiles of nNOS isoforms were analysed in six randomly selected VL biopsies conducting RT-PCR. The 5′-UTR of the mRNA for nNOS was determined by combining an exon 5 reverse primer with forward primers derived from the four major mRNA variants of 5′-UTR (1a, 1c, 1f and 1g) that are generated by alternative splicing of the nNOS gene. When exon 1c was used as the upstream primer in the RT-PCR analysis, a single cDNA band (1,500 base-pairs) was detected. When any of the other three exon-variants (1a, 1f or 1g) were used as the forward primer, no cDNA was amplified (Fig. 1a). Nucleotide sequencing confirmed the 1c–2–3–4–5 exon-composition of the PCR-amplificate (data not shown).

Six additional pairs of exon-specific primers were designed, which covered the complete cDNA-coding sequence of nNOS downstream from exon 4. Using five of

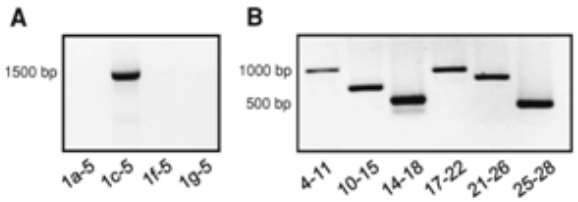

Fig. 1 mRNA expression profiles of nNOS isoforms in the human vastus lateralis (VL) muscle. **a** A representative agarose gel after RT-PCR on biopsies of human VL using nNOS-specific forward primers for the human exons 1a, 1c, 1f and 1g and a reverse primer for exon 5. A cDNA band (1,500 base-pairs in length) was amplified only when using exon 1c as the forward primer. **b** Evaluation of the mRNA expression patterns of nNOS isoforms using overlapping primers from exons 4 to 28. The identity of each band was confirmed by nucleotide sequencing. Note that two bands were amplified when using the nucleotide sequence between exons 14 and 18

the six primer-pair combinations, a single cDNA band was amplified by RT-PCR (Fig. 1b). This finding indicates that no alternative splicing of the mRNA for nNOS occurs between exons 4–15 and exons 17–28. The molecular-weight characteristics of the cDNA bands indicated that they contained no introns; nucleotide sequencing confirmed this to be the case (data not shown). When using a primer pair that covered the nNOS nucleotide sequence between exons 14 and 18, two cDNA bands were amplified by RT-PCR (Fig. 1b); each encoded an nNOS isoform with a 14–15–16–17–18 exon-composition, as determined by nucleotide sequencing (data not shown). In the larger molecular-weight band, an additional nucleotide segment, 102 base-pairs in length (exon mu), was inserted between exons 16 and 17. Thus, two mRNA isoforms of nNOS are co-expressed in the human VL. Both variants contain exons 1c and 2 and are thus alpha isoforms; they differ in the absence or the presence of the exon mu. Since the larger molecular-weight band had an approximately tenfold higher optical density than the smaller one, the mu isoform of nNOS is more abundantly expressed in human VL than the non-mu variant.

Angiogenesis as a function of nNOS expression and exercise in human VL biopsies

The quantification analysis of VL biopsies in the light microscope revealed exercise to elicit significant ($P \leq 0.05$) and concomitant increases in the C/F-ratio (21–31%) and the numerical density of capillaries (4–42%) in five of the ten individuals, who were assigned to the so-called "angiogenesis responder" (AR) group (Table 1). In the other five subjects [assigned to the "non-angiogenesis responder" (NR) group comprising the subjects that showed no numerical increase in capillarization with training], the pre- and post-exercise values for these parameters did not differ significantly from each other ($P > 0.05$).

Table 1 Morphometric data relating to structural parameters in the human VL biopsies before and after moderate long-term exercise

Subject		C/F-ratio (*n*)	Capillary density (mm^{-2})	MCSFA (μm^2)	Group
1	Pre-exercise	1.62	342	4741	AR
	Post-exercise	2.00	484	4136	
2	Pre-exercise	1.48	404	3663	AR
	Post-exercise	1.82	519	3511	
3	Pre-exercise	1.11	342	2926	AR
	Post-exercise	1.45	355	4091	
4	Pre-exercise	1.95	478	4080	NR
	Post-exercise	1.70	408	4161	
5	Pre-exercise	1.88	510	3688	NR
	Post-exercise	2.26	504	4485	
6	Pre-exercise	1.82	581	3125	NR
	Post-exercise	1.72	515	3343	
7	Pre-exercise	1.30	460	2814	AR
	Post-exercise	1.70	599	2838	
8	Pre-exercise	1.30	484	2686	NR
	Post-exercise	1.29	482	2684	
9	Pre-exercise	1.42	592	2400	AR
	Post-exercise	1.72	638	2696	
10	Pre-exercise	1.59	421	3787	NR
	Post-exercise	1.35	390	3462	

Subjects in whom both the C/F-ratio and the capillary density were concomitantly increased after the exercise were assigned to the angiogenesis responder (AR) group, and those in whom no concomitant exercise-induced increases in these parameters occurred to the group showing no numerical increase in capillarization with training (NR)

C/F-ratio number of capillaries per number of fibres, *capillary density* number of capillaries per skeletal muscle area, *MCSFA* mean cross-sectional fibre area

The mitochondrial volume density which is usually increased after endurance exercise as an established hallmark of the oxidative metabolism was higher after training (+14%; $P < 0.05$; AR: +14% and NR: +13%) and non-significantly related to VO_2max ($r = 0.2$; $P > 0.05$) and capillarity ($r = 0.2$; $P > 0.05$) and nNOS mRNA levels ($r = 0.4$; $P > 0.05$), if all 10 subjects of the study were included in the analysis (data not shown). The corresponding correlation coefficients for the AR and NR groups are: oxidative capacity and VO_2max: AR: $r = 0.50$; $P > 0.05$ and NR: $r = 0.42$; $P > 0.05$; oxidative capacity and capillarity: AR: $r = 0.30$; $P > 0.05$ and NR: $r = 0.53$; $P > 0.05$; oxidative capacity and nNOS mRNA expression: AR: $r = 0.67$; $P > 0.05$ and NR: $r = 0.72$; $P > 0.05$. Albeit the mitochondrial volume density was increased after endurance exercise, it was not significantly related to nNOS expression in both the AR and the NR cohorts.

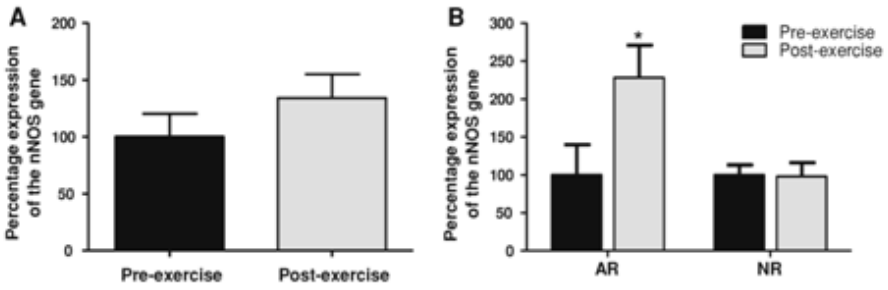

Fig. 2 Exercise-induced up-regulation of the nNOS mRNA expression in VL biopsies derived from subjects of the angiogenesis responder (AR) group but not from the group showing no numerical increase in capillarization with training (NR). **a** When the group of subjects was considered as a whole, the mRNA levels of nNOS (determined by the real-time PCR technique) were not significantly elevated after exercise (+34%, $P > 0.05$). **b** In the AR group subjects, exercise induced a significant elevation in the mRNA level of nNOS (+128%, $P \leq 0.05$), whereas in the NR group of individuals, this parameter was not significantly influenced by the training regime (−2%, $P > 0.05$). Mean values ± SEM. **a** $n = 10$; **b** $n = 5$ in each of the two groups. The pre-exercise values were set as 100%

The real-time PCR analysis of the VL biopsies disclosed training to elicit no significant increase (+34%; $P > 0.05$) in the mRNA level of nNOS for the group of participants as a whole (Fig. 2a). However, when the AR group was analysed separately, the nNOS gene revealed to be significantly up-regulated (+128%; $P \leq 0.05$) in response to exercise. In the NR group, the mRNA level of nNOS was not significantly increased (−2%; $P > 0.05$) by exercise (Fig. 2b). Quantifying the mRNA levels in the biopsies collected before and after the training period, VEGF-A expression was only non-significantly altered if referred to all 10 subjects, those of the AR or the NR group (data not shown). As shown in Fig. 3, a significant correlation existed between the exercise-induced elevation in the mRNA level of nNOS and the exercise-induced increase in the C/F-ratio ($r = 0.8$; $P \leq 0.01$). The nNOS expression was also positively but non-significantly correlated to VO_2max ($r = 0.2$; $P > 0.05$) and mitochondrial volume density ($r = 0.4$; $P > 0.05$; data not shown).

Conducting immunoblotting, nNOS is predominantly demonstrated as a 165 kDa band (Fig. 4a). If the VL biopsies were subjected to quantitative immunoblotting, the nNOS protein levels were significantly higher in the samples collected after than before the exercise in the subjects of the AR group (+82%; $P \leq 0.05$) but not in those of the NR group (−16%; $P > 0.05$), as shown in Fig. 4b. The levels of nNOS protein were positively correlated to C/F-ratio ($r = 0.52$; $P \leq 0.05$; AR: $r = 0.21$; NR: $r = 0.38$) and to those of nNOS mRNA ($r = 0.86$; $P \leq 0.01$; AR: $r = 0.66$; NR: $r = 0.66$).

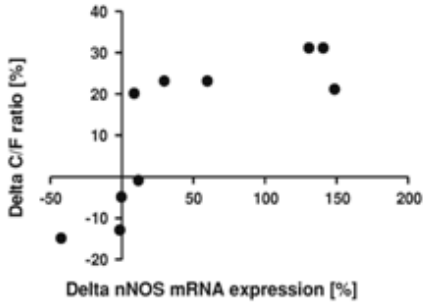

Fig. 3 Relationship between the exercise-induced differences in C/F-ratio and mRNA levels of nNOS in human VL biopsies. A significant positive correlation ($r = 0.8$; $P \leq 0.01$) existed between the exercise-induced differences in C/F-ratio and nNOS expression

Discussion

Since the signalling enzyme nNOS undergoes alternative splicing in many tissues (Wang et al. 1999), we first wished to identify its isoform-specific expression pattern in the human VL. The RT-PCR analysis revealed the expression of two mRNA isoforms in these samples. Both mRNA variants express exon 2 and are thus nNOS alpha isoforms; they differ in the absence or presence of exon mu, which inserts between exons 16 and 17 and consists of 102 base-pairs (Silvagno et al. 1996). This finding indicates that exon 2 and exon mu might be simultaneously co-expressed within an individual mRNA molecule of nNOS. Consequently, alpha and mu isoforms of nNOS do not represent different proteins, as has been previously contended (Bradley et al. 2007; Laine and de Montellano 1998). In the real-time PCR analysis, we did not discriminate between the various isoforms of nNOS since the variants do not differ in either their catalytic properties or their sites of localization (Silvagno et al. 1996).

In five of the ten individuals, the C/F-ratio and the numerical density of capillaries were concomitantly higher after than before exercise, thereby indicating that the absolute number of capillaries increased without a corresponding increase in the mean fibre cross-sectional area (Hudlicka 1998). Hence, in these individuals (assigned to the AR group), the VL responded to the moderate regime of

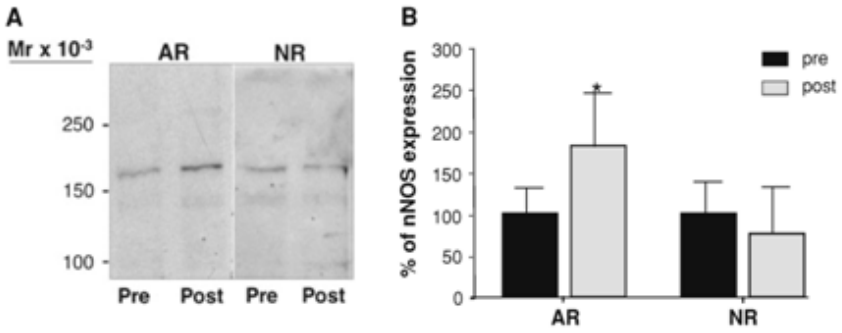

Fig. 4 Exercise-induced up-regulation of the nNOS expression at the protein level in VL biopsies derived from angiogenesis responder (AR) group but not from the group showing no numerical increase in capillarization with training (NR). **a** Quantitative immunoblotting revealed the nNOS band (165 kDa) to be markedly up-regulated in the VL muscle biopsies of individuals of the AR but not the NR group collected after (post) than before (pre) the exercise. **b** In the AR group subjects, exercise induced a significant elevation in the protein level of nNOS (+82%, $P \leq 0.05$), whereas in the NR group of individuals, this parameter was not significantly influenced by the training regime (−16%, $P > 0.05$). Mean values ± SEM. $n = 4$ in each of the two groups. The pre-exercise values were set as 100%

exercise with a process of angiogenesis. In the other five subjects (assigned to the NR group), a similar regime of exercise elicited no significant change in either the C/F-ratio or the numerical density of capillaries. According to the available documentation, the 10 subjects showed different responses in capillarity, although they have underwent a training of identical intensity and duration. The best way to converge these observations is to hypothesize that the individuals exhibited a different degree of malleability. Such a heterogeneous response to the same training stimulus is a well-documented phenomenon (Bouchard and Rankinen 2001; Bray et al. 2009; Timmons and Sundberg 2006), and is presumed to reflect individual differences in genetic predisposition. The overtly non-consistent reactivity of the capillary system might also be influenced by the assortment of the study participants (rather untrained) and the training load (rather moderate), because an induction of angiogenesis in skeletal muscle is observed usually in response to endurance exercise (Gavin 2009).

The real-time PCR analysis revealed a significant exercise-induced increase in the mRNA level of nNOS only in the AR group of individuals, and in these subjects, this parameter was significantly positively correlated with the capillarity. Accordingly, the nNOS protein expression was significantly increased in the VL muscle biopsies of specifically those subjects that underwent angiogenesis as determined by quantitative immunoblotting. On the basis of these findings, we conclude that the expression of nNOS increases at the mRNA and protein level only if the VL responds to exercise with angiogenesis. Correspondingly, increases of C/F-ratio were accompanied by an up-regulation of sarcolemmal nNOS expression in response to exercise performed during bed rest (Rudnick et al. 2004; Salanova et al. 2008).

In two previously published studies, exercise-induced changes in the protein concentration of nNOS within homogenates of VL biopsies that had been derived from subjects with anthropometrically similar characteristics to ours were investigated by immunoblotting (Frandsen et al. 2000; McConell et al. 2007). In one of these studies (Frandsen et al. 2000), the protein levels of nNOS and its catalytic activity were non-significantly up-regulated in response to exercise, whereas in the other (McConell et al. 2007), a 10-day, high-intensity training regime elicited a significant 29% increase in the protein levels of nNOS. The evidently contradictory observations made in these two studies can be merged with the conclusion that the expression of nNOS increases in human VL muscle in response to endurance exercise only if angiogenesis is simultaneously noticeable, as we have drawn in this investigation.

However, our experimental data do not permit us to ascertain whether the regulation of nNOS expression is a cause or consequence of angiogenesis, or indeed an independent coincidence. Although animal studies demonstrated an up-stream signalling function of higher available levels of nNOS-derived NO on exercise-induced angiogenesis in skeletal muscle (Vassilakopoulos et al. 2003; Williams et al. 2006), it remains to be clarified whether nNOS exerts such a function in humans.

Two explanations of the influence of nNOS on exercise-induced angiogenesis in skeletal muscle are plausible. According to the first, higher concentrations of nNOS-generated NO would directly or indirectly raise the expression or activity of PGC-1alpha inside the skeletal muscle fibres, which, as an autocrine signalling molecule, would regulate not only mitochondrial biogenesis but also VEGF-A-dependent angiogenesis in response to exercise (Arany et al. 2008; Chinsomboon et al. 2009; Leick et al. 2009). Because VEGF-A mRNA is only transiently induced in adaptation to exercise (Gustafsson et al. 2007), we suggest that its expression was already down-regulated at the time point the biopsies were taken.

Alternatively, since all alpha isoforms of nNOS (either with or without exon mu) are localized to the sarcolemma, the NO-product of their catalysis diffuses into the extramysial milieu, where, as a paracrine signalling molecule, it would influence the contractility of vascular smooth muscle cells (Lau et al. 2000). In consequence to this supposition, up-regulated levels of the alpha isoforms of nNOS would also increase the endomysial availability of NO, thereby enhancing the vasodilation of larger feeding vessels (Sarelius and Pohl 2010). The consequent increase in blood flow would then raise the shear stress in the downstream capillaries. This mechanical signal would represent a relevant one for angiogenesis (Baum et al. 2004), which is triggered by endothelial VEGF-A (Da Silva-Azevedo et al. 2002). According to this assumed sequence of events, nNOS would indirectly influence the degree of training-induced skeletal muscle angiogenesis by promoting vasodilation. In contrast, the chemical inhibition of nNOS activity did not influence the muscle hyperaemic response to exercise in rats (Copp et al. 2010) underling that the relationship of nNOS/vasodilation in skeletal muscle has to be further investigated.

Consistent with this hypothesis, the loss of sarcolemmal nNOS from the dystrophic muscles of Duchenne muscular dystrophy patients, as well as from the skeletal muscles of mdx- and nNOS-knockout mice, was accompanied by exaggerated fatigue after exercise (Kobayashi et al. 2008; Percival et al. 2008; Wehling-Henricks et al. 2009). In the light of the data gleaned from these loss-of-function studies in dystrophic muscles, our observation that nNOS is up-regulated after training might represent an additional evidence for the functional impact of up-regulated nNOS on exercise-induced angiogenesis in the skeletal muscles of healthy subjects.

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Conflict of interest There is no conflict of interest.

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2.2 Project II

Characterization of the expression and structure of neuronal nitric oxide synthase (nNOS) isoforms in mice skeletal muscle

In skeletal muscles from mice, various isoforms of nNOS are known: the alpha isoform (160 kDa), the beta isoform (136 kDa), the gamma isoform (125 kDa) and the mu isoform (165 kDa). Currently a detailed characterization of the expression of these nNOS isoforms at protein level is missing. Moreover, the primary structure of these variants and their localisation has not yet been elucidated.

Based on this reason, the aim of the second project of my PhD thesis was to characterise the nNOS isoforms in skeletal muscles from mice. Therefore, the nNOS protein level was analysed by immunoblotting and the localisation of the nNOS isoforms was detected by immunofluorescence. Moreover, the expression and structure of the different nNOS isoforms were investigated at the mRNA level and further analysed by nucleotide sequencing.

As the results of my second project are not yet published, the methods and results are summarized in the following sections.

2.2.1 Methods

Animals

The animal protocol was approved by the Animal Protection Commission of the Canton Bern, Switzerland (no.: 48/08). Six month old male C57Bl/6 mice were housed in a temperature-controlled room (21°C) with a 12:12 h light-dark cycle. Animals were allowed food and water ad libitum.

Immunoblotting

Immunoblotting was performed as previously described in Huber-Abel et al. (in press).

Immunofluorescence histochemistry

For immunofluorescence histochemistry, the cryosections were chemically fixed in a mixture of methanol and acetone (50:50) for 5 min at -20°C. Non-specific enzymatic activity was blocked by treatment for 1 h at ambient temperature with 5% BSA in PBS. The sections were exposed for 2 h to a polyclonal antibody against nNOS (1409-1429, Sigma; diluted 1:20.000 with 5% BSA in PBS), and then for 1 h, likewise at ambient temperature, to a goat anti-rabbit Cy5-conjugated secondary antibody (Sigma; diluted 1:500 with 5% BSA in PBS). As a negative control, the primary antibody was excluded from the incubation medium. Immunofluorescence images were recorded in a Zeiss 510 Metalaser-scanning microscope (Axiovert 200 M, Lasers: HeNe (absorbance wavelength: 633 nm), HeNe (absorbance wavelength: 543 nm), Ar (absorbance wavelength: 488 nm)) and were processed with IMARIS software (Bitplane, Zurich, Switzerland).

RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed as previously described in Huber-Abel et al. (in press).

RT-PCR

The reverse transcription (RT)-PCR was performed using GoTaq Hot Start Polymerase (Promega, Dübendorf, Switzerland). nNOS exon-specific primer pairs were designed using Primer3 software and purchased from Microsynth (Balgach, Switzerland). Sequences of the primer pairs used in figure 10: exon 1a: 5'-CAG ATG GCA GCA GAC AGG TA-3'; exon 1c: 5'-AGG GCT GCT GAT TAG AGC TG-3'; exon 1f: 5'-CGT TTC AGC GGT GAT AGG AT-3'; exon 1g: 5'-AAC CCG GAA AGT TCA CCT CT-3'; exon 2 5'-CTT GGC TTG GAG GTC TTC TG-3'; exon 5 forward: 5'-ACC AGC TCT TCC CTC TAG CC-3'; exon 5 reverse: 5'-GGC TAG AGG GAA GAG CTG GTC C-3'; exon 6: 5'-TCC TTG AGC TGG TAG GTG CT-3'; exon 10: 5'-GGC GTT CGT GAT TAC TGT GA-3'; exon 11: 5'-TTA ATC TCC ACC AGG GCT TG-3'; exon 14: 5'-CTG TGC GAG ATC TTC AAG CA-3'; exon 15: 5'-TCC AGG TGC ACG ATG TCA TA-3'; exon 17: 5'-ACA AGG TCC GAT TCA ACA GC-3'; exon 18: 5'-AGC TCA TCT CCC TCC CTC AT-3'; exon 21: 5'-GTG GTG AAG GTG GAA ATG CT-3'; exon 22: 5'-CCG CTG CTT CTC TTT CTC AT-3'; exon 25: 5'-CTT CCG AAG TTT TTG GCA AC-3'; exon 26: 5'-TCT CTG AAG ACG CCC TTG TT-3'; exon 28: 5'-CTC AGA TCT AAG GCG GTT GG-3'. Sequences of the primers used in figure 14: exon rho/sigma: 5'-CAA CCC AAC GTC ATT TCT GTC C-3'; exon rho/4: 5'-CAA CCC AAC GAA CAG TCT CCC-3'; exon rho/tau: 5'-ATC CAA CCC AAC GAG AGA CCT-3'; exon 5: 5'-GGC TAG AGG GAA GAG CTG GTC C-3'. The protocol was used as described in Huber-Abel et al. (in press). PCR fragments were visualized by 0.8-1% agarose gel electrophoresis supplemented with 0.5 µg/ml ethidium-bromide.

2.2.2 Results

In mice skeletal muscles, four protein bands were detected using immunoblotting with a nNOS C-terminal specific antibody (figure 7). The two larger bands, at 165 and 160 kDa, were the alpha isoform, one with the alternatively spliced mu exon and the other one without the mu exon. The two lower bands, at 140 and 135 kDa, were the beta isoform of nNOS, with and without the mu exon. According to this, four different protein isoforms of nNOS are co-expressed in mice skeletal muscle.

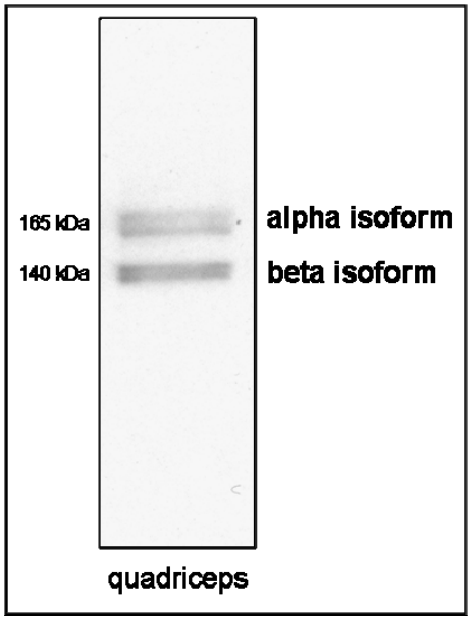

Figure 7: nNOS protein expression in skeletal muscle from mice. The nNOS alpha isoform, with and without the mu exon, is shown at 165 / 160 kDa and the nNOS beta isoform, with and without the mu exon, at 145 / 140 kDa.

The nNOS is linked to the dystrophin glycoprotein complex and thereby to the sarcolemma via the PDZ domain at the N-terminal end (Brenman et al. 1995). The localization of the nNOS alpha and beta isoforms to the sarcolemma of skeletal muscle fibres was demonstrated using a nNOS C-terminal specific antibody detected by immunofluorescence (figure 8).

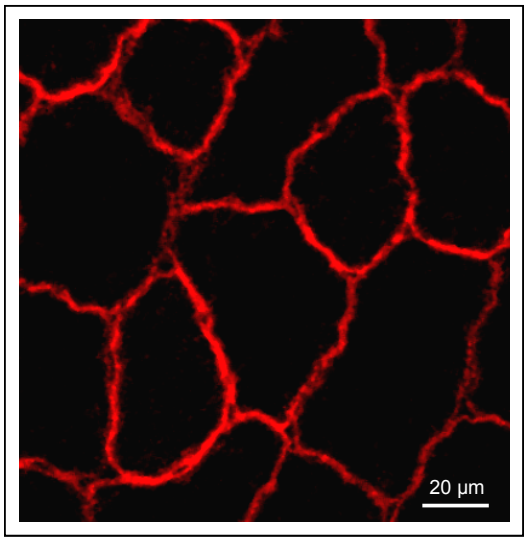

Figure 8: Localisation of nNOS alpha and beta isoforms at the sarcolemma of skeletal muscle fibres. The nNOS alpha and beta isoforms were stained using a nNOS C-terminal specific antibody detected by immunofluorescence.

Covering the complete nNOS coding sequence, eight exon specific over-lapping primer pairs were designed: exon 1a, c, f or g - exon 5, exon 2 - exon 6, exon 5 - exon 11, exon 10 - exon 15, exon 14 - exon 18, exon 17 - exon 22, exon 21 - exon 26 and exon 25 - exon 28 (figure 9).

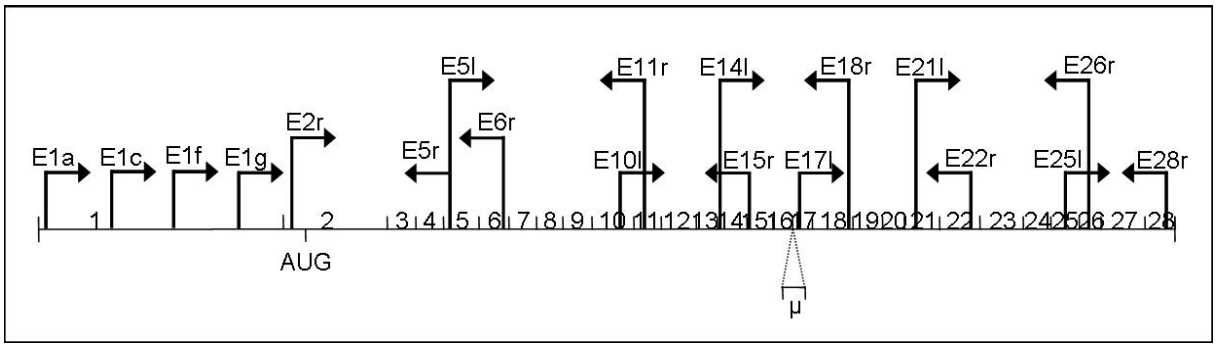

Figure 9: RT-PCR strategy to cover the complete nNOS sequence with eight exon specific over-lapping primer pairs.

The 5' UTR of the nNOS mRNA was determined by using an exon 5 reverse primer and four different exon 1 forward primers. These four major mRNA variants of the 5' UTR (exon 1a, c, f and g) were derived by alternative splicing of the nNOS gene (Wang et al. 1999). When the exon 1c forward primer was used in the RT-PCR analysis, three different cDNA variants were detected in C57Bl/6 mice (figure 10A). When any of the other three forward primers (1a, f or g) were used, no cDNA was amplified.
In nNOS-knockout mice, two different cDNA variants were detected using the primer pair specific for exon 1c and exon 5 (figure 10C). As in C57Bl/6 mice, the other three primer pairs (exon 1a, 1f and 1g - exon 5) detected no cDNA variant in nNOS-knockout mice.

To cover the complete coding sequence of nNOS from exon 2 to exon 28, seven additional exon-specific primer pairs were designed. Using six of the seven primer pair combinations, a single cDNA variant was amplified by RT-PCR in TA muscle from C57Bl/6 mice (figure 10B). This finding indicated that no alternative splicing of the nNOS mRNA occurs between exons 2-15 and exons 17-28. Two cDNA variants were amplified by RT-PCR using the primer pair that covered the nNOS nucleotide sequence between exons 14 and 18 (figure 10B).
In nNOS-knockout mice, a similar expression pattern was amplified (figure 10D). One cDNA variant was amplified using primer pairs that cover the nNOS nucleotide sequence from exons 2-15 and exons 17-28. As in C57Bl/6 mice, two cDNA variants were amplified between exons 14-18. However, using the primer pair specific for exon 2 and exon 6, no cDNA band was detected as exon 2 is deleted in the nNOS-knockout mice (Huang et al. 1993).

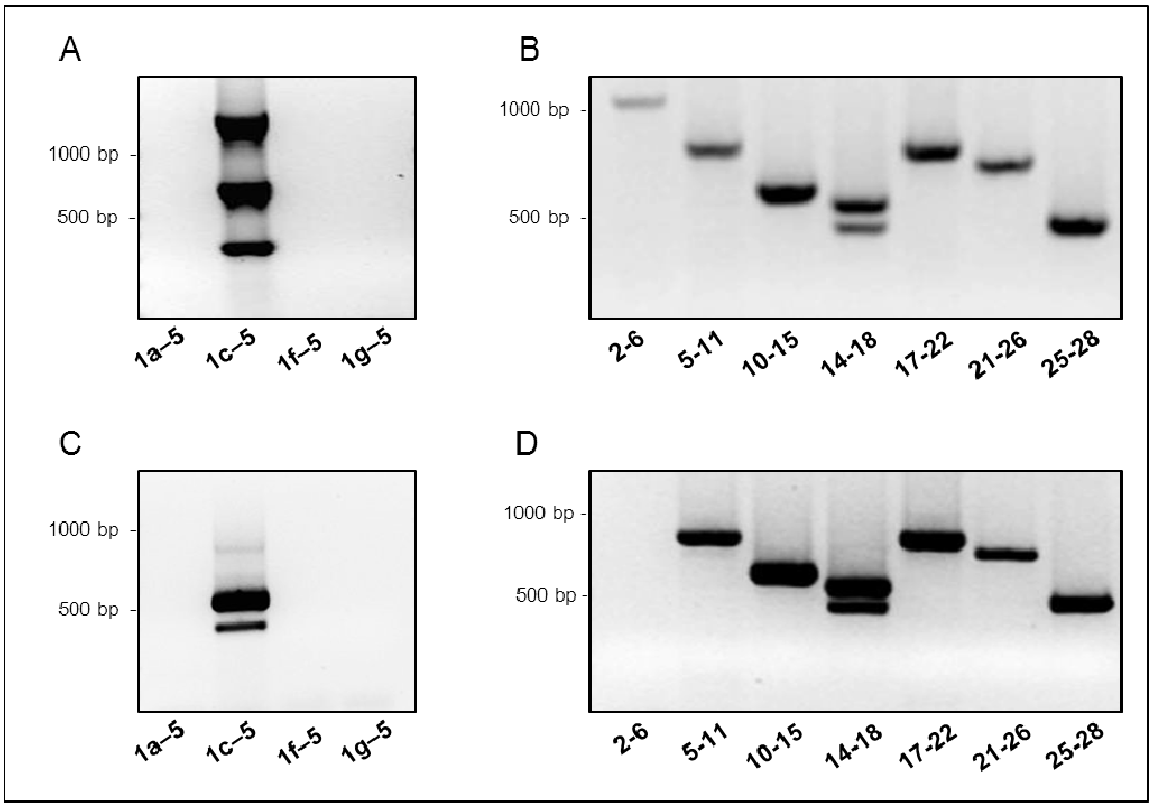

Figure 10: Amplification of the complete nNOS mRNA sequence with different exon specific over-lapping primer pairs in C57Bl/6 (**A** and **B**) and nNOS-knockout (**C** and **D**) mice. **A:** Using the primer pair specific for exon 1c and exon 5, three mRNA variants were detected. **B:** One cDNA band was detected using primer pairs that covered the nNOS sequence between exons 2-15 and exons 17-28. Using the primer pair specific for exon 14 and exon 18, two cDNA variants were amplified. **C:** Using the primer pair specific for exon 1c and exon 5, two mRNA variants were detected. **D:** One cDNA band was detected using primer pairs that covered the nNOS sequence between exons 5-15 and exons 17-28. Using the primer pair specific for exon 14 and exon 18, two cDNA variants were amplified. Using the primer pair specific for exon 2 and exon 6 no cDNA band was amplified.

Nucleotide sequencing and molecular weight characteristics indicated that the cDNA variants contained no introns (data not shown). Additionally, nucleotide sequencing revealed that the lower cDNA variant, amplified between exons 14 and 18, encoded a nNOS isoform with a 14–15–16–17–18 exon composition. In the larger cDNA variant, an additional nucleotide segment, 102 base-pairs in length, was inserted between exons 16 and 17 called exon mu. Thus, six mRNA isoforms of nNOS are co-expressed in the skeletal muscles from C57Bl/6 mice and four mRNA isoforms of nNOS are co-expressed in the skeletal muscles from nNOS-knockout mice.

For further analysis, all 5' UTR bands were sequenced and compared to the previously published gene structure (Brenman et al. 1996).
This analysis revealed a new gene structure with three distinctive differences shown in figure 11:

1.) A 150 bp long nucleotide sequence was detected between exons 1 and 2, which is designated exon pi (the use of a Greek capital indicates that the exon is alternatively spliced).
2.) The original exon 2 was found to actually consist of two different exons, a 270 bp long exon rho containing the initiation codon and a 660 bp long exon sigma.
3.) The original exon 3 is also alternatively spliced and consequently re-named exon tau.

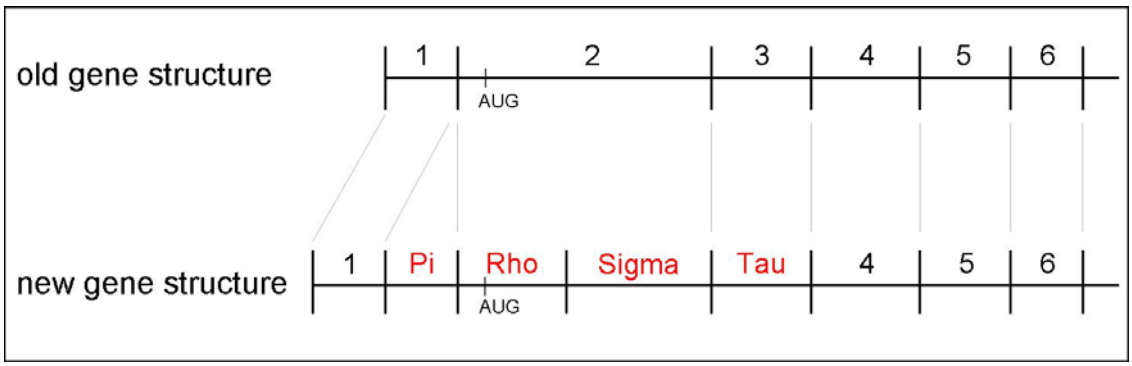

Figure 11: Comparison of the previously published gene structure and the newly detected gene structure of nNOS in skeletal muscle from mice.

According to these findings, the newly detected 1500 bp PCR amplificate consisted of exon 1, exon pi, exon rho (containing the initiation codon), exon sigma, exon tau, exon 4 to exon 29 in C57Bl/6 mice (figure 12). The 650 bp PCR amplificate was missing exon sigma and exon tau. The sequencing of the third band (300 bp) revealed the gene structure from exon 1 to exon 29, missing all four alternatively spliced exons and without any initiation codon. Thus, only two of the three mRNA variants in C57Bl/6 mice might be translated, the nNOS alpha isoform (1500 bp) and the nNOS beta isoform (650 bp), each with and without exon mu.

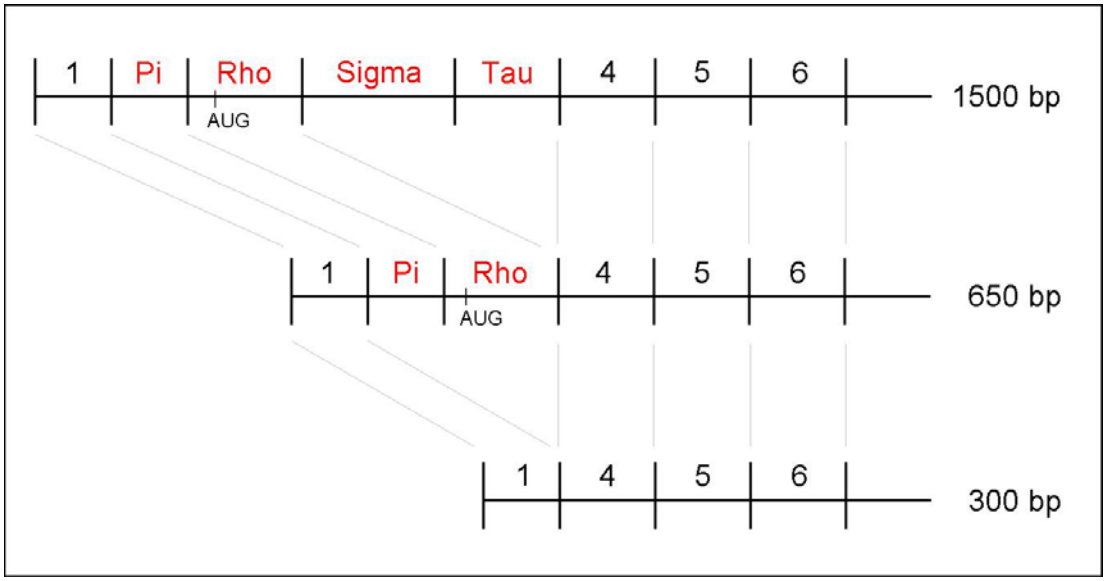

Figure 12: Newly detected gene structure of the nNOS alpha isoform (1500bp) and nNOS beta isoform (650 bp) in skeletal muscle from C57Bl/6 mice. The third nNOS isoform (300 bp), without initiation codon, is not translated.

In nNOS-knockout mice, two mRNA variants were detected and sequenced. The 450 bp sequence consisted of exon 1, exon tau and exon 4 to exon 29 (figure 13). The second mRNA variant consisted of exon 1 to exon 29, missing all alternative spliced exons. Exon rho containing the initiation codon was missing in both mRNA variants in skeletal muscle from nNOS-knockout mice. Therefore, these mRNA variants in nNOS-knockout mice are not translated.

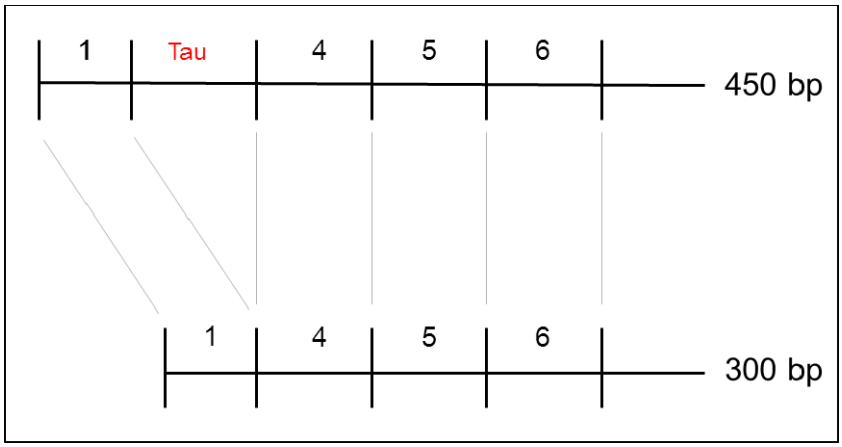

Figure 13: Gene structure of the nNOS isoforms detected in skeletal muscles from nNOS-knockout mice. As the initiation codon is missing in both mRNA variants, they were not translated.

In order to validate the new nNOS gene structure more precisely, RT-PCR with exon-exon over-spanning primer pairs was performed in TA muscles from C57Bl/6 mice (figure 14).

One forward primer covering the boundary between exons rho and sigma was used as a positive control for the nNOS alpha isoform, and another forward primer covering the boundary between exons rho and 4 was used as a positive control for the nNOS beta isoform. Performing RT-PCR with these two forward primers and an exon 5 reverse primer amplified one single cDNA variant. As a negative control, a forward primer covering the boundary between exons rho and tau was also used and detected no cDNA variant in the TA muscles from C57Bl/6 mice (figure 14).

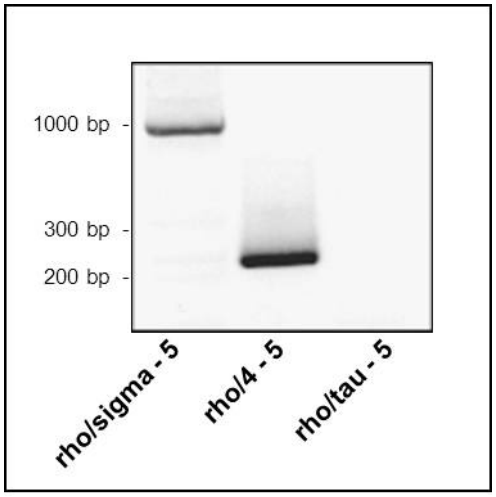

Figure 14: Validation of the new gene structure of the nNOS isoforms by RT-PCR in TA muscle from C57Bl/6 mice. One cDNA band was detected using the exon-exon over-spanning primer pairs specific for exon rho/sigma and exon 5 and for exon rho/4 and exon 5. No cDNA band was amplified using the primer pair specific for exon rho/tau and exon 5.

Altogether six nNOS mRNA variants were detected in skeletal muscles from C57Bl/6 mice. However, only four of these mRNA variants are obviously translated. The new nNOS isoforms in C57Bl/6 mice are the nNOS alpha isoform, with and without the mu exon, and the nNOS beta isoform, with and without the mu exon. In skeletal muscles from nNOS-knockout mice, four mRNA variants were detected but none of them were translated.

2.3 Project III

Characterization of the functional impact of the neuronal nitric oxide synthase (nNOS) alpha isoform in mice skeletal muscle

The third project of my PhD thesis focuses on the characterization of the functional impact of the nNOS alpha isoform in skeletal muscle from mice. Due to our results of the human study (Project I) and further unpublished data, an association between nNOS expression and angiogenesis in response to exercise was expected.

Therefore, the full-coding nNOS alpha sequence was isolated, cloned into a vector and over-expressed in TA muscles from mice by the use of gene electrotransfer. Afterwards, the mice were separated into one sedentary and one exercise group. The efficiency of the transfection and the nNOS mRNA and protein level were analysed by immunofluorescence and real-time PCR.

As the analyses of my third project are not yet complete, the methods and results are summarized in the following section.

2.3.1 Methods

Animals

The animal protocol was approved by the Animal Protection Commission of the Canton Bern, Switzerland (no.: 48/08). Six month old male C57Bl/6 mice were housed in a temperature-controlled room (21°C) with a 12:12 h light-dark cycle. Animals were allowed food and water ad libitum.

Immunofluorescence histochemistry

For immunofluorescence histochemistry, the cryosections were chemically fixed in a mixture of methanol and acetone (50:50) for 5 min at -20°C. Non-specific enzymatic activity was blocked by treatment for 1 h at ambient temperature with 5% BSA in PBS. The sections were exposed for 2 h to a polyclonal antibody against nNOS (1409-1429, Sigma; diluted 1:20.000 with 5% BSA in PBS), and then for 1 h, likewise at ambient temperature, to a goat anti-rabbit Cy5-conjugated secondary antibody (Sigma; diluted 1:500 with 5% BSA in PBS). As a negative control, the primary antibody was excluded from the incubation medium. Immunofluorescence images were recorded in a Zeiss 510 Metalaser-scanning microscope (Axiovert 200 M, Lasers: HeNe (absorbance wavelength: 633 nm), HeNe (absorbance wavelength: 543 nm), Ar (absorbance wavelength: 488 nm)) and were processed with IMARIS software (Bitplane, Zurich, Switzerland).

RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed as previously described in Huber-Abel et al. (in press).

RT-PCR

The reverse transcription (RT)-PCR was performed using GoTaq Hot Start Polymerase (Promega, Dübendorf, Switzerland). nNOS exon-specific primer pairs were designed using Primer3 software and purchased from Microsynth (Balgach, Switzerland): EcoRI + exon 1c: 5'-TAG GAA TTC AGG GCT GCT GAT TAG-3'; exon 5: 5'- ACC AGC TCT TCC CTC TAG CC-3'; exon 7: 5'-GGG CTG TGG TGC AGT CTC-3'; exon 19: 5'-GGC GAA CAA TTC CCT CAT TA-3'; exon 22: 5'-CCG CTG CTT CTC TTT CTC AT-3'; exon 29 + NsiI: 5'-TGG TAC GTA TTA GGA GCT GAA AAC CT-3'. The protocol was used as described in Huber-Abel et al. (in press). PCR fragments were visualized by 0.8-1% agarose gel electrophoresis supplemented with 0.5 µg/ml ethidium-bromide.

Plasmids

Two different expression vectors were used: pGEM®-T easy Vector (Promega, Dübendorf, Switzerland) and pIRES2-ZsGreen1 Vector (Clontech, Heidelberg, Germany).

The synthesis of the nNOS alpha isoform (4676 bp), the cloning into the expression vector pIRES2-ZsGreen1 (pIRES2-ZsGreen1-nNOS) and the endotoxin-free isolation was outsourced to Entelechon (Regensburg, Germany) and PlasmidFactory (Bielefeld, Germany). As control, an empty pIRES2-ZsGreen1 vector was used.

Restriction digest

For the restriction digest, NEB restrictions enzymes and their compatible buffers were used (New England BioLabs, Ipswich, USA). The total reaction volume of 10 µl consisted of 10x NEBuffer, 100x BSA, enzyme, cDNA template (100 ng) and double-distilled water. The digest was incubated at 37°C for 60 min with a following heat inactivation at 80°C for 20 min.

Cloning

The cloning of the full-coding nNOS alpha isoform sequence into the pGEM®-T Easy Vector was performed as described in the manufacturer's instructions (Promega, Dübendorf, Switzerland).

Miniprep

The plasmid preparation was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Dübendorf, Switzerland) in accordance with the manufacturer's instructions.

Gene electrotransfer

Intramuscular gene transfer was achieved via injection of plasmid DNA and subsequent electric pulse delivery in both legs essentially as described previously (Durieux et al. 2002). The mice were individually anaesthetized with isoflurane and the lower limbs were shaved. 30 µg of expression plasmid in 30 µl physiological saline solution (0.9% NaCl) was injected with a sterile 0.3 ml syringe into the tibialis anterior (TA) muscle. After 5 min, electric pulses (3 strains of 100 pulses of 100 µs each at 50 mA) were delivered at two different locations in the central portion of the muscle using GET42 pulser with needle electrodes (E.I.P. Electronique et Informatique du Pilat, Jonzieux, France). This technique typically results in over-expression of the vector for more than one week (Flück et al. 2008). Mice recovered rapidly from this procedure and began to move freely 30 min after the intervention.

Training protocol

After the gene electrotransfer, all mice were familiarized with treadmill running using a low treadmill speed (6 m/min, 1.5° incline and 10 min) prior to performing the exercise.

The mice were divided into two groups, containing of seven and eight mice. All mice were euthanized after seven days. The TA muscles were prepared and frozen at isopentane and liquid nitrogen.

Group A: Control remaining sedentary group.

Group B: Endurance exercise group. One day after the gene electrotransfer, mice were trained on a treadmill for 1 h per day at 16 m/min and with an incline increasing every second day from 9° to 15° for six days.

Real-time PCR

Real-time PCR was performed using the ABI Prism 7900 HT sequence detection system and SYBR Green PCR Master Mix for quantitative PCR (Applied Biosystems, Rotkreuz, Switzerland). Primers were designed using the software Primer Express (Applied Biosystems) and qPrimerDepot: nNOS exon 2 forward: 5'-CTT GGC TTG GAG GTC TTC TG-3'; nNOS exon 2 reverse: 5'-GAT GAT CAC CGG GGG CTT-3'; 18S rRNA forward: 5'-GCT TAA TTT GAC TCA ACA CGG GA-3'; 18S rRNA reverse: 5'-AGC TAT CAA TCT GTC AAT CCT GTC-3'. Measurement of 18S rRNA expression was used for normalisation. The total reaction volume of 15 µl consisted of 2x SYBR Green PCR Master Mix, primers (500 nM), cDNA (50 ng) and double-distilled water. Following a 15 min denaturation step at 95°C, specific cDNAs were amplified through 40 cycles at 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. For recording of the dissociation curves the following protocol was used: 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. To analyse the mRNA expression, cycling reports and melting curves were evaluated after setting of a baseline cycle threshold. Data were evaluated by the relative quantification method ($2^{-\Delta CT}$).

Statistics

All numerical data are expressed as mean values (± SEM). Significance values relating to the real-time PCR data were calculated by a two-way ANOVA. The significance level was set at $P \leq 0.05$.

2.3.2 Results

To investigate whether the nNOS alpha gene has a functional impact on mice skeletal muscles, the full-coding nNOS alpha sequence was isolated to be over-expressed in hind limb muscles from C57Bl/6 mice by gene electrotransfer. To isolate the gene, the entire sequence was subdivided into three fragments: fragment I from exon 1c to exon 7, fragment II from exon 5 to exon 22 and fragment III from exon 19 to exon 29. The fragments were amplified with specific primer pairs by RT-PCR (figure 15).

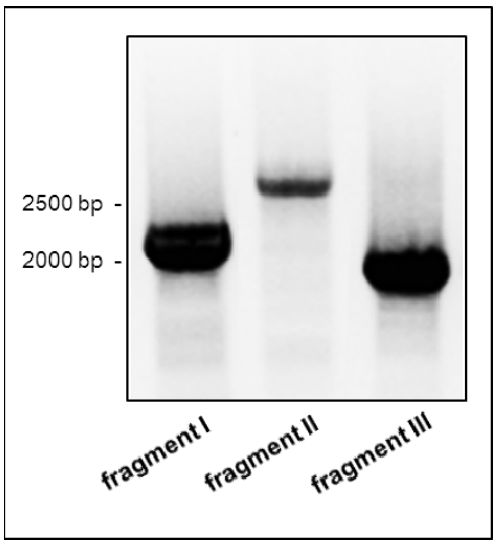

Figure 15: Amplification of fragment I, II and III of the nNOS mRNA by RT-PCR. Fragment I: exons 1c-7, fragment II: exons 5-22, fragment III: exons 19-29.

To clone the complete nNOS alpha coding sequence into the pGEM®-T Easy Vector, the fragments had to be digested with specific restriction enzymes (EcoRI, NsiI, BamHI and PvuI) (figure 16). The restriction sites for EcoRI, 5' UTR upstream of the nNOS sequence, and NsiI, 3' UTR downstream of the nNOS sequence, were incorporated with specific primers during the RT-PCR. The restriction sites BamHI and PvuI were present in the nNOS sequence.

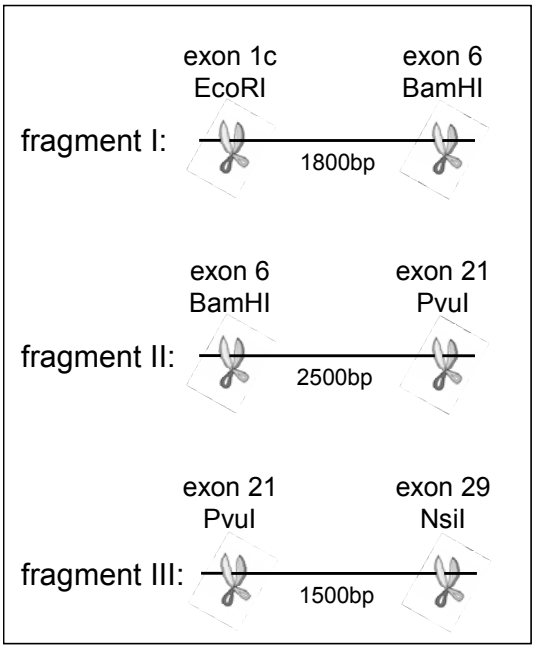

Figure 16: Isolation of the full-coding nNOS alpha isoform. Different restriction enzymes were used to prepare the three nNOS alpha fragments, in order to clone them into the pGEM®-T Easy Vector.

To achieve higher amounts of the three nNOS cDNA fragments, each of them was cloned into a pGEM®-T Easy Vector. Therefore, the fragments and the vectors were digested with the specific restriction enzymes and subsequently ligated, to transform high efficiency competent cells. The transformed cells containing the plasmid with fragment I, II or III were selected by blue-white screening and the plasmid DNA was prepared using a miniprep kit. The correct sequence of the fragments was confirmed by RT-PCR and nucleotide sequencing (data not shown). In the next steps, the complete nNOS cDNA sequence had to be cloned into a pGEM®-T Easy Vector. Therefore, the three nNOS fragments were digested with the restriction enzymes, ligated with one vector and finally transformed into high efficiency competent cells. The completeness of the nNOS alpha coding sequence was checked by restriction digests, RT-PCRs and nucleotide sequencing (data not shown). The validation of the nNOS sequence revealed that at least one fragment was missing, so that none of the samples contained the complete nNOS cDNA sequence.

As the cloning of the nNOS alpha isoform into the pGEM®-T Easy Vector was not successful, the gene synthesis and the production of endotoxin-free expression vectors were outsourced. Finally, the nNOS alpha isoform was cloned into the pIRES2-ZsGreen1 Vector. The gene synthesis and the correct assembly of the nNOS alpha isoform sequence into the plasmid were confirmed by nucleotide sequencing and RT-PCR (data not shown).

The pIRES2-ZsGreen1-nNOS plasmid and the control pIRES2-ZsGreen1 plasmid were induced into the TA muscles of both hind limbs from C57Bl/6 mice by gene electrotransfer (figure 17).

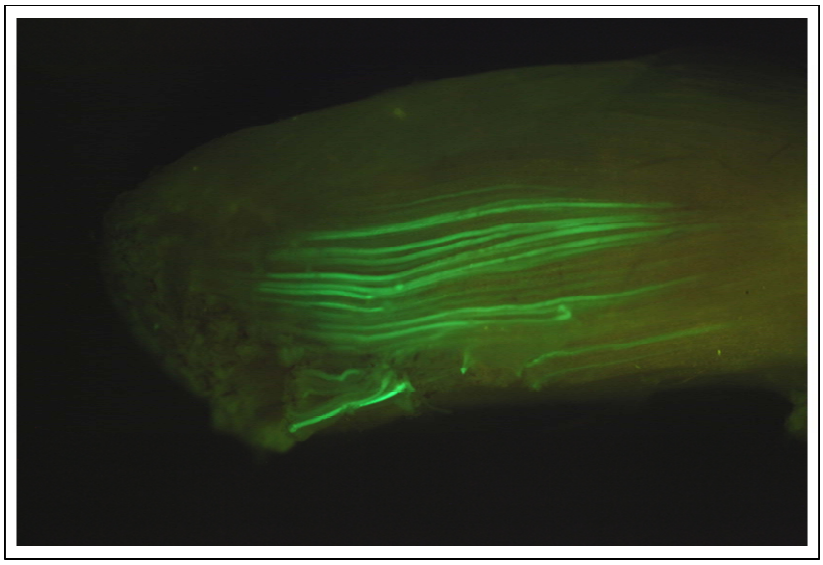

Figure 17: TA muscle from a mouse one week after the gene electrotransfer. Skeletal muscle fibres induced with pIRES2-ZsGreen1-nNOS plasmid were visible due to the fluorescence detection of ZsGreen1 (green fluorescence; 493 nm).

After the gene electrotransfer, all mice were assigned to two groups (group A and B). Group A was the control group which remained sedentary. Group B performed endurance exercise on a treadmill for one week.

The success of the gene electrotransfer was investigated by immunofluorescence histochemistry. Skeletal muscle fibres containing the control pIRES2-ZsGreen1 plasmid (figure 18A) and the pIRES2-ZsGreen1-nNOS plasmid (figure 18D) could be detected due to the expression of ZsGreen1 (green fluorescence; 493 nm).

A clear over-expression of nNOS was not visible in the muscle fibres transfected with the pIRES2-ZsGreen1-nNOS plasmid (figure 18E) compared to the control muscle fibres (figure 18B). Expression of nNOS was shown using a nNOS-specific antibody. There was no difference in the immunoreactivity pattern detectable between the sedentary (group A) and the exercise (group B) mice (data not shown).

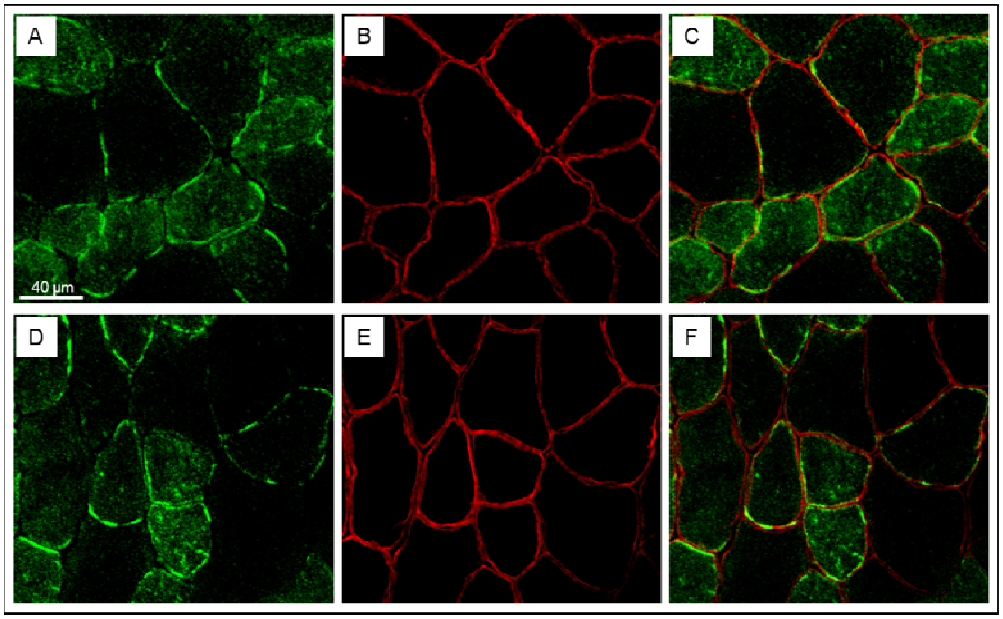

Figure 18: Skeletal muscle fibres transfected with pIRES2-ZsGreen1 plasmid (A, B, C) or pIRES2-ZsGreen1-nNOS plasmid (D, E, F). Plasmid-containing fibres were visible due to the fluorescence detection of ZsGreen1 (green fluorescence; 493 nm) (A, D). nNOS isoform was stained with a nNOS-specific antibody (red fluorescence; 649 nm) (B, E). Co-localisation of ZsGreen1 and nNOS (C, F).

The real-time PCR analysis confirmed that the nNOS mRNA expression was not up-regulated in the TA muscle with the pIRES2-ZsGreen1-nNOS plasmid compared to the TA muscle with the pIRES2-ZsGreen1 plasmid in the sedentary control as well as exercise group (figure 19).

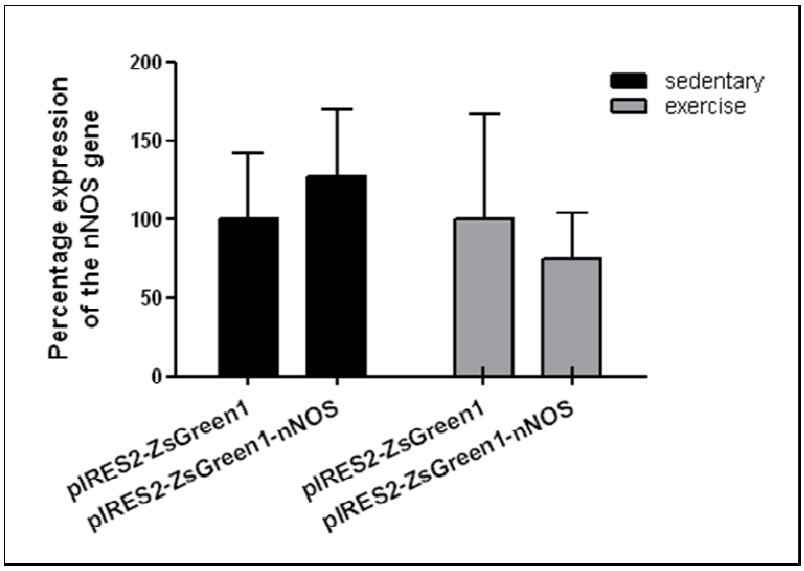

Figure 19: nNOS mRNA level was analysed in the TA muscles from mice by real-time PCR. No up-regulation of the nNOS gene in the TA muscle with the pIRES2-ZsGreen1-nNOS plasmid compared to the TA muscle with the control pIRES2-ZsGreen1 plasmid in the sedentary (n=7) as well as exercise group (n=8). Mean values ± SEM. $P \geq 0.05$.

Thus, nucleotide sequencing and RT-PCRs confirmed that the cloning of the nNOS alpha isoform sequence into the plasmid was correct and did not incur a frame-shift. The fluorescence of ZsGreen1 revealed that the gene electrotransfer with the pIRES2-ZsGreen1 and the pIRES2-ZsGreen1-nNOS plasmid was successful. However, the nNOS alpha isoform was not up-regulated in the TA muscles as a result of transfection with the pIRES2-ZsGreen1-nNOS plasmid. Consequently, a further characterization of the function of the nNOS alpha isoform in mice skeletal muscle has not yet been completed.

3 Discussion and Outlook

The two main aims of this study were to investigate the functional impact of nNOS in skeletal muscle from mice and humans in association with angiogenesis and exercise (Projects I and III) and to characterize the expression pattern and the structure of different nNOS isoforms at mRNA and protein levels in skeletal muscles from mice (Project II) and humans (Project I).

Functional impact of nNOS

The analysis of the human biopsies revealed that the C/F ratio as well as the numerical density of capillaries was higher after than before exercise in five of the ten individuals. Consequently, the VL muscles of these five individuals (assigned to the AR group) responded to endurance exercise with a process of angiogenesis. In contrast, angiogenesis was not detected in the other five individuals (assigned to the NR group) as the C/F ratio as well as the capillary density in the VL biopsies had not changed after training. The process of angiogenesis is confirmed as the absolute number of capillaries increased without a corresponding increase in the mean fibre cross-sectional area (Hudlicka 1998). A different response of individuals to the same training stimulus, intensity and duration, as it was seen in our study, is a well-documented phenomenon and is presumed to reflect individual differences in genetic predisposition (Bray et al. 2009; McPhee et al. 2010; Timmons 2011).

The increase in formation of new blood vessels in skeletal muscle due to exercise typically correlates with an exercise-induced fibre type transition (2B / 2X → 2A). The process of angiogenesis is necessary to support a more oxidative muscle phenotype of exercise (Gavin 2009). An increase in C/F ratio and in capillary density was seen after long-term voluntary running of mice (Waters et al. 2004). However the fibre type transformation was detected subsequent to the process of angiogenesis. Endurance exercise leads to angiogenesis in type 2B / 2X fibres before switching to type 2A fibres (Waters et al. 2004).

Moreover, in TSP-1 null mice a higher endurance exercise capacity and increased capillarity was measured. TSP-1 plays a role in the balance between positive and negative angiogenic regulators through direct and indirect effects on VEGF (Malek et al. 2009).

The nNOS protein level was significantly up-regulated in the VL muscle biopsies of the AR group in response to six months endurance exercise, whereas no difference in nNOS expression was detectable in the biopsies of the NR group. The real-time PCR analysis also revealed a significant exercise-induced increase in the mRNA level of nNOS only in the subjects of the AR group. These findings lead to the conclusion that the expression of nNOS increases at the mRNA and protein level only if the VL responds to exercise with an increase in C/F ratio. The increase in C/F ratio was significantly positively correlated with the increase in nNOS expression. An increase in C/F ratio in association with an increase in nNOS expression due to exercise was also found in previous 60 to 90 day bed rest studies (Rudnick et al. 2004; Salanova et al. 2008).
The regulation and functional impact of nNOS on angiogenesis in response to endurance exercise in human skeletal muscle remains controversial (Frandsen et al. 2000; McConell et al. 2007). Exercise-induced changes in the nNOS protein levels were analysed in VL muscle biopsies that had been derived from subjects with anthropometrically similar characteristics to ours. In one of these studies, the protein levels of nNOS and its catalytic activity were not significantly up-regulated in response to six weeks of endurance exercise (Frandsen et al. 2000). In the other study, the nNOS protein level was significantly increased in endurance-trained athletes compared to sedentary individuals as well as after ten days of intense exercise training (McConell et al. 2007). The analysis of these two apparently contradictory studies and our study lead to the conclusion that the expression of nNOS increases in human skeletal muscle in response to exercise only if angiogenesis is simultaneously noticeable.

Accordingly, the association of increased nNOS activity or nNOS protein expression with chronic treadmill exercise for four weeks was also detected in rodents (Vassilakopoulos et al. 2003). Unpublished data from our lab revealed that the nNOS mRNA and protein levels were significantly increased in mice skeletal muscle in response to endurance exercise after treadmill training for five weeks (Meier, Renga, Baum; unpublished data). Additionally, an endurance exercise study where mice were administered the specific chemical nNOS-inhibitor SMTC revealed a less pronounced increase in exercise-induced peak performance, although this inhibitory effect of SMTC was statistically not significant (unpublished data). In conclusion, the previous published study and our investigations suggest that nNOS plays an important role in the angiogenic adaptation of skeletal muscle to exercise in mice and humans.
To confirm the hypotheses that the nNOS expression is connected to angiogenesis in response to exercise and is additionally involved in the adaptation of the skeletal muscle to exercise, further investigations looking at nNOS expression in response to endurance exercise training should be performed on humans and rodents.

In order to further characterise the influence of nNOS expression on angiogenesis and molecular metabolism in skeletal muscle, the nNOS alpha isoform gene was used to transfect TA muscles of mice by gene electrotransfer. The gene electrotransfer of the plasmids in the TA muscles was successful, but no significant increase in the nNOS mRNA and protein expression was detected by real-time PCR and immunofluorescence. Therefore, further experiments to explore and potentially explain these results were conducted. Performing RT-PCR with nNOS- and vector-specific primer pairs revealed that the pIRES2-ZsGreen1-nNOS plasmid is detectable in the TA muscles. Nucleotide sequencing and further sequence controls confirmed the correct reading of the nNOS sequence including the right reading frame. Despite these control experiments we currently have no evidence to explain, why nNOS over-expression was not detected.

We hypothesised that an increase in C/F ratio and in capillary density would be detected after seven days of nNOS over-expression in the TA muscles of all mice. Accordingly, increased nNOS expression and C/F ratio should be detected in the TA muscles of the mice that underwent the endurance exercise training compared to the sedentary mice. A significant positive correlation between up-regulated nNOS expression and increased C/F ratio was shown in our human study (Huber-Abel et al. in press) and in previous rodent studies (Vassilakopoulos et al. 2003; Kobayashi et al. 2008). Whether a further up-regulation of the nNOS level in the exercise mice due to the gene electrotransfer is detected, is unknown. It seems possible that the nNOS level is further up-regulated due to the exercise training and the gene electrotransfer, but there might also be some inhibitory mechanisms, which prevents further production of NO. One possible inhibitory mechanism might be an up-regulation of the ROS metabolism, which represents a controlled adaptive response to compensate for the increased nNOS activity (Da Silva-Azevedo et al. 2009). Additionally, the binding of the protein inhibitor of nNOS (PIN) leads to a destabilization of the nNOS dimer and consequently to a decrease in nNOS activity (Jaffrey et al. 1996).

The influence of nNOS on angiogenesis is also discussed in diverse skeletal muscle diseases. In skeletal muscle from mdx mice, nNOS is dislocated from the sarcolemma as dystrophin is missing in the dystrophin glycoprotein complex (Brenman et al. 1995). Training studies showed that mdx mice had a decreased spontaneous running activity and fatigue was exaggerated after exercise, although the muscle force was similar to C57Bl/6 mice (Kobayashi et al. 2008). The loss of nNOS from the sarcolemma and the deficient signalling for increased vasodilation causes muscle ischemia which contributes to increased muscle damage (Kobayashi et al. 2008; Wehling-Henricks et al. 2009; Percival et al. 2010). In biopsies from patients with myopathic disorders such as DMD, Becker muscular dystrophy and LGMD 2C, 2D and 2E, nNOS was also dislocated from the sarcolemma or not detectable (Chao et al. 1996; Crosbie et al. 2002; Kobayashi et al. 2008). The loss of nNOS from the sarcolemma causes deficient angiogenesis in skeletal muscle which leads to fatigue after exercise. A re-introduction of nNOS in dystrophic skeletal muscles would potentially restore normal vascularisation only if nNOS were localized

at the sarcolemma (Thomas et al. 2003; Percival et al. 2008). This point might be essential for new effective gene therapies.
The results of the gene electrotransfer experiment would provide important insight into the integration of nNOS expression, exercise and angiogenesis in healthy and pathological muscles. As such, the repeating of this nNOS over-expression experiment by gene electrotransfer in mice skeletal muscle is strongly recommended.

One possibility to explain the direct influence of nNOS expression on angiogenesis might be the PGC-1alpha pathway. Due to exercise and the excitation-contraction coupling, the Ca^{2+} levels were increased and together with calmodulin activate the expression of nNOS (Percival et al. 2010). The increased nNOS-produced NO level leads to a higher expression or activity of PGC-1alpha inside the skeletal muscle fibres due to an up-regulation of AMPK phosphorylation and activity (Lira et al. 2010; Powers et al. 2011). As an autocrine signalling molecule, the change in PGC-1alpha expression regulates mitochondrial biogenesis as well as VEGF-dependent angiogenesis. PGC-1alpha induces Ang-2, PDGF-B and VEGF in skeletal muscle. This pathway is independent of HIF-1alpha, a known hypoxia-induced transcription factor that also regulates VEGF expression. PDGF-B and VEGF are both induced by the coactivation of ERR-alpha. PGC-1alpha coordinates adaptations in both, muscle capillarisation and oxidative capacity (Arany et al. 2008; Chinsomboon et al. 2009; Gavin 2009; Leick et al. 2009). In order to determine the connection between nNOS-produced NO and the PGC-1alpha pathway in more detail, the activity and regulation of various involved proteins, like VEGF, Ang-2 and ERR-alpha, have to be analysed under different conditions as for example in mice with nNOS deletion or over-expression in skeletal muscle.

Another possible explanation might be an indirect effect of nNOS-produced NO in the extramysial and endomysial milieu of skeletal muscle fibres. As all nNOS isoforms are localized at the sarcolemma of skeletal muscle fibres, the diffusion distance between the site of NO production and the site of action is minimized. Therefore, NO diffuses easily into the extramysial milieu and as a paracrine signalling molecule, influences the contractility of vascular smooth muscle cells by activating a cGMP-dependent relaxation cascade (Lau et al. 2000; Grange et al. 2001). Additionally, the

nNOS-produced NO is also increased in the endomysial milieu and influences the vasodilation of larger vessels. Consequently, blood flow increases which leads to higher shear stress in the capillaries. This signal activates the endothelial VEGF-dependent angiogenesis (Da Silva-Azevedo et al. 2002; Baum et al. 2004). Additionally, the increased shear stress increases the eNOS concentration and activity in vascular endothelial cells (Fleming et al. 2003). However, the vasoconstriction in contracting muscle was impaired in skeletal muscles of nNOS-knockout and mdx mice in which nNOS but not eNOS is reduced (Thomas et al. 1998). Therefore, the nNOS-produced NO mainly serves as paracrine regulator of vasodilation in skeletal muscle and thereby indirectly influences the degree of angiogenesis. Some contradictory results are shown in the work of Copp et al. (2010) as the nNOS inhibition with SMTC reduced the muscle blood flow at rest but showed no inhibitory effect at blood flow during exercise (Copp et al. 2010).

Taken together, nNOS with its reaction product NO seems to be a critical regulator for angiogenesis and exercise capacity in skeletal muscle. Additionally, the localisation of nNOS at the sarcolemma is important. Therefore, the functional role of nNOS and its reaction product NO in the process of angiogenesis in healthy and pathological skeletal muscle has to be further investigated.

Expression and structure of nNOS

The expression and structure of the nNOS isoforms were characterized in skeletal muscles of mice and humans.

Due to the results obtained by nucleotide sequencing of the nNOS alpha and beta isoforms in human and rodent skeletal muscles, we suggest that the previous exon structure of the nNOS gene has to be corrected. Currently, nNOS is organized in 29 exons with an additional mu exon between exons 16 and 17. We have suggested that exons which are alternatively spliced be re-named with Greek characters in accordance with the designation of the mu exon (Silvagno et al. 1996). Consequently, in mice skeletal muscle the nNOS alpha isoform and the nNOS beta isoform consist of the following exon structures: 1c-pi-rho-sigma-tau-4 to 29 and 1c-

pi-rho-4 to 29 (see figure 12). In nNOS-knockout mice, no cDNA variant is translated. In human skeletal muscle, the nNOS alpha isoform consists of the following exon structure: 1c-pi-rho-sigma-tau-4 to 29. The pi exon represents an exon located 5' upstream of the former exon 2, which is now divided into the rho and sigma exons. The tau exon is the former exon 3. The re-organization of the exon structure is confirmed by results from data base search. The new pi exon has been identified in mouse tissues as expressed sequence tag (EST).
The RT-PCR analysis revealed the co-expression of nNOS mRNA species with and without the mu exon. In contrast, all nNOS isoforms express the rho exon. This finding indicates that the rho exon and the mu exon might be simultaneously co-expressed in the skeletal muscles of mice and humans. Previous studies suggest that the nNOS mu isoform is a special isoform expressed only in skeletal and cardiac muscles (Silvagno et al. 1996; Lainé et al. 1998; Bradley et al. 2007). Contrary to these studies, we claim that the alpha and mu isoforms do not represent different proteins but are simultaneously co-expressed in skeletal muscles.

Wang et al. (1999) studied the alternative splicing of nNOS and discovered the existence of nine unique exon 1 variants of nNOS in diverse tissues. We assessed the presence of four exon 1 variants (1a, c, f and g). These four variants are highly expressed in skeletal muscle and brain tissue (Wang et al. 1999). Performing RT-PCR, only exon 1c was detected in skeletal muscles from mice and humans. This might be explained by the activation of various promoters which would lead to a benefit of exon 1c in these skeletal muscles. The presence of different exon 1 variants might play a role in the regulation of translational efficiency. The alternative use of different first exons and tissue-specific promoters was also shown in a number of other genes such as for the genes encoding aromatase or glucocorticoid receptor (Harada et al. 1993). Consistent with this, a previous study showed that the translation of exon 1a was increased in differentiated C2C12 murine skeletal myocytes in comparison to the translation of exon 1c (Wang et al. 1999).

A co-expression of the nNOS alpha and beta isoforms has been reported only in rat skeletal muscle (Planitzer et al. 2001), in rat kidney (Smith et al. 2009) and intestine (Saur et al. 2002) so far. In the current study, four different nNOS variants were detected by immunoblotting in skeletal muscles of C57Bl/6 mice. Due to the molecular weights, the 165 / 160 kDa double-band was identified as the nNOS alpha isoform with and without the additional mu exon (intervening exons 16 and 17), while the 145 / 140 kDa double-band encoded for the nNOS beta isoform with and without the mu exon.

The simultaneous expression of such a variety of isoforms of nNOS in skeletal muscle has not been described so far. We suggest that some studies have used N-terminal antibodies against nNOS resulting in the demonstration of a single 160 kDa band and not recognizing the nNOS beta isoform (Crosbie et al. 2002; Thomas et al. 2003). In other cases, the existence of the second nNOS-immunoreactive band was explained with the co-presence of mu and non-mu isoforms (Wehling et al. 1998) or with proteolytic digestion (Nguyen et al. 2003).

So far, most investigations concerning nNOS isoforms focused on the nNOS alpha isoform in contrast to the nNOS beta isoform, a circumstance that reflects the non-availability of a specific antibody against this nNOS-isoform. The nNOS beta isoform was originally identified in skeletal muscles of nNOS-knockout mice (Brenman et al. 1996). To ablate the expression of nNOS in the nNOS-knockout mice, exon 2 of the nNOS gene was deleted. This deletion results in skipping of an 807 bp long stretch of the mRNA containing the initial AUG codon that is used to transcribe the alpha isoform (Huang et al. 1993). Consequently, two shorter mRNA variants of nNOS (designated beta and gamma) isoforms were detected in skeletal muscle of these nNOS-knockout mice. Since the reading frame of the coding sequence is shifted, the transcription of the beta isoform is presumed to be exceptionally initiated at a CTG codon in exon 1a, whereas that of the truncated gamma variant reputedly begins at a second AUG codon in exon 5 (Brenman et al. 1996).

These findings revealed a new gene structure for the nNOS beta isoform (see figure 12). The previous exon 2 (with the initiation codon) is divided into the rho and the sigma exon thereby the nNOS beta isoform is just missing the sigma exon. As the initiation codon ATG is localised in the rho exon, which is still present in the nNOS

beta isoform, the alternative initiation codon CTG in exon 1 is not necessary (Brenman et al. 1996). Additionally, in the third nNOS mRNA transcript no second AUG codon in exon 5 exists as it was shown in the gamma variant of Brenman et al. (1996). Although this third nNOS mRNA variant is not translated, the presence and function should be clarified.

In addition, the nNOS alpha isoform contains a PDZ domain and β-hairpin loop (residues 100-230) at the N-terminus, which via interaction with α-syntrophin are necessary for the localisation of nNOS at the sarcolemma (Brenman et al. 1996; Abdelmoity et al. 2000). As the N-terminus of the nNOS alpha and beta isoforms is equal, these presumptive sarcolemma-binding domains are still expressed in the nNOS beta isoform. The localisation of both nNOS isoforms at the sarcolemma of skeletal muscle fibres was confirmed by immunofluorescence in this study. Additionally, the co-localization of the nNOS alpha and beta isoforms was further approved by immunoblotting and immunoprecipitation with three different nNOS-specific antibodies (Baum et al. 2011).

It seems that both the alternative splicing and the alternative promoter usage lead to nNOS mRNA transcripts with complex 5' UTRs. These variations in length and sequence of the 5' UTRs may affect mRNA processing, localization or stability as well as nNOS protein levels via different posttranscriptional mechanisms (Xie et al. 1995; Lee et al. 1997).
Further studies are needed to clarify the nNOS exon structure, expression pattern and gene regulation. Moreover, the sarcolemmal localization and the different binding partners of the nNOS alpha and beta isoforms should be analysed in detail.

5 References

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Curriculum Vitae

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Education

1993 – 2002	Grammar School and Graduation, Schwabmünchen, Germany
2002 – 2005	Bachelor study “Biological Sciences”, University of Konstanz, Germany
July – Sept 2005	Bachelor thesis, Laboratory of Prof. Dr. C. Stürmer, Chair of Neurobiology, University of Konstanz, Germany “Cloning of zebrafish prion proteins, PrP-1 and PrP-2, in the expression vector pQE30, for bacterial expression”
2005 – 2008	Master study “Biological Sciences”, University of Konstanz, Germany
Apr 2007 – Jan 2008	Master thesis, Laboratory of Prof. Dr. A. Görlach, Experimental Paediatric Cardiology, German Heart Center Munich, Germany “Role of NADPH oxidase in tumor progression”
July 2008 – Dec 2011	PhD study and thesis, Laboratory of Prof. Dr. H. Hoppeler, Functional Anatomy, Institute of Anatomy, University of Bern, Switzerland

Scientific congresses and classes

2008	Microcirculation Congress, Budapest, Hungary
	Applied Biosystems Real-Time PCR class, Rotkreuz, Switzerland
2009	USGEB, Interlaken, Switzerland
	Muscle Symposium, Basel, Switzerland
	CBCS/ECS Summer School, Nice, France
	Laboratory Animal class, Bern, Switzerland
2010	JEB Meeting, Mürren, Switzerland
	ECSS, Antalya, Turkey
	SGAHE Meeting, Bern, Switzerland
	SSEM Meeting, Magglingen, Switzerland
	Stereology class, Bern, Switzerland
2011	Muscle Symposium, Basel, Switzerland

Scientific awards

2008	Award of the “Verein der Ehemaligen der Universität Konstanz (VEUK e.V.)” for the best graduation in the Department of Biology at the University of Konstanz, Germany
2009	Award of the “Gesellschaft für Mikrozirkulation und Vaskuläre Biologie (GfMVB e.V.)” for the CBCS/ESC Summer School on Cardiovascular Sciences: “From Basic Mechanisms to Clinical Application” in Nice, France

Publications

Huber-Abel, F.A., Gerber, M., Hoppeler, H. and Baum, O. (in press) "Exercise-induced angiogenesis correlates with the up-regulated expression of neuronal nitric oxide synthase (nNOS) in human skeletal muscle." European Journal of Applied Physiology, doi: 10.1007/s00421-00011-01960-x.

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Vaughan, D., Brogioli, M., Hoppeler, H., **Huber-Abel, F.A.**, Graber, F., Waldron, S., Rittweger, J. and Flueck, M. (submitted) “Angiotensin 2 production exerts system control over fuel handling in skeletal muscle.” Physiological Genomics

Declaration of Originality

Last name, first name: **Lauer, Felicitas**

Matriculation number: **08-119-596**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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