Involvement of neuronal NO synthase in collateral artery growth

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To evaluate the role of neuronal nitric oxides synthase (nNOS) in collateral artery growth (arteriogenesis), we analyzed the expression pattern of nNOS at distinct time points on RNA and protein levels in a rabbit and a murine model of peripheral arteriogenesis. In the rabbit model, Northern blot analyses revealed a significant upregulation of nNOS at 6 h (1.6-fold), 12 h (2.2-fold) and 24 h (2.0-fold) after induction of arteriogenesis via femoral artery ligation, when compared to the sham operated side. In mice, an upregulation of nNOS was also detected using Northern blot (at 6 h, 12 h) and qRT-PCR (12 h: 2.4-fold). On the protein level, nNOS was found to be upregulated 24 h after femoral artery ligation. Immunohistochemical staining showed that nNOS was localized in endothelial and smooth muscle cells of collateral arteries, as well as in skeletal muscle and nerves. In summary, our data provide evidence that nNOS is not constitutively expressed, but is induced during arteriogenesis, playing a role in supplying reactive oxygen species such as H$_2$O$_2$ and low levels of NO.

Keywords: Neuronal NOS, Arteriogenesis, NO, Collateral artery growth

The uncharged free radical nitric oxide (NO), an important regulator of vascular tone and blood flow\textsuperscript{1} is also an essential vascular signaling molecule that influences various physiological processes, such as vasodilatation, prevention of platelet aggregation and the immune response to infection\textsuperscript{2}. Three major NO-synthase (NOS) isoforms generate NO from L-arginine, NADPH and molecular oxygen. Inducible NOS (iNOS) also termed NOS II whose expression is induced by gene regulation at the transcriptional level largely accounts for NO derived from macrophages as part of the oxidative burst during the inflammatory response\textsuperscript{3,4}. Endothelial NOS (eNOS or NOSIII) and neuronal NOS (nNOS or NOS I) whose activity is regulated by intracellular Ca\textsuperscript{2+} concentrations\textsuperscript{5}, generate NO in blood vessels and mediate vasodilatation in murine\textsuperscript{6} and human\textsuperscript{6,7} arterial vessels\textsuperscript{8}.

As NO and the NOS isoforms modulate pathophysiological conditions such as diabetes and atherosclerosis\textsuperscript{9,10}, NO produced by either one of the three NO synthases has been considered as an interesting candidate to study in collateral artery growth (arteriogenesis). Arteriogenesis is the remodelling of pre-existing arterio-arteriolar anastomoses to functional arteries. It is the only natural way to compensate for a stenotic artery and occurs as an adaptive response to increased shear stress after arterial occlusion. Increased fluid shear stress (FSS), one of the main stimuli of arteriogenesis\textsuperscript{11} is shown to upregulate the expression of eNOS and iNOS isoforms \textit{in vitro} and \textit{in vivo} augmenting NO production\textsuperscript{12,13}. Moreover, FSS induces the formation of reactive oxygen species (ROS) such as H$_2$O$_2$, which in turn are important messengers mediating collateral artery development\textsuperscript{14-16}. Interestingly, nNOS generates rather low levels of NO and relatively more H$_2$O$_2$\textsuperscript{5}.

Previous work has evidenced that long-term administration of a NO donor furthers collateral artery growth\textsuperscript{13} and pharmacological, non-selective NOS inhibition impairs collateral artery formation\textsuperscript{17}. iNOS and eNOS isoforms have already been studied in collateral growth, although results remain controversial\textsuperscript{18-20}. But, so far, the involvement of nNOS in arteriogenesis has not been evaluated. In this

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study, we have performed femoral artery ligation in rabbits and mice, in order to induce peripheral arteriogenesis and analyzed the expression pattern of nNOS at the area of collateral artery growth after distinct time points using Northern blot, quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western blot. Here, we report for the first time that nNOS is not constitutively expressed, but is upregulated during arteriogenesis accounting for increased NO and H₂O₂ levels.

Materials and Methods

Animals

Animals were kept in normed cages at 12:12 h light-dark cycle with chow and water provided ad libitum. Male rabbits (New Zealand White rabbit, n = 6, average weight 3.0 kg) and male C57Bl/6 mice (age 6 to 8 weeks, n = 6 per group) underwent unilateral femoral artery ligation in order to induce arteriogenesis as previously described²¹ (Fig. 1). In brief, the right femoral artery was exposed, separated from the surrounding tissue and ligated distal from the branching of the arteria profunda femoris and proximal to the branching of the arteria genu descendens. The left femoral artery underwent sham operation. None of the animals died and no gangrene was observed during the subsequent observation period. All animal experiments were approved and controlled by the Local Ethics Committee and carried out according to the guidelines of the German law for protection of animal life.

Tissue sampling

At distinct time points after femoral artery ligation, the peripheral vasculature was perfused with 0.9% saline solution, followed by contrast medium via a canula placed in the aorta. In rabbits, a contrast medium based on bismuth and gelatine was used according to the formula developed by Fulton²². For murine tissue, the contrast medium latex (Chicago Latex) was used for improved discrimination. Tissue samples from the adductor muscle containing collateral arteries were isolated (Fig. 2), snap-frozen in liquid nitrogen and stored at -80°C until further processing for Northern blot, qRT-PCR or Western blot analyses.

RNA Isolation, Northern blot and qRT-PCR

Frozen tissue was homogenized and total RNA was isolated according to the method of Chomczynski and Sacchi²³. Northern blots were performed according to standard procedures²⁴. For nNOS detection, the blot was hybridized with a ⁴²P-dCTP randomly prime-labelled cDNA probe (Rediprime™ II labeling system, Amersham Pharmacia Biotech) complementary to rat nNOS (accession: X59949). Signals were quantified using a PhosphorImager. For normalization, blots were rehybridized with an oligonucleotide specific for 18S rRNA as previously described²⁵ and the data of each hybridization signal of nNOS was divided by the value of the matching 18S rRNA signal.

Quantitative RT-PCR was carried out on a Light Cycler (Roche) using a Master SYBR Green I Kit (Roche) using 50 pmol of primers (nNOS: 5'-TGGGCAGATCCAGCTAATGTGG-3', 5'-GGGATCTGAAAGAGTTCAGGGTC-3'; 18S: 5'-GGAGGATTGACAGATTGATAG-3', 5'-CTCGTGTTCTTGATCGGAATTAC-3') and 0.1 µg cDNA that

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**Fig. 1**—Peripheral arteriogenesis model [Images showing the murine adductor muscles 7 days after femoral artery ligation or sham operation. Vessels are filled with latex contrast medium allowing a high contrast view of collateral vessels. A: Arteriogenesis was induced by femoral artery ligation. Grown collateral arteries appear in typical corkscrew formation; and B: depicts the sham operated side with small pre-existent collateral arteries]

**Fig. 2**—Site of tissue sampling [The image displays collateral vessels in the rabbit adductor muscle filled with contrast medium. On the right, the dissected tissue area is shown in magnification]
was transcribed from total RNA using random primers. The cycling parameters were 10 min 95°C, 40 x (10 s 95°C, 5 s 62°C, 15 s 72°C) for nNOS, and for 18S rRNA 10 min 95°C, 40 x (10 s 95°C, 5 s 64°C, 15 s 72°C). To ensure that only one specific product was amplified, melt curve analyses were performed. The threshold value Ct was determined. The data obtained on 18S rRNA expression were used for normalization of the target data.

**Western blot**

Western blot analyses were performed according to standard procedures. Equal amounts of proteins (10 µg per lane) were separated on a 4–20% Tris-glycine gel (Serva). A nNOS polyclonal rabbit antibody was used as first antibody and a HRP-linked anti-rabbit antibody served as secondary (both Cell Signaling). Blots were repeated at least three-times and for internal control the first antibody was omitted.

**Immunohistochemistry**

Tissue samples from the murine adductor muscles containing collateral arteries were dissected, embedded in Tissue-Tek® mounting media (Sakura) and cryopreserved with liquid nitrogen-cooled isopentane (Sigma). Cryosections were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) at pH 7.4 and washed with PBS. After quenching the endogenous peroxidase with 3% hydrogen peroxide, unspecific binding of the antibodies was blocked with blocking solution (1% sheep serum, 0.5% bovine serum albumin in PBS). As antibody, we used a rabbit polyclonal anti-nNOS (Abcam), which was immunogenic to aa 724-739 of rat nNOS. Incubation of sections overnight was followed by washing with PBS and incubation with peroxidase-conjugated anti-rabbit IgGs (Sigma). Peroxidase activity was detected using 3`3-diaminobencidine solution (Sigma). Sham-operated animals served as controls.

**Statistical analysis**

All experimental values were expressed as mean ± SEM. For comparison of two groups, we used unpaired student’s t-test. Mean values between more groups were compared with one-way-ANOVA and subsequent Bonferroni correction for multiple pairwise comparisons. Results were considered to be statistically significant at P<0.05.

**Results and Discussion**

In the present study, we investigated the role of nNOS in collateral artery growth using the femoral artery ligation model of arteriogenesis in rabbits and mice. Occlusion of the femoral artery leads to the growth of collateral arteries located in the adductor muscle. Northern blot analyses revealed a significant upregulation of nNOS mRNA on the occluded side, when compared to the sham operated side at 6, 12 and 24 h after femoral artery ligation in rabbits (1.6-fold at 6 h, 2.2-fold at 12 h and 2.0-fold at 24 h; occ vs. sham) (Fig. 3). In the murine model, comparable results were obtained. Northern blot analyses evidenced an upregulation of nNOS at 6 and 12 h after femoral artery ligation (Fig. 4A). In qRT-PCR analyses, a 2.4-fold upregulation was observed at 12 h (Fig. 4B).

Increased fluid shear stress, the initial trigger for arteriogenesis has previously been shown to induce eNOS and iNOS mRNA expression in vitro and in vivo. Our study revealed for the first time that during arteriogenesis nNOS mRNA levels are upregulated, thus indicating that constitutively expressed nNOS isoform can also be regulated at the transcriptional level. To further confirm our results, we performed Western blot analyses. Our results evidenced increased nNOS protein levels (occluded side compared to sham side) in murine adductor muscles 24 h after femoral artery ligation (Fig. 5A). Immunohistochemical staining showed that nNOS...
was localized in endothelial cells and smooth muscle cells of the collateral vessel wall, as well as in skeletal muscle cells and nerves of mouse adductor muscles (Fig. 5B). Altogether, the differential expression of the nNOS isoform during the early phase of arteriogenesis in two different species points to a strong demand for nNOS-derived NO and H₂O₂ at the area of ongoing collateral artery growth.

Arteriogenesis relies on perivascular accumulation of leukocytes and macrophages supplying growth factors and cytokines⁵⁻¹⁰, which promote the proliferation of endothelial and vascular smooth muscle cells¹¹⁻¹³. Since NO has been described to inhibit smooth muscle cell proliferation¹⁳, it seems at first unlikely that an upregulation of any NOS isoform might be beneficial. It is, however, well established that NO is required during arteriogenesis and that the application of NO donors enhance collateral artery growth significantly¹⁴. A study has investigated the most effective therapy regimen of the NO donor sodium nitrite to promote angiogenesis and arteriogenesis¹⁵. Moreover, ROS such as H₂O₂ are critical mediators of collateral artery growth¹⁶. We, therefore, conclude that H₂O₂ production and release of low levels of NO provided by nNOS and not the other NOS isoforms, is an important mechanism to promote arteriogenesis.

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References
