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ARTICLE

Specificity of Antibodies to Nitric Oxide Synthase Isoforms in Human, Guinea Pig, Rat, and Mouse Tissues

Wilko Coers, Wim Timens, Claudia Kempinga, Pieter A. Klok, and Han Moshage

Departments of Pathology (WC,WT,CK,PKK) and Gastroenterology and Hepatology (HM), University Hospital Groningen, Groningen, The Netherlands

SUMMARY Ten commercially available rabbit polyclonal anti-NOS antibodies were tested for their immunohistological applicability in normal human, guinea pig, rat, and mouse organs. Most antibodies reacted as expected and described in the literature with various tissues of the investigated species. Several antibodies did not react with the expected cell populations in a certain species, or reacted in previously unknown patterns. In addition, different antibodies to the same isoform rarely detected identical cell populations, even within one species. Most of these unexpected immunoreactivities were observed in bronchial epithelial, glomerular epithelial, and vascular smooth muscle cells. These unexpected results usually occurred when the antibodies were tested in other organs or species than that to which they were originally raised. We therefore strongly recommend the use of anti-NOS antibodies only after careful immunohistological and biochemical analysis of their reactivity in the organ and species to be studied. **(J Histochem Cytochem 46:1385–1391, 1998)**

KEY WORDS
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The gaseous product nitric oxide (NO) has an enormous variety of (patho)physiological functions. Therefore, the expression and function of the various isoforms of nitric oxide synthases (NOS) under normal and disease conditions are widely studied. NOS enzymes are a family of at least three isoforms: neuronal ("n"), endothelial ("e"), and inducible ("i") NOS that can be distinguished by aspects such as their expression patterns and dependency on calcium ions for activation. At the cDNA level, a particular isoform can be up to 98% homologous among various species. The homology among the different isoforms is around 50%, even within one species (Robinson et al. 1996). At present, for the study of human (patho)physiology, a wide variety of antibodies that specifically recognize NOS isoforms are commercially available. Because of the high degree of evolutionary conservation of the three NOS isoforms, these antibodies can be expected to crossreact with their counterparts in various animal models. However, this crossreactivity among species is only sparsely documented or not known at all. Such a comparison is essential to adequately interpret the information obtained in studies of animal models. We therefore performed a comparative study with a selection of widely used commercially available rabbit polyclonal antibodies to the neuronal (n = 3), endothelial (n = 3), and inducible (n = 4) NOS isoforms using immunohistological and immunoblotting techniques (Table 1). These antibodies were tested on human (n = 6), guinea pig [Duncan Hartley (DH) and outbred], rat [Brown Norway (BN), Wistar Utrecht (WU), Lewis (LEW), Sprague–Dawley (SD), Fisher (F344)], and mouse [Balb/c, C57Black/6, DBA2, Swiss (SE)] cerebrum and cerebellum, lung, spleen, liver, and kidney.

The goals of our study were to establish the immunohistological reactivity patterns of the commercially available isoform specific anti-NOS antibodies in these species; to explain potential differences in staining patterns among these species; and to determine the applicability of commercially available anti-NOS antibodies for animal models.

Correspondence to: Prof. Dr. W. Timens, Dept. of Pathology, University Hospital Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.

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Table 1 Investigated commercially available rabbit polyclonal anti-NOS antibodies

NOS isoform	Species	Antigen	Amino acid	Dilution	Source[a]
nNOS	Rat	Synthetic peptide; mid-region	724–739	1:250	ABR
nNOS	Human	Synthetic peptide; N-terminus	37–56	1:200	SC
nNOS	Human	22-kD fragment; C-terminus	1095–1289	1:50	TL
eNOS	Bovine	Synthetic peptide; mid-region	599–613	1:250	ABR
eNOS	Human	Synthetic peptide; N-terminus	4–23	1:150	SC
eNOS	Human	20-kD fragment; C-terminus	1030–1209	1:50	TL
iNOS	Mouse	Synthetic peptide; C-terminus	1131–1144	1:250	ABR
iNOS	Human	Synthetic peptide; C-terminus	1135–1153	1:150	SC649
iNOS	Mouse	Synthetic peptide; C-terminus	1126–1144	1:200	SC650
iNOS	Mouse	21-kD fragment; N-terminus	961–1144	1:50	TL

[a]ABR, Affinity Bioreagents (Golden, CO); SC, Santa Cruz Biotechnology (Heidelberg, Germany); TL, Transduction Laboratories (Lexington, KY).

Materials and Methods

Sources of Human, Guinea Pig, Rat, and Mouse Organs and Tissues

Human tissues were obtained from normal donor organs that were disapproved for transplantation for technical reasons, or from autopsy material obtained within 6 hr of death from a total of six different individuals. The tissue donors were not suffering from any kind of systemic disease and the organs used in this study were considered histologically normal.

The most commonly used laboratory animals and strains were selected for this study. Two mature male individuals of about 3 months of age in two strains of outbred guinea pigs were tested (DH and Harlan outbred). The rats and mice investigated in this study were all sexually mature male animals at the age of about 10 weeks (generously provided by Harlan; Horst, The Netherlands). Four commonly used mouse (Balb/c, C57Bl/6, DBA2, SE) and five commonly used rat (BN, LEW, F344, SD, WU) strains were investigated. To exclude intrastrain variability, four animals were tested per strain.

The immunoreactivities of the NOS antibodies with the above-mentioned species were tested on the cerebrum, cerebellum, lung, spleen, kidney, and liver. These organs were chosen because they all should demonstrate specific patterns of expression of at least one of the NOS isoforms. In addition, this choice of organs covers main areas of research interest and allows comparison with the majority of published data on NOS expression (Robinson et al. 1996). Human tissues were snap-frozen in isopentane immediately after resection. The organs from the guinea pigs, rats, and mice were removed under halothane anesthesia after sacrificing the animals. Bleeding was performed to remove as much serum as possible from the organs to minimize unwanted immunological crossreactivities. The removed organs were snap-frozen in isopentane and stored at −80C for the preparation of cryostat sections.

Antibodies to NOS Isoforms

In this study, a selection of widely used antibodies from three different commercial sources was tested. To evaluate the crossreactivity of the antibodies in several species and to compare the antibodies with each other, we chose to investigate only rabbit polyclonal antibodies to NOS isoforms (Table 1). The antibodies were titrated in the species to which they were directed on organs in which immunoreactivity could be expected. For example, the immunoreactivity of anti-human eNOS from Transduction Laboratories (Lexington, KY) was tested on human lung which contained enough vessels for proper judgment. The proper antibody concentration was the one with clearly distinguishable positive reactivity with the expected cells and lowest nonspecific background reactivity. In general, this titration resulted in comparable IgG concentrations of 1–10 μg/ml, and these concentrations were used throughout the study in all species.

Immunohistological Techniques

Frozen sections were cut on a cryostat microtome at 4 μm thickness. These sections were wrapped in aluminum foil and stored for approximately 2 weeks at −20C. Sections of several organs were collected on the same slide. After thawing, the sections were fixed in 100% acetone for 10 min at room temperature (RT), washed with PBS, and preincubated with 10% normal goat serum in PBS. After removal of the preincubation serum, the rabbit anti-NOS polyclonals were added in PBS containing 1% bovine serum albumin (BSA; Serva Feinbiochemica, Heidelberg, Germany) for 60 min at RT. After this, endogenous peroxidase was blocked by incubation for 30 min in 0.075% H_2O_2 in PBS. To detect bound antibodies, the slides were washed with PBS and then incubated for 30 min with affinity-purified, peroxidase-conjugated goat anti-rabbit antibodies (Dakopatts; Glostrup, Denmark) containing 1% (v/v) normal serum of the species from which the tissue was tested. Peroxidase reactivity was developed using 3-amino-9-ethylcarbazole (Aldrich Chemical; Milwaukee, WI) and H_2O_2, after which the slides were counterstained with hematoxylin. For identification of NOS reactivity in the human lung and brains from all species, double stainings with the anti-macrophage antibody CD68 (KP-1; Dakopatts) and vimentin (V9; Dakopatts) were performed, respectively. The latter antigens were identified with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Southern Biotechnology Associates; Birmingham, AL) and the hematoxylin counterstain was omitted. The alkaline phosphatase label was developed with naphthol AS-MX, Fast Blue BB, and levamisol (all from Sigma; St Louis, MO) in 0.1 M Tris-HCl (pH 8.2).

The sections were scored independently by two observers as strongly positive (++), weakly positive (+), or negative (blank) immunoreactivity.

Immunoblotting Technique

Immunoblotting using positive controls and tissue homogenates were used to test the specificity of the NOS antibodies.

Lysates of IFN-γ- and LPS-stimulated mouse macrophages, human aortic endothelial cells, and a rat brain pituitary tumor (all from Transduction Laboratories) were used as positive controls for iNOS, eNOS, and nNOS, respectively.

Tissue homogenates were prepared as described previously for rat liver (Vos et al. 1997). Positive controls for NOS isoforms and tissue homogenates were fractionated on 10% SDS-PAGE and transferred to nitrocellulose (Amersham International; Poole, UK), using a semidry blotting system according to the manufacturer's instructions (Pharmacia; Uppsala, Sweden). Transfer of proteins was checked by Ponceau Red staining of the nitrocellulose filters after blotting. Molecular weight standards (BDH; Poole, UK) were used as marker proteins. The blots were incubated with polyclonal NOS antibodies in PBS containing 4% skim milk powder and 0.1% Tween-20, subsequently incubated with horseradish peroxidase-labeled swine anti-rabbit IgG (dilution 1:2000; Dakopatts), and finally developed using the ECL Western blotting system (Amersham).

Results

Immunohistology

Our results were generally in agreement with expected and previously published staining patterns in different species (Springall et al. 1992; Kobzick et al. 1993; Bredt and Snyder 1994; Dinerman et al. 1994; Tracey et al. 1994; Bachmann et al. 1995; Sancesario et al. 1996; Xue et al. 1996a,b), but important exceptions were found (see below). The Affinity Bioreagents antibodies all gave high background reactivity, making it difficult to titrate these antibodies. The following antibodies did not allow proper titration because of absence of specific immunoreactivity with the expected positive control cell population: anti-rat nNOS from Affinity Bioreagents; anti-human nNOS from Santa Cruz; anti-human eNOS from Santa Cruz; anti-mouse iNOS from Affinity Bioreagents; and anti-mouse iNOS from Santa Cruz. In the cases where there was no expected reactivity, we titrated the antibodies on cell types displaying specific positive immunoreactivity. For example, anti-human nNOS from Santa Cruz did not react with human neuronal cells but did react with smooth muscle cells in the vessel wall in all tissues of all species and with rat kidney glomeruli. The optimal concentration in the latter cell types was then titrated and chosen for further study.

The anti-rat nNOS antibody from ABR showed some reactivity in neuronal cells in the cerebrum and cerebellum of all tested species except the rat. This antibody did show reactivity with rat bronchial epithelium and diffusely with glomeruli in the rat kidney. The anti-human nNOS from SC reacted with the smooth muscle cell layer of all vessels in all species, in the human lung diffusely with the alveolar walls, in the rat lung with bronchial epithelial cells, and in the human kidney with parietal epithelial cells of the glomerulus. The anti-human nNOS from TL reacted only with neuronal cells in the human brain, and reacted diffusely with mouse kidney glomeruli and strongly with podocytes in guinea pig glomeruli (Figure 1A). Contrary to several reports, none of the anti-nNOS antibodies reacted with the renal macula densa (Tojo et al. 1994; Bachmann et al. 1995).

The anti-bovine eNOS antibodies from ABR showed, apart from high backgrounds, strong luminal reactivity in all vessels in all species, which was not restricted to the endothelium. The anti-human eNOS from SC did not show endothelial immunoreactivity with any of the tested tissues, but this antibody did show strong reactivity with mouse lung bronchial epithelial cells (Figure 1C). The anti-human eNOS antibodies from TL demonstrated endothelial reactivity only in human tissues and in mouse spleen and liver. This antibody also reacted strongly in the glial cell in the cerebrum and cerebellum of all species (Figures 1E and 1F) and sometimes with the smooth muscle cell layer of vessels.

The anti-mouse iNOS antibodies from ABR showed dot-like reactivity in all cells in the mouse lung and reactivity with some isolated cells in human organs. This antibody also showed weak reactivity with vessels in human tissues and was reactive only with the WU strain of rats, specifically with smooth muscle cells in the vessels and with endothelial cells in the liver. The anti-human iNOS from SC (649) showed reactivity with the glomerulus in human and with tubuli in the rat kidney. This antibody was also reactive with guinea pig endothelium and with human hepatocytes and macrophages in the spleen. The anti-mouse iNOS from SC (650) was strongly reactive with parietal epithelial cells of the glomerulus in the kidney of the SE mouse and in all mouse lungs with bronchial epithelial cells and nerves. This antibody was also strongly reactive with rat kidney tubuli, the red pulp in the spleen, and with hepatocytes. Weak reactivity was found with vessel walls in the human kidney and with guinea pig macrophages. The anti-human iNOS from TL was strongly reactive with human lung macrophages (Figure 1B), with guinea pig and mouse podocytes in the kidney, with bronchial epithelial cells of the guinea pig and rat (Figure 1D), and with mouse liver dendritic cells.

There were only small differences in intensity of the staining in the individual human specimens, and no differences could be detected among the individual animals of a particular strain. However, the aberrant staining patterns with several antibodies in the WU rat and the SE mouse, in comparison with other rat and mouse strains, demonstrates that interstrain differences in immunoreactivites may exist.

On the basis of our own experience as well as suggestions in the literature (Gonzalez–Hernandez et al. 1996), the antibodies were also tested on their positive control tissues using fixation with 2% and 4% buff-

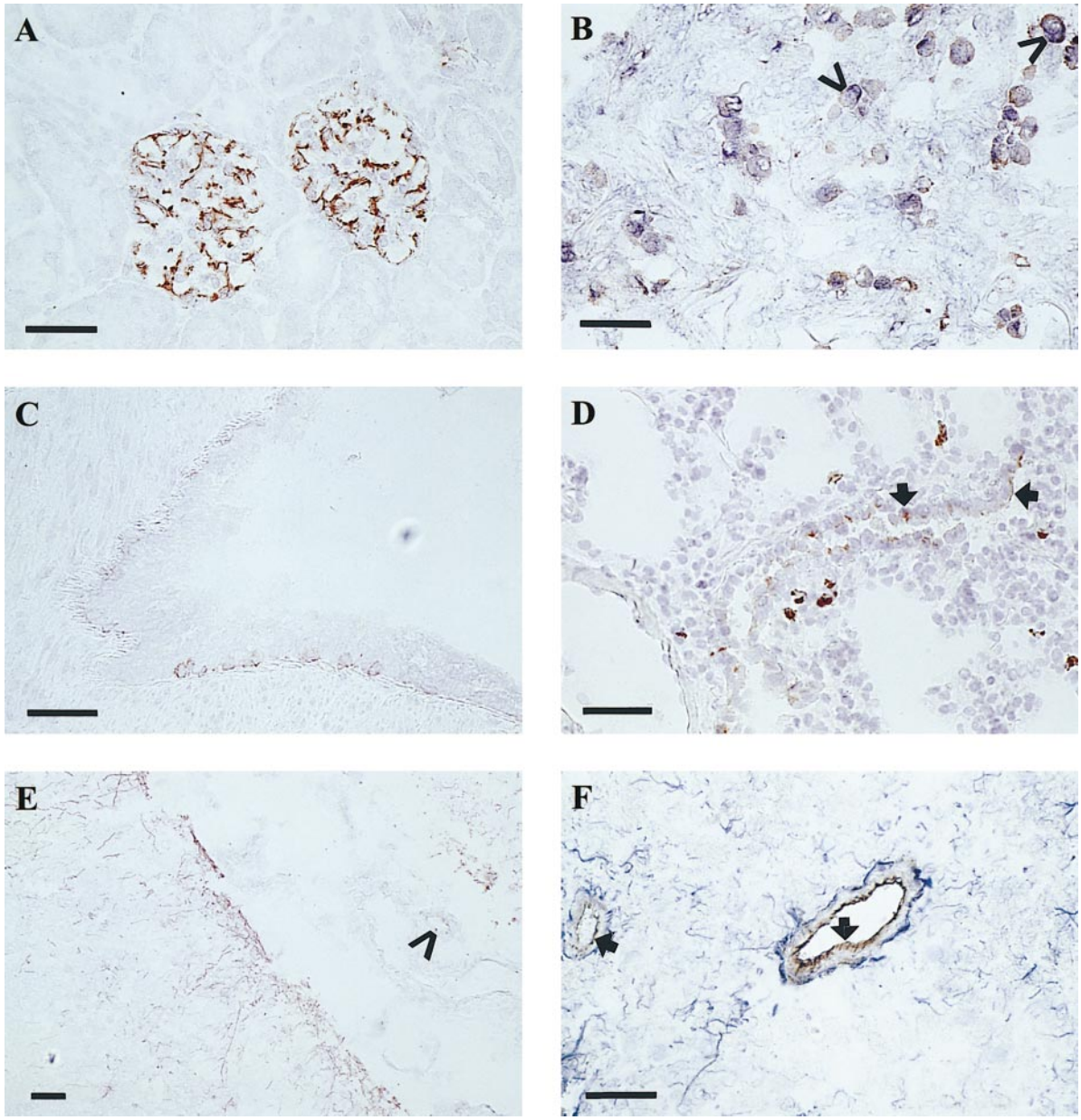

Figure 1 Selection of immunohistological staining results with NOS antibodies. Frozen sections of tissue were incubated with NOS antibodies and processed for peroxidase staining only (**A,C–E**) or in a double stain with alkaline phosphatase (**B,F**). (**A**) Podocytes in the guinea pig kidney staining intensely with anti-human nNOS from Transduction Laboratories. (**B**) Double staining of human lung macrophages with anti-mouse iNOS from Transduction Laboratories (blue; alkaline phosphatase) and CD68 (red; peroxidase). iNOS-positive macrophages are indicated by an arrowhead. (**C**) Basal epithelial cells in mouse bronchioles staining with anti-human eNOS from Santa Cruz. (**D**) Bronchial epithelial cells in the rat lung staining with anti-mouse iNOS from Transduction Laboratories (arrow). (**E**) Glial cell staining in the human cerebral cortex with anti-human eNOS from Transduction Laboratories. Note the positive staining in the endothelial cells as well (arrowhead). (**F**) Double staining of rat cerebral cortex with anti-human eNOS from Transduction Laboratories (blue; alkaline phosphatase) and anti-vimentin (red; peroxidase). Note the absence of eNOS staining in the endothelial cells (arrowhead), whereas the perivascular glial cells are strongly reactive with eNOS. Bars = 25 μm.

ered paraformaldehyde, but this generally resulted in a decrease or loss of immunoreactivity (not shown). In addition, amplification of the signals by third-step antibody incubations or the avidin–biotin method resulted in stronger but not other or more specific signals.

Immunoblotting

Most antibodies detected the proper protein band in Western blots of the positive control preparations (Figure 2). The high background in immunohistology demonstrated by the Affinity Bioreagents antibodies

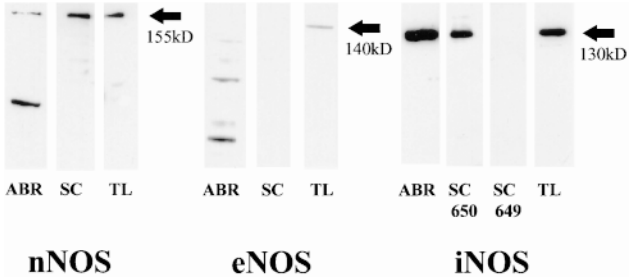

Figure 2 Immunoblot showing reactivity of NOS antibodies against corresponding commercially available positive control preparations. SDS-PAGE and immunoblotting were performed as described in Materials and Methods. Antibodies used are as described in Table 1. Molecular weights were verified by running molecular weight markers in lanes adjacent to the positive controls.

against eNOS and nNOS therefore probably results from the lack of affinity purification.

All antibodies against the iNOS isoform recognized a single band of approximately 130 kD, with the exception of the Santa Cruz antibody against human iNOS (#649). This antibody is raised against a C-terminal peptide of the human sequence and may not recognize the murine iNOS present in the positive control owing to the low homology between the murine and human iNOS in this region. The antibody from Transduction Laboratories against eNOS recognized a single band of approximately 140 kD in the endothelial cell lysate. In contrast, the anti-eNOS antibody from Santa Cruz did not react at all with the endothelial cell lysate, whereas the anti-eNOS antibody from ABR recognized several low molecular weight proteins but not a protein at the proper molecular weight. Finally, the antibodies against nNOS from Transduction Laboratories and Santa Cruz reacted with a single protein of approximately 155 kD in the rat pituitary lysate. In contrast, the anti-nNOS from ABR predominantly recognized lower molecular weight proteins.

In contrast to the satisfactory Western blot results with the commercially available positive control lysates, we rarely found the expected molecular weights using tissue homogenates. This was unexpected because the tissue homogenates comprised human, rat, and mouse lung and kidney, which were selected on the basis of positive immunohistological results with eNOS and iNOS antibodies from ABR and TL. As shown in Figure 3, the NOS antibodies, particularly the eNOS antibodies, recognized a variety of proteins in different tissue homogenates. Most of the recognized proteins were around or below the 56-kD molecular weight marker. The pattern of recognized proteins in, e.g., tissue homogenates, varied among species and among the sources of antibodies. This is illustrated in Figure 3 for lung homogenates and eNOS antibodies. This inconsistency was also seen, albeit to a lesser extent, with the iNOS antibodies.

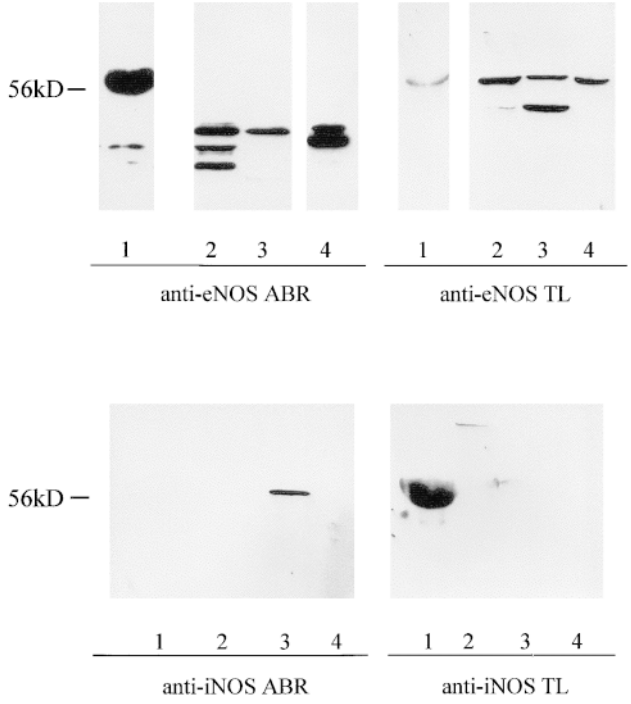

Figure 3 Immunoblot showing reactivity of selected NOS antibodies against a selection of tissue homogenates. SDS-PAGE and immunoblotting were performed as described in Materials and Methods. Antibodies used are eNOS antibodies (upper panel) and iNOS antibodies (lower panel) from ABR (left) and TL (right). The position of the 56-kD molecular weight marker is shown to indicate the molecular weights of the proteins recognized by the antibodies. Lane 1, human lung; Lane 2, rat lung; Lane 3, rat kidney; Lane 4, mouse lung.

Discussion

This report describes for the first time a thorough comparison of the immunoreactivity of several commercially available antibodies to NOS in human, guinea pig, rat, and mouse tissues. Most antibodies reacted as expected in the species from which the antigen used for immunization was originally derived, in a pattern characteristic for the cell types known to express the specific isoform. In addition, most antibodies identified a protein with the expected molecular weight in the Western blots with the commercially available positive control extracts. The demonstration of specificity of antibodies using immunohistological and immunoblotting techniques is normally considered sufficient for further research applications. In our study, however, we also observed unexpected crossreactivities and recurring patterns of aberrant immunoreactivities that are discussed below.

First, anti-NOS antibodies detect different structures in different species. For example, we found, as expected, that anti-human nNOS from Transduction Laboratories recognized human neuronal cells. However, the reactivity of this antibody with guinea pig glomerular epithelial but not neuronal cells is striking and is difficult to explain. If the anti-human nNOS antibody from Transduction Laboratories would be species-specific, causing absence of neuronal recognition in the guinea pig, then it remains to be explained why the antibody reacts so strongly with guinea pig glomeruli. This explanation goes beyond the scope of this study and will be the subject of further study (manuscript in preparation). It could imply that a human-like nNOS is expressed in glomerular epithelial cells in the guinea pig or that this antibody recognizes a NOS different from nNOS in guinea pig tissues. Interestingly, similar glomerular staining patterns can be obtained with the anti-mouse iNOS from Transduction Laboratories in the mouse kidney. Another example of species-specific differences in staining is the anti-human iNOS from Santa Cruz which showed weak renal apical tubular reactivity in the rat, whereas in the guinea pig these antibodies reacted with endothelial cells.

Second, unrelated antibodies can recognize identical structures in different species. Bronchial epithelial cells in the rat are recognized by the iNOS antibody from Transduction Laboratories. The same cell type in the rat is also recognized by the nNOS antibody from Santa Cruz. However, the latter antibody does not recognize neuronal cells in the rat brain. The iNOS antibody from Transduction Laboratories also recognizes bronchial epithelial cells in the guinea pig, but only in the apical region of the cells. Other investigators have described the apical expression of eNOS in rat (Xue et al. 1996a) and human (Shaul et al. 1994) bronchial epithelial cells, but it is unlikely that the anti-mouse iNOS from Transduction Laboratories would recognize eNOS in the guinea pig becauses no endothelial reactivity was identified in guinea pigs with this antibody. In contrast, the anti-mouse iNOS from Santa Cruz does react with endothelial cells in the guinea pig, suggesting that eNOS recognition by iNOS antibodies may occur.

Third, different antibodies raised against the same isoform of the same species rarely result in similar staining patterns. Specifically, the anti-human nNOS from Transduction Laboratories identifies neuronal cells in human neuronal tissues but the anti-human nNOS antibody from Santa Cruz fails to detect these cells, even in high concentrations. However, immunoblotting of the latter antibody with rat pituitary extract demonstrated its ability to recognize nNOS. Whether or not the specific vascular immunoreactivity of this Santa Cruz nNOS antibody should be interpreted as recognizing neuronal or another NOS isoform in the vasculature remains to be established.

Finally, we cannot explain the recurrent patterns of the aberrant immunoreactivities. Bronchial epithelial, glomerular epithelial, and vascular smooth muscle cells were remarkably often the cell types that reacted unexpectedly with the antibodies. The significance of this finding remains to be established.

These inconsistencies in immunohistological results were paralleled by the immunoblotting results. Although we performed our blots on a far from complete selection of tissues, it was nevertheless unexpected that in none of the lysates proper molecular weights were detected, despite their selection on the basis of positive immunohistology. The main reason for this may be that in normal, nondiseased tissue homogenates the dilution of NOS proteins is such that it remains below the detection level of the Western blots. We have increased the sensitivity of the Western blots to be able to see bands at all, but the observed bands are not necessarily proof of lack of specificity. It could very well be possible that specific NOS proteins can be detected with Western blots only in homogenates of purified cell populations or under induced conditions (Vos et al. 1997).

In general, the unexpected results occurred when the antibodies were used to detect their corresponding antigens in other species or organs than those to which they were originally raised. Until recently, the expression patterns of NOS isoforms appeared rather simple. Neuronal and endothelial NOS were constitutively expressed in neuronal and endothelial cells, respectively, and inducible NOS was expressed in activated macrophages and was therefore also called macrophage NOS (Stuehr et al. 1991; Bredt and Snyder 1994). The expression patterns of the NOS isoforms were subsequently found to be less exclusive (Dinerman et al. 1994; Shaul et al. 1994; Tojo et al. 1994; Gonzalez–Hernandez et al. 1996; Sancesario et al. 1996). Thus, iNOS was identified in and cloned from hepatocytes (Geller et al. 1993), vascular smooth muscle cells (Geng et al. 1994), and chondrocytes (Charles et al. 1993). In addition, co-expression of different NOS isoforms in a single cell type was described (Dinerman et al. 1994). The significance of our unexpected antibody reactivities in other species therefore remains to be established.

The potential relevance of NOS expression in many (patho)physiological situations has led to an enormous number of publications in the past few years. Several of these studies demonstrated the expression of NOS isoforms by immunohistochemical techniques, using the same antibodies as those we tested in this study. Frequently, the specificities of these antibodies were demonstrated in Western blots on extracts of other cell types and/or other species than were studied

in immunohistochemistry. Our results demonstrate that the detection of a specific band on purified positive control lysates does not justify immunohistochemical application on tissues without further study on tissue homogenates.

In conclusion, our findings demonstrate that (commercially available) NOS antibodies should be used carefully and critically. We discourage the use of anti-NOS antibodies for research applications on species and organs other than those to which these antibodies were raised. We recommend the following strategy to verify the immunohistological applicability of anti-NOS antibodies. Only if the immunohistological results on a certain tissue corresponds with the expression as well as the proper molecular weight of the protein in immunoblotting on its homogenate, do we believe that it is safe to use the antibody. If immunoblotting does not confirm the immunohistological result, we recommend the use of preferably antibody-independent techniques such as mRNA detection to verify the results. Alternatively, if an antibody-independent technique cannot be used, the tissue homogenates under study should be enriched for the studied cell type, e.g., for iNOS studies after or followed by induction of enhanced expression with LPS and cytokines. Again, only when the bands in these blots have the proper molecular weight and confirm the expression can the antibodies be applied in immunohistochemistry.

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Literature Cited

Bachmann S, Bosse HM, Mundel P (1995) Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. Am J Physiol 268:F885–F898

Bredt DS, Snyder SH (1994) Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 63:175–195

Charles IG, Palmer RMJ, Hickery MS, Bayliss MT, Chubb AP, Hall VS, Moss DW, Moncada S (1993) Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proc Natl Acad Sci USA 90:11419–11423

Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH (1994) Endothelial nitric oxide synthase localised to hippocampal pyramidal cells: implications for synaptic plasticity. Proc Natl Acad Sci USA 91:4214–4218

Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Di Silvio M, Wang SC, Nakayama DK, Simmons RL, Snyder SH, Billiar TR (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc Natl Acad Sci USA 90:3491–3495

Geng Y, Almqvist M, Hansson GK (1994) cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. Biochim Biophys Acta 1218:421–424

Gonzalez–Hernandez T, De La Cruz MAP, Mantolan–Sarmiento B (1996) Histochemical and immunohistochemical detection of neurons that produce nitric oxide: effect of different fixative parameters and immunoreactivity against non-neuronal NOS antisera. J Histochem Cytochem 44:1399–1413

Kobzick L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localisation. Am J Respir Cell Mol Biol 9:371–377

Robinson LJ, Ghanouni P, Michel T (1996) Posttranslational modifications of endothelial nitric oxide synthase. Methods Enzymol 268:436–448

Sancesario G, Reiner A, Figueredo–Cardenas G, Morello M, Bernardi G (1996) Differential distribution of nicotinamide adenine dinucleotide phosphate-diaphorase and neural nitric oxide synthase in the rat choroid plexus. A histochemical and immunocytochemical study. Neuroscience 72:365–375

Shaul PW, North AJ, Wu LC, Wells LB, Brannon TS, Lau KS, Michel T, Margraf LR, Star RA (1994) Endothelial nitric oxide synthase is expressed in cultured human bronchiolar epithelium. J Clin Invest 94:2231–2236

Springall DR, Riveros–Moreno V, Buttery L, Suburo A, Bishop AE, Merrett M, Moncada S, Polak JM (1992) Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms. Histochemistry 98:259–266

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc Natl Acad Sci USA 88:7773–7777

Tojo A, Gross SS, Zhang L, Tisher CC, Schmidt HHHW, Wilcox CS, Madsen KM (1994) Immunocytochemical localisation of distinct isoforms of nitric oxide synthase in the juxtaglomerular apparatus of normal rat kidney. J Am Soc Nephrol 4:1438–1447

Tracey WR, Xue C, Klinghofer V, Barlow J, Pollock JS, Förstermann U, Johns RA (1994) Immunochemical detection of inducible NO synthase in human lung. Am J Physiol 266:L722–727

Vos TA, Gouw ASH, Klok PA, Havinga R, van Goor H, Huitema S, Roelofsen H, Kuipers F, Jansen PLM, Moshage H (1997) Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. Gastroenterology 113:1323–1333

Xue C, Botkin SJ, Johns RA (1996a) Localization of endothelial NOS at the basal microtubule membrane in ciliated epithelium of rat lung. J Histochem Cytochem 44:463–471

Xue C, Reynolds PR, Johns RA (1996b) Developmental expression of NOS isoforms in fetal rat lung: implications for transitional circulation and pulmonary angiogenesis. Am J Physiol 270:L88–100