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Expression of neuronal nitric oxide synthase splice variants in atherosclerotic plaques of apoE knockout mice

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Abstract

Objective—We previously reported that deletion of brain type neuronal nitric oxide synthase- α (nNOS- α) accelerates atherosclerosis in apolipoproteinE (apoE) knockout (ko) mice. The regulation of nNOS expression is complex, involving the generation of mRNA splice variants. The current study investigates occurrence and distribution of nNOS variants in atherosclerotic lesions of apoE ko and apoE/nNOS- α double ko (dko) animals.

Methods—Mice were fed a high fat diet for 20 weeks. Immunohistochemistry and Western blot analysis were performed using antibodies detecting the carboxy terminal-, or amino terminal-residue of the nNOS protein. Confocal microscopy and in situ hybridization were used to identify the compartment of cellular expression.

Results—In situ hybridization revealed the presence of nNOS- α and - γ mRNA variants in apoE ko plaques, while only nNOS- γ was detectable in apoE/nNOS dko plaques. Consistent with mRNA expression nNOS- α protein can be detected in the neointima of apoE ko, but not apoE/nNOS dko animals. In contrast, the carboxy terminal antibody stained the neointima and media in apoE ko vessels and showed residual nNOS immunoreactivity in apoE/nNOS dko lesions. Confocal microscopy showed predominant nNOS expression in vascular smooth muscle cells, while colocalization with macrophages was less pronounced.

Conclusions—Our study shows that nNOS- α and - γ splice variants are expressed in atherosclerotic plaques of apoE ko mice. nNOS variants colocalized with markers for vascular smooth muscle cells and macrophages but not for endothelial cells. Since nNOS- α is atheroprotective, other nNOS splice variants which differ in enzyme kinetic and subcellular localization may also influence plaque formation.

Keywords

Atherosclerosis; Neuronal nitric oxide synthase; Splice variants; Nitric oxide; nNOS variants

1. Introduction

Nitric oxide is an autocrine and paracrine signalling molecule which is generated by three different nitric oxide synthase (NOS) isoforms [1]. While only endothelial NOS (eNOS) is detectable in healthy human vessels with standard techniques, all three isoforms are present in atherosclerotic lesions [2]. The importance of eNOS for vascular function is well documented and eNOS deletion in apoE ko mice leads to increased atherosclerosis [3]. The inducible isoform (iNOS) is expressed in inflammatory cells and vascular smooth muscle cells and has proatherogenic effects, since deletion of iNOS decreases plaque development in apoE ko mice [4]. eNOS and nNOS activity depends on the intracellular Ca^{2+} concentration, whereas iNOS is regulated by modulation of enzyme expression. In the human atherosclerotic plaque nNOS is expressed in smooth muscle cells, macrophages and endothelial cells [2]. Recently, we have addressed the role of nNOS in atherogenesis and showed that the deletion of nNOS- α leads to an increase in atherosclerosis in apoE ko mice [5]. In the latter study we detected nNOS- α and nNOS- γ mRNA variants in total RNA from vessels of C57/Bl6 and apoE ko animals by RT-PCR but did not investigate the compartment of splice variant expression.

The neuronal NOS was first detected in nitrinergic nerves and brain tissue [6–8]. Regulation of nNOS expression is complex through alternative splicing, deletion and insertion of exons and multiple promoter usage [9–11]. In mice, four different nNOS protein variants have been detected in non-vascular tissue (Fig. 1). The nNOS- α - and nNOS- μ -variants contain the PDZ (postsynaptic density-95/discs large/zona occludens-1 homology) domain which allows binding of the enzyme to the cell membrane and fast activation through calcium influx [12]. Multiple transcripts encode for nNOS- α , which has high catalytic activity [13]. nNOS- μ which has an identical kinetic constant compared to nNOS- α is predominantly expressed in differentiated skeletal muscle and differs from other nNOS variants by a 102 bp insertion between exon 16 and 17 [14]. nNOS- β and nNOS- γ , which lack the membrane PDZ-binding-domain, are cytoplasmic enzymes. The latter isoforms are expressed at low levels in brain tissue of nNOS ko mice and contribute to residual nNOS catalytic activity [15]. In vitro studies revealed that enzyme activity for nNOS- β is approximately 80% of nNOS- α , while the activity for nNOS- γ is low (3%) [12]. While the differences in catalytic activity between the variants is one aspect, the presence of the PDZ domain in the enzymes determines specific binding sites for PIN (protein inhibitor of NOS) [16], the NMDA receptor [17], syntrophin [12] or CAPON (carboxyl terminal PDZ ligand of NOS) [18]. These protein-protein interactions may regulate NO production and signal transduction. So far, nNOS splice variants have been detected in the human and rat gastrointestinal tract and nNOS- β was shown to mediate penile erection [19–21]. Furthermore, nNOS- γ and nNOS- β are upregulated in amyotrophic lateral sclerosis and may mediate oxidative damage [22].

In the present study we used apoE ko and apoE/nNOS (Exon 2 deletion) dko mice to investigate the distribution of nNOS mRNA and protein variants in the atherosclerotic plaques.

2. Methods

All procedures performed conformed with the policies of the University of Würzburg, the NIH guidelines and an independent governmental committee for care and use of laboratory animals.

2.1. Animals

apoE ko mice came from The Jackson Laboratory. nNOS ko animals (Exon 2 deletion) came from Paul Huang's laboratory [23]. ApoE/nNOS dko mice were generated as described earlier [5], weaned at 3 weeks and fed a standard western type diet (42% of total calories from fat; 0.15% cholesterol; Harlan Teklad) for 20 weeks. In general, animals were anesthetized with pentobarbital (80 $\mu\text{g/kg}$ i.p.). The aorta was rinsed with ice-cold PBS buffer through the left

ventricle and the aorta was removed quickly and snap-frozen in liquid nitrogen. For immunohistochemistry tissues were fixed in 10% phosphate buffered formalin and embedded in paraffin or directly frozen in Tissue-Tek™ (Sakura Finetek).

2.2. In situ hybridization

In situ hybridization using ³³P-end-labelled oligonucleotides was performed as described [15]. Following oligonucleotide sequences, specifically targeting nNOS splice variants were used: 5'-CAG CCA TCA GCC AGC AAA GAC CAG CCA TTA GCA GTA GAC AGG GTC-3' for nNOS-α and 5'-GCC CCA GCC ACC TTG CCT TCC AGA GAC CTC GAC GGC AAA CTG CAC A-3' for nNOS-γ. The probe for nNOS-γ recognizes the junction of exon 1b and exon 3 of nNOS [15]. The probe for nNOS-α targets exon 2. Probes were ³³P-labelled using terminal deoxynucleotidyltransferase and 2.5 μCi were applied per slide. Cryosections of the aortic root (*n* = 4 per genotype, duplicate slides per animal) were hybridized overnight at 45 °C, washed in SSC and dipped in NTB-2 emulsion (Kodak). Slides were then developed for 14 days, fixed and counterstained with hematoxylin/eosin. Probes corresponding to the sense strand of the splice variants were processed according to the standard labelling protocol and used as a negative control.

2.3. Western blot analysis

Aortas were homogenized under liquid nitrogen and total protein was extracted. Proteins were separated by SDS-PAGE. Antibodies raised against the amino terminal end of the nNOS protein, specific for nNOS-α and nNOS-μ (1:250, US Biological, for aorta; 1:500, Stress gene, for brain), or its carboxy terminal end, detecting nNOS-α, -β, -γ and -μ variants (1:500, BD Biosciences) were used to probe for nNOS. As secondary antibody rabbit IgG TrueBlot™ (eBioscience) was used and staining was visualized by an ECL-Kit (Amersham). Brain homogenates were used as positive controls.

2.4. Immunohistochemistry

For immunohistochemistry serial sections of paraffin or Tissue-Tek™ embedded aortic roots and aortic arches were used to detect nNOS expression in atherosclerotic lesions of apoE ko and apoE/nNOS dko mice. Briefly, endogenous peroxidase and biotin activity was blocked with 0.03% hydrogen peroxide. Then, sections were incubated with the primary antibodies raised against the carboxy- or amino terminus of the nNOS protein. Sections were subsequently incubated with abiotinylated secondary antibody (BD Biosciences) and freshly prepared ABC-reagent (Vectastain; Vector) following the manufacturer's protocols. The secondary antibody was detected using glucose oxidase-3,3-diaminobenzidine (DAB). Hematoxylin was used for counterstaining. Secondary antibody alone served as a negative control.

2.5. Confocal microscopy

To investigate the cellular compartment of nNOS splice variant expression the amino- and carboxy terminal anti-nNOS antibodies (US Biological 1:50, BD Bioscience 1:20) were incubated on acetone fixed cryosections of the aortic arch of apoE ko mice. Following appropriate blocking procedures, cross reactivity of secondary antibodies with the alternating primary antibodies was ruled out.

Colocalization of nNOS protein with cell markers of macrophages, endothelial cells and smooth muscle cells was examined by double immunofluorescence. Slides were incubated with rat anti-nNOS primary (BD Bioscience 1:20) followed by the biotinylated rabbit anti-rat secondary antibody (Vector 1:200) and subsequently stained with streptavidin–Texas red complex. After washing, slides were incubated with the antibodies directed against macrophages (MOMA-2, Chemicon 1:25), endothelial cells (CD 31, BD Bioscience 1:100) or

vascular smooth muscle cells (α -smooth-muscle actin, Sigma 1:60). The latter antibodies were either directly labelled with fluoresceinisothiocyanate (FITC) or a secondary FITC-labelled antibody was used. Finally, all sections were mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting media and examined with a confocal microscope (Zeiss).

3. Results

3.1. Detection of nNOS- α and nNOS- γ mRNA by in situ hybridization

In order to localize nNOS mRNA splice variants in atherosclerotic lesions we performed in situ hybridization with splice variant specific probes. Using this technique nNOS- α mRNA is localized in the media and the neointima of lesions in aortic roots of apoE ko mice (Fig. 2a). In contrast, NOS- α mRNA was not detectable in plaques of apoE/nNOS dko animals (Fig. 2b). The nNOS- γ hybridization probe revealed staining in the media and the neointima of apoE ko and apoE/nNOS dko mice (Fig. 2d, e). No staining was observed with all negative control sense probes as shown for nNOS- α and nNOS- γ on sections of an apoE ko aorta (Fig. 2c, f, sense ko).

3.2. Residual nNOS protein in apoE/nNOS dko mice

Immunostaining with the carboxy terminal antibody revealed strong nNOS expression in the media and neointima of apoE ko plaques (Fig. 3a). In apoE/nNOS dko animals nNOS protein expression appeared less robust (Fig. 3b). Employing the amino terminal antibody which recognizes the first 139 amino acids of nNOS- α , no staining was observed in apoE/nNOS dko animals (Fig. 3d), whereas specific expression of nNOS- α is shown in the intima and media of apoE ko animals (Fig. 3c). Western blot analysis of nNOS protein isolated from the total aorta revealed the presence of protein bands consistent with nNOS- α (155 kDa) and nNOS- γ (125 kDa) (Fig. 4a). In agreement with the results from the immunohistochemistry, no nNOS- α protein (155 kDa) was detected in aortas from apoE/nNOS dko animals. The absence of a protein band of higher molecular weight suggests that nNOS- μ (164 kDa) expression is not relevant in apoE ko aortas. Similar results were obtained using brain lysates from both genotypes (Fig. 4b).

3.3. Colocalization of nNOS protein with macrophages, endothelial cells and smooth muscle cells

Immunofluorescence studies show colocalization of the staining with the amino terminal and the carboxy terminal antibodies, suggesting that nNOS splice variants are co-expressed in the same cellular compartment in the plaque (Fig. 5a). In control experiments cross reactivity of secondary reagents to the two primary anti-nNOS antibodies was ruled out (data not shown).

Additionally, confocal microscopy was used to identify the cell types expressing nNOS proteins in atherosclerotic lesions of apoE ko mice (Fig. 5b). α -Smooth-muscle actin-positive vascular smooth muscle cells, primarily detected in the media and to a lesser extent in the neointima showed robust nNOS expression (Fig. 5b). Several, but not all MOMA-2-positive macrophages showed nNOS immunoreactivity, while CD31-positive endothelial cells did not reveal nNOS staining (Fig. 5b).

4. Discussion

The present study revealed three major findings. First, nNOS- α and nNOS- γ splice variants are expressed in the atherosclerotic plaque. Second, nNOS- α and nNOS- γ splice variants colocalize in the same cellular compartment. Third, in apoE ko vessels nNOS is expressed in vascular smooth muscle cells and macrophages in the neointima and media.

Wilcox et al. previously reported nNOS mRNA and protein expression in early and advanced human atherosclerotic plaques and absence of nNOS in healthy human vessels [2]. More recent studies using high sensitivity immunohistochemistry document the existence of nNOS in vascular smooth muscle cells, endothelial cells and perivascular nerve fibres in healthy vascular tissue of rats, pigs and humans [24–26]. Furthermore, nNOS expression is increased in rat vascular smooth muscle cells under conditions of high intraluminal pressure, following hypoxia and stimulation with platelet derived growth factor [27–31]. In a carotid artery ligation model, Morishita et al. found accelerated neointima formation and constrictive vascular remodelling in nNOS ko mice, suggesting that nNOS expression protects from vascular injury. In addition, these authors report absence of nNOS in carotid arteries of wild type mice [32].

Previous work from our laboratory suggests that nNOS- α protects apoE ko mice from spontaneous development of atherosclerosis [5]. In the latter study, deletion of nNOS (Exon 2) in apoE ko animals led to an increase in atherosclerotic plaque area compared to apoE ko control mice. Since we observed residual nNOS expression in aortas of apoE/nNOS dko animals, we here investigated the presence and localization of nNOS splice variants in the atherosclerotic plaque. We document expression of nNOS protein in the neointima and the plaque bordering media of atherosclerotic lesions from apoE ko and apoE/nNOS dko animals using a carboxy terminal antibody (Fig. 3a, b). In order to localize nNOS- α protein we used the antibody directed against the amino terminal end of nNOS. And indeed, we obtained a robust staining of apoE ko plaques, while apoE/nNOS dko plaques show no residual staining (Fig. 3c, d), suggesting the existence of nNOS splice variants in atherosclerotic lesions.

In brain tissues three differently spliced nNOS proteins have been described [12]. In the latter study residual catalytic nNOS activity (5% of wildtype) in brain tissue of nNOS (exon 2) ko mice was due to the presence of nNOS- β and - γ variants. In vitro catalytic activity of the nNOS- γ variant is low, reaching approximately 3% of nNOS- α , while the activity of the nNOS- β variant is about 80% of nNOS- α [12]. The nNOS- μ , variant has similar catalytic activity compared to nNOS- α . While nNOS- μ , is predominantly expressed in skeletal muscle, recent reports localized low amounts of nNOS- μ , in the media and adventitia of rat and human vascular tissue [33–35]. Schwarz et al. reported that inhibition of nNOS following endothelial denudation enhanced agonist mediated vasoconstriction in rat aortic rings, indicating a role for nNOS in regulation of vascular tone [34].

We confirm the presence of nNOS variants of different molecular weight in the atherosclerotic aorta using immunoblot analysis and demonstrate the presence of an additional nNOS protein band at the predicted molecular weight of nNOS- γ (125 kDa). No larger protein band at the expected molecular weight of nNOS- μ (164 kDa) was detected in aortic tissues by Western blot in both genotypes and nNOS- μ and nNOS- β mRNA was not detectable by RT-PCR in our previous study [5].

Using in situ hybridization we detect mRNA of nNOS- α and nNOS- γ splice variants in atherosclerotic lesions (Fig. 2). In situ hybridization experiments employing appropriate negative control sense oligonucleotides revealed no unspecific staining for the two probes. Since nNOS- μ , and nNOS- β were not detectable by Western blot and RT-PCR we conclude that nNOS- μ and nNOS- β are not expressed in the atherosclerotic plaque and did not perform in situ hybridization for these splice variants.

We see strong signals of nNOS- γ on the mRNA level in apoE ko mice. While nNOS- α is involved in the regulation of receptor and ion channel functions by binding to membrane proteins, the functional role of cytoplasmic nNOS- γ with low in vitro enzyme activity in vascular tissue is presently unclear, but might be independent of the existence and function of nNOS- α . This would be in agreement with the finding that the lack of nNOS- α does not change

mRNA expression levels of nNOS- γ in apoE/nNOS dko aortas compared to apoE ko aortas [5]. nNOS activity is regulated by several posttranslational mechanisms including enzyme activation through calcium-calmodulin, phosphorylation and by protein interactions [36]. In this respect, activation through interaction with membrane proteins like PDZ-binding domains or caveolin-3 have been described [37–39]. Taking in mind, that nNOS- γ lacks the PDZ domain which is essential for membrane binding, it seems reasonable to speculate that its regulation and function in cell signalling differs from the PDZ containing splice variants. The truncated splice variants may maintain basal nitric oxide production. Moreover, the nature of the N-terminal modifications may change the enzymes ability to produce reactive oxygen [40] species along with NO production in various cellular compartments and influence dimerization, required for catalytic activation. Therefore, the presence of nNOS gamma in the plaque may influence oxidative stress and impact on substrate and cofactor availability required for proper function of the atheroprotective isoform nNOS alpha. Interestingly, primitive NOS protein variants which lack N-terminal elements have been identified [41,42] pointing towards conservation of nNOS splice variant like proteins throughout evolution, underscoring the potential importance of this regulation.

In eNOS ko mice, nNOS was shown to be responsible for flow induced dilatation of coronary arteries [43]. In this study Huang et al. detected nNOS in the endothelial cell layer of coronary arteries of eNOS ko, but not in wild type mice. Our results are consistent with the latter study, since confocal microscopy showed absence of nNOS immunostaining in endothelial cells of apoE ko animals (Fig. 5b) and we previously reported that eNOS expression is comparable in apoE ko and apoE/nNOS dko mice [5]. In contrast, Loesch and Burnstock found nNOS protein in endothelial cells of the rat basilar artery using immuno-gold labelling [24]. This difference might be due to the different assays, or the diverse models used.

As expected, nNOS protein colocalizes to smooth muscle cells in the media and neointima. Here, only a few cells show positive α -smooth muscle actin staining. As proposed by Wilcox et al. smooth muscle cells in the neointima may dedifferentiate to mesenchymal appearing intimal cells, which are not detected by specific markers for macrophages, endothelial cells and vascular smooth muscle cells [2]. These cells express nNOS mRNA and protein and thus are likely to contribute to nNOS activity in atherosclerotic plaques [2]. Consistent with previous studies our data show low nNOS expression levels in lesional macrophages [2].

5. Conclusion

In summary, nNOS- α and nNOS- γ splice variants are expressed in atherosclerotic lesions of apoE ko mice. While nNOS expression was localized in vascular smooth muscle cells and fewer lesional macrophages, it was absent in endothelial cells. Our previous studies show that nNOS- α protects from plaque development. Thus, the presence of nNOS splice variants, their differences in enzyme kinetics and subcellular localization could mediate various functional properties relevant to plaque formation.

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Fig. 1.

Map of nNOS splice variant exon structures (gray boxes, E1a-E29) and protein regions targeted by the antibodies used. nNOS- α and nNOS- μ contain a PDZ domain (PDZ) encoded in exon 2. nNOS- β and nNOS- γ lack the PDZ-domain and have different translational start sites. The amino terminal antibody used in this study detects the first 139 amino acids of nNOS- α containing the PDZ domain. In contrast, the carboxy terminal antibody recognizes all splice variants. Binding sites for the different oligonucleotide probes used for in situ hybridization experiments are indicated.

Fig. 2.

In situ hybridization using splice variant specific radio labelled probes for nNOS- α (a, b) and nNOS- γ (d, e). Cryosections of aortic roots of apoE ko (ko) (a, c, d, f) and apoE/nNOS dko (dko) (b, e) animals were incubated with antisense (a, b, d, e) or sense control (c, f) probes. Subsequently radioactivity was detected by developing sections in NTB-emulsion. Positive staining areas show dark grains. nNOS- α mRNA is found in lesions of apoE ko mice (a) and is absent in apoE/nNOS dko animals (b). Furthermore, we find strong nNOS- γ staining in apoE ko (d) and apoE/nNOS dko (e) sections. Using sense control probes, no signal was detected as shown for nNOS- α (c) and nNOS- γ (f) in apoE ko mice. Scale bar: 100 μ m.

c-term **n-term** **Fig. 3.**

Immunostaining for nNOS in atherosclerotic lesions of apoE ko (ko) and apoE/nNOS dko (dko) mice. Robust nNOS staining is detectable with the carboxy terminal (c-term) antibody (a) and the amino terminal (n-term) antibody (c) in the neointima and media of apoE ko vessels. In contrast, the carboxy terminal antibody shows residual nNOS staining in apoE/nNOS dko mice (b), while no staining is detectable in the latter animals using the amino terminal antibody (d). Scale bar, 50 μ m.

Fig. 4.

(a) Western blot analysis of total aortic lysates from apoE ko (ko) and apoE/nNOS dko (dko) animals using an amino terminal (n-term) and a carboxy terminal (c-term) antibody. nNOS- α protein (155 kDa) is present in apoE ko aortas and absent in apoE/nNOS dko aortas. Using the carboxy terminal antibody (c-term) one additional protein band of lower molecular weight is visible at a size corresponding to nNOS- γ (125 kDa) in both genotypes. (b) Western blot analysis of brain lysates of apoE ko (ko) and apoE/nNOS dko (dko) animals. The amino terminal antibody detects nNOS- α protein (155 kDa) in apoE ko mice, whereas no nNOS- α protein is seen in dko mice (upper panel). Residual nNOS protein is found in brain lysates using the carboxy terminal antibody (lower panel) corresponding to nNOS- γ (125 kDa).

Fig. 5.

Fluorescence microscopy of nNOS protein in atherosclerotic lesions. (a) Sections of the aortic arch of apoE ko animals were incubated successively with the carboxy terminal (C-term, green) and the amino terminal (N-term, red) anti-nNOS antibody. Both antibodies target the same cells. Please note: the elastic lamina shows red autofluorescence. (b) Colocalization of nNOS (carboxy terminal antibody, red) with endothelial cells (CD31, green), smooth muscle cells (α -sm-actin, green) and macrophages (MOMA-2, green). Endothelial cells show no nNOS staining. nNOS-positive smooth muscle cells and macrophages localize in the neointima (arrows). (For interpretation of the references to color in Fig. 2, Fig. 3 and Fig. 5, the reader is referred to the web version of the article.)