Effect of Scrapie Infection on the Activity of Neuronal Nitric-oxide Synthase in Brain and Neuroblastoma Cells*

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Nitric-oxide synthase (NOS) is responsible for the synthesis of nitric oxide which serves as a neural messenger in the central nervous system. NOS activity was markedly inhibited in brains of mice and hamsters and neuroblastoma cells infected with scrapie (ScN2a). The decrease in activity was in accordance with decreased NADPH-diaphorase-positive cells and decreased staining of NOS-positive cells demonstrated by specific anti-NOS antibodies. However, the specific nNOS mRNA in ScN2a was elevated when compared with normal neuroblastoma cells (N2a). Immunoblotting of fractions from these cell lines with an anti-nNOS monoclonal antibody revealed a band of nNOS from N2a and two bands with a lower molecular weight in ScN2a cells. Furthermore, NOS in ScN2a cells was insoluble in nondenaturing detergents. This insolvibility is one of the landmark properties of PrPSc. It is, therefore, possible that nNOS in scrapie-infected cells and brains is aberrantly folded, resulting in an insoluble and inactive enzyme.

Since the discovery of nitric oxide (NO) as the endothelium derived relaxing factor, acting in nonadrenergic noncholinergic relaxation of blood vessels (1, 2), this gaseous free radical was implicated in macrophage cytotoxicity (3) and in neurotransmission (4, 5). In the nervous system, NO fulfills criteria of neurotransmitters and play roles in synaptic transmission, release, or inhibition of release of other neurotransmitters, N-methyl-D-aspartate-related currents, long term depression and potentiation, animal learning, and hippocampal plasticity (6–9). NO is the product of the conversion of arginine to citrulline catalyzed by nitric-oxide synthase (NOS). The activation of this enzyme, at least in neurons, is the result of glutamate receptor coupling with specific agonists and influx of Ca²⁺ (10).

Because of its short half-life time and chemical instability, NO cannot readily be localized in tissues and most of the studies describing NO disposition have come from measurements of NOS activity, immunoreaction with specific anti-NOS antibodies, and expression of specific mRNA transcripts. Several NOS isoenzymes were characterized and cloned. Two forms are constitutive that require calmodulin and Ca²⁺ for their activity and located mainly in specialized neurons (10), in endothelia of blood vessels (11–13), and in skeletal muscles (14). One form of NOS, which is induced in macrophages (3), neutrophils (15), glial cells (16), and hepatocytes (17), does not require calmodulin and Ca²⁺ for its activation. It is assumed that, when NO is produced in large quantities and in combination with hydroxyl free radicals, it may result in neurotoxicity and may damage neighboring neurons (18).

An interesting and puzzling feature of NOS-positive neurons is their selective resistance to insults occurring in neurodegenerative diseases such as Huntington's disease (19, 20) and Alzheimer's disease (21). Furthermore, these neurons are well protected after massive ischemic event (22, 23) or neurotoxin administration (24, 25).

Prion diseases are a group of neurodegenerative transmissible disorders that affect both humans and animals. The microscopic features that characterize prion diseases are spongiform degeneration of neurons, severe astrocytic gliosis, and amyloid plaque formation (26). The putative infectious agent, denominated prion, may be composed only of PrPSc, which is an aberrant isoform of a cellular protein, PrPc (27). The only difference between PrPSc and PrPc seems to be conformational, since differences in amino acid sequence as well as posttranslational modifications have been ruled out (28). Prion diseases are sometimes considered as models for other more common neurodegenerative disorders, like Alzheimer's disease, since they share clinical and biochemical characteristics (29). As opposed to Alzheimer's disease which lacks a perfect animal model, prion diseases have well characterized models in rodents and even in cell culture (30, 31). Although, much is known about the molecular biology, biochemistry, and metabolism of PrP, the pathogenesis of prion diseases remains a mystery (32, 33).

Our results summarized below suggest that NOS activity was markedly inhibited in brains of mice and hamsters infected with scrapie as compared with normal brains. The activity of NOS was totally abrogated in neuroblastoma cells infected with scrapie. However, there was an increased accumulation of mRNA of NOS in ScN2a cells infected with scrapie as compared with N2a cells. Immunoblotting of fractions from these cell lines showed that NOS in ScN2a cells, as opposed to NOS in N2a cells, was insoluble in nondenaturing detergents. It is, therefore, possible that nNOS in scrapie-infected cells and brains is aberrantly folded, resulting in an insoluble and inactive form of the enzyme.

**EXPERIMENTAL PROCEDURES**

N-Nitro-l-arginine methyl ester (L-NAME), and β-nicotinamide dinucleotide phosphate, reduced form (NADPH), were purchased from Sigma. Dowex AG 50W-X8 (100–200 mesh, Na⁺ form) was purchased from Bio-Rad. [2,3-³H]-Arginine (43.5 Ci/mmol) was purchased from DuPont NEN. Calmodulin was purchased from Boehringer Mannheim. Antibodies to neuronal, endothelial, and inducible nitric-oxide synthase were purchased from Transduction Laboratories, Lexington, KY.

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1 The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; L-NAME, N-nitro-L-arginine methyl ester; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction.
Brains of normal as well as scrapie-infected brains of mice and hamsters were kindly supplied by Dr. Prusiner (San Francisco, CA).

Cell Culture—Normal and scrapie-infected neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. Three independently scrapie-infected N2a clones were used in this study. The original clone was infected 8 years ago according to Butler et al. (31). The other two clones were infected 2 years ago using the same procedure (31). All clones were positive for scrapie infectivity and for the presence of the pathological isoform PrPsc. In addition each of these clones as well as N2a cells were subdoubled in our laboratory, and 10 subclones were used throughout the experiments.

Nitric-oxide Synthase Activity—The enzyme activity both in brains and cell lines was measured, as described elsewhere (10), by monitoring the conversion of [3H]arginine to [3H]citrulline. Briefly, cerebella of three mice, and 5 × 10⁶ neuroblastoma cells were homogenized in 50 mM Tris buffer (pH 7.4), 1 mM EDTA, 1 mM EGTA. The homogenates were spun at 10,000 × g for 3 min at 4°C, and the supernatants were collected and passed through a mini-column containing 0.5 ml of Dowex 50W-X8 (Na⁺ form) in order to remove the endogenous arginine. Aliquots of the eluted material (25 μl) were incubated with 10 μM NADPH (reduced form), 10 μM CaCl₂, and [3H]arginine (0.5 μCi). After 15-min incubation at ambient temperature, the enzymatic reaction was terminated by adding 3 ml of Hepes (pH 7.5) with 2 mM EDTA and then passed on a column of Dowex. 0.5 ml of the 3 ml of eluted [3H]citrulline was quantified by liquid scintillation spectroscopy. Results were expressed as [3H]citrulline formed per min/mg of protein.

Arginase Activity—Soluble lysates of N2a and ScN2a cells were prepared, and arginase activity was determined exactly as described elsewhere (34).

Lactate Dehydrogenase Activity—Lysates of N2a and ScN2a cells were prepared in PBS pH 7.4 and tested in a standard procedure where the rate of conversion of NADH to NAD, in the presence of pyruvate, was measured in a spectrophotometer at 340 nm.

Other cytosolic enzymes, such as glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and creatine phosphokinase were measured in an automatic analyzer (Monarch plus).

Diaphorase Staining—Frozen sections of normal and scrapie-infected mice were fixed with 4% formalin in PBS for 60 min. The sections were rinsed thoroughly in PBS and then incubated for 1–3 h, at 37°C in complete darkness, with a substrate mixture containing 0.1 M Tris buffer (pH 7.8), 1 mM NADPH, 0.1 mM nitro blue tetrazolium, and 0.5% Triton X-100. The sections were rinsed and fixed in Kaiserglycerol gelatin solution.

Immunohistochemistry—Frozen sections of mouse cerebellum were immersed in 5% bovine serum albumin solution for 15 min and then reacted with mouse monoclonal anti-nNOS for 1 h and subsequently reacted with goat anti-mouse IgG-biotin (Jackson Immunoresearch Laboratories, West Grove, PA). After thorough washings with PBS, the sections were incubated with 0.3% peroxidase in 80% methanol. The reaction was stopped with 1% sodium perboride and aminoethanol carbazide was done according to the manufacturer's instruction (Sigma, Israel). N2a and ScN2a cells were fixed to plastic plates with 4% formalin (for the use with polyclonal antisera) and with 1.5% formalin (for the use with monoclonal antibodies). The cells were washed with PBS and treated with 1 μg glycine for 3 min followed by 0.1% Triton X-100. The fixed cells were then blocked with 1% bovine serum albumin solution and incubated with anti-nNOS antibodies for 16 h. The cells were then incubated with either anti-mouse or rabbit antisera conjugated with alkaline phosphatase and developed with the appropriate substrates.

RT-PCR of nNOS and PrP mRNA—Total RNA was isolated from N2a and ScN2a cells using Trisreagent™ LS (Molecular Research Center, Cincinnati, OH). RNA was treated with DNase I and then amplified by using the RT-PCR method. cDNA was synthesized with poly(dT)₃₀ as a primer and then amplified with NOX-specific primers CAG-GTG-CCT-CTC-GTG-GTC (NOS-3820, sense) and AGA-TGT-CCT-GGT-ACC-GGT-GTG (NOS-4258, antisense) to generate a 462-base pair fragment and with PrP-specific primers ATG-ACA-CTC-TCA-ACA-CTC-T (PrP-599, sense) and CCA-TCA-GTG-GCA (PrP-995, antisense) to generate a 415-base pair fragment. Each PCR product was characterized by digestion with specific restriction enzymes: PvuII, Alw26I, and BsaH I for NOS and Alw26I and Cfd for PrP.

Immunoblotting of Cell Extracts and Pellets—N2a and ScN2a cells (10⁶ cells) were extracted in 1 ml of lysis buffer containing 10 mM Tris (pH 7.5), 100 mM NaCl, and 1% Nonidet P-40. The samples were centrifuged at 3000 rpm for 15 min. Half of the supernatant was concentrated by methanol precipitation. 2% Sarkosyl was added to the other half of each sample followed by centrifugation at 10,000g x g for 40 min. The pellets (methanol and high speed centrifugation) were immunoblotted with monoclonal anti-nNOS.

RESULTS

The activity of NOS in cerebellar extracts of normal and scrapie sick mice and hamsters was measured by monitoring the formation of [3H]citrulline from [3H]arginine. In both species, NOS activity in the extracts of scrapie-infected brains was significantly reduced as compared with normal brain extract samples of the same age and sex (Table I).

When NOS activity was measured in several independently scrapie-infected N2a clones (ScN2a) and neuroblastoma cells (N2a), the difference was even more striking. In N2a cells, the activity was very high and was found to correlate with the amount of protein extracted from the cells (Fig. 1A). In contrast, no activity could be detected in all the three clones and the subclones of ScN2a that were tested in different occasions. In N2a cells the formation of [3H]citrulline was catalyzed by constitutive NOS, since the enzyme activity was gradually and specifically inhibited by the addition of -NAME (Fig. 1B). Furthermore, NOS activity was markedly decreased (more than 70%) when N2a cells were grown in arginine-free medium (Table I). We have tested several cytosolic enzymes in ScN2a cells and compared their activity with lysates of N2a cells. Values of lactate dehydrogenase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and creatine phosphokinase activities were similar in both types of cells. We conclude that some of the constitutive cytosolic enzymes were not affected in ScN2a clones (Table II). A series of experiments were conducted in order to investigate a series of possible factors which may cause the abrogation of NOS activity in ScN2a cells. To rule out the possibility that NOS in ScN2a cells remains attached to the Dowex resin during the absorption of endogenous arginine, scrapie and normal neuroblastoma cells were cultured in arginine-free medium, so that the absorption step in the activity assay could be omitted. Nevertheless, NOS activity in ScN2a could not be detected (Table I).

Another possibility for decreased NOS activity could be due to competition. It is well known that arginine can metabolize arginine and cause depletion of the substrate and its availability for NOS (35). Arginase activity was tested in both N2a and ScN2a cells, and no activity could be detected in cell lysates. Therefore, this possibility was ruled out.

In order to investigate whether that ScN2a cells or scrapie-infected brains contain a putative NOS inhibitor, extracts of ScN2a cells were mixed with extracts of N2a cells, before and after Dowex absorption of both samples. Extracts of scrapie-infected mouse brain and purified mouse PrP were also mixed with N2a extracts. No significant inhibition of the N2a extract NOS activity was observed in any of these experiments (data not shown), suggesting that the decreased NOS activity in the scrapie samples was not due to an endogenous NOS inhibitor.
The presence of calphostine (100 nM), a known phosphorylation inhibitor (36), N2a and ScN2a cells were also cultured in the presence of increasing concentrations of L-NAME. Values represent the mean ± S.D. of four different experiments.

**TABLE I**
Cytosolic enzymes activities in neuroblastoma cell lines
Enzyme activities were expressed as milliunits/mg of protein. The values represent the mean ± S.D. of three independent lines of cells.

|                | N2a | ScN2a |
|----------------|-----|-------|
| Creatine phosphokinase   | 15.7 ± 5.6 | 17.8 ± 7.7 |
| Oxaloacetic transaminase | 126.3 ± 24.2 | 92.3 ± 35.8 |
| Glutamic pyruvic transaminase | 21.5 ± 9.6 | 24.2 ± 11.2 |
| Lactate dehydrogenase    | 12.7 ± 5.6 | 16.4 ± 7.3 |

We tested whether NOS activity in ScN2a cells can be restored by the addition of calmodulin, which is a crucial cofactor for constitutive NOS activity. Addition of calmodulin did not induce NOS activity in ScN2a extracts. Since NOS activity is inhibited by phosphorylation and activated by phosphorylation inhibitors (36), N2a and ScN2a cells were also cultured in the presence of calphostine (100 nM), a known phosphorylation inhibitor (37). However, no increase of activity in the scrapie samples was observed after these treatments (Table I).

A well documented method to visualize NOS activity in tissue sections is the NADPH-diaphorase staining (38). A blue staining develops when NOS catalyzes the reduction of nitro blue tetrazolium by NADPH. Diaphorase staining represents total activity of neuronal, endothelial, and inducible NOS. When brain sections from normal and scrapie-infected mice were stained by this method, a strong blue signal appeared in neurons from normal brains (Fig. 2B), while no staining was present in the scrapie-infected brains (Fig. 2A). We have noticed also that only nNOS was affected in the scrapie brains, since blood vessels expressing endothelial NOS reacted as diaphorase-positive (Fig. 2A).

To further substantiate the finding obtained with diaphorase staining, we reacted the frozen cerebellum sections of normal and scrapie-infected mice with monoclonal antibodies specific for nNOS. Neuronal cells in the scrapie-infected cerebellum did not react with the antibody (Fig. 3A), as opposed to neurons in the normal tissue (Fig. 3B). Similar results were obtained when the neuroblastoma cell lines were reacted with anti-NOS antibodies. N2a cells reacted positively to both the monoclonal antibody (Fig. 4A) and the polyclonal anti-nNOS (Fig. 4C). ScN2a cells reacted very weakly with these antibodies (Fig. 4, B and D).

In order to investigate whether the absence of NOS activity in ScN2a cells may be due to reduced nNOS mRNA gene expression, we performed a semi-quantitative RT-PCR for total mRNA samples extracted from four clones of ScN2a and N2a cell lines. The amplification of cDNA with primers specific for murine nNOS and PrP (as control gene) is shown in Fig. 5. As expected, PrP mRNA amplification in clones of N2a (lanes 1–4) and ScN2a (lanes 5–8) were practically identical (39). ScN2a clones expressed a noticeable excess of nNOS mRNA (lanes 5–8) over normal N2a cell lines (lanes 5–8). Similar results were obtained while performing Northern blot analyses of specific NOS mRNA in cell extracts (not shown).

The existence of specific NOS mRNA transcripts and practically no protein molecules detected either by enzyme activity or by antibodies raised two possibilities. First, the process of translation might be defective or inhibited. Second, NOS may be translated successfully, but its conformation and solubility in detergents may change as in the case of the scrapie prion protein. We have tested the later possibility by subjecting brain and cell extracts to Western blot analysis and immunoreaction. The results of such analysis are presented in Fig. 6. Protein extracts of N2a cells subjected to 6% SDS-polyacrylamide gel electrophoresis and blotted on nylon filter reacted with anti-nNOS to give a single band at a range of 155 kDa as expected for nNOS (Fig. 6A, upper panel, lane 1). However, the antibody reacted with two bands in the ScN2a extracts (Fig. 6A, upper panel, lane 2). These bands migrated faster and had probably a lower molecular weight. These bands reacted specifically with the antibody directed against nNOS, since other antibodies raised against endothelial NOS (eNOS) and the inducible NOS (iNOS) reacted with neither N2a extracts (Fig. 6B, upper panel, lanes 1 and 4) nor ScN2a extracts (Fig. 6B, lanes 2 and 5). It was of crucial importance to show that additional bands could be detected by the anti-nNOS in scrapie-infected brains also. Indeed, that was the case in scrapie-infected hamster brain. The antibody detected the main NOS band in normal and infected brain (Fig. 6B, lower panel, lanes 7 and 8, respectively), but also two additional weak bands in the scrapie-infected brain (lane 8). The existence of both normal and aberrant nNOS in tissue is expected, since not all cells in the brain are affected by the scrapie infection.

The extracts of ScN2a and N2a cells were reacted with 2% Sarkosyl, separated by ultracentrifugation, and the pellets

**TABLE III**
NOS activity in neuroblastoma cell lines following miscellaneous treatments
Values given as (dpm/mg protein/min). ND = not detected.

| Treatment               | N2a    | ScN2a   |
|-------------------------|--------|---------|
| Untreated               | 125,060 ± 3700 | ND |
| Calmodulin (0.5 μM)    | 110,750 ± 4550 | ND |
| Calphostin (100 nM)    | 95,500 ± 2700 | ND |
were subjected to electrophoresis. As seen in Fig. 6A (upper panel, lanes 3 and 4), the NOS reactive bands in the scrapie samples were found in the insoluble pellet. For comparison, we demonstrated the insolubility of the PrPSc in Sarkosyl (Fig. 6A, lower panel, lane 4). In scrapie-infected brains NOS is only partially pelleted in the presence of Sarkosyl. We assume that in scrapie-infected brains not all the NOS protein is found in insoluble fraction just as in the case of PrP and PrPSc.

In summary, NOS in ScN2a cells, as opposed to N2a cells, is present at a different cellular compartment, having two major polypeptides with a lower molecular weight. This may suggest that NOS in ScN2a cells is aberrantly synthesized, and that may cause the enzyme to be inactive and insoluble.

DISCUSSION

In the present study, we found that nNOS activity was completely abolished in ScN2a cells as compared with noninfected N2a cells. We found also a significant decrease in nNOS activity in cerebella of mice and hamsters infected with scrapie. By using NADPH-diaphorase method, that represents NOS activity (40), we observed a significant reduction in diaphorase-positive cells in cerebella of scrapie-infected mice. Furthermore, we noticed a marked decrease in staining of NOS neurons, using specific anti-nNOS antibodies. Decrease in nNOS activity was reported after infection with noncytolytic neurotropic viruses (41) and in a case of spinocerebellar ataxia (42). But in other neurodegenerative disorders like Huntington’s and Alzheimer’s disease, NOS neurons were shown to be spared (19, 21). Scrapie infection of both cell lines and brains was shown, in several instances, to modulate some enzymatic pathways and neurotransmitter metabolism. Scrapie infection of PC12 altered cholinergic related enzymes, choline acetyltransferase and acetylcholinesterase, but the activity of tyrosine hydroxylase was unaffected (43). Activity of glutamic acid decarboxylase was reduced in cerebellum of infected experimental animals (44). We have tested several cytosolic enzymes such as lactate dehydrogenase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase, and creatine phosphokinase and found that their activity was unaffected. In no one of the enzymes tested by us or others, there was a total abrogation of activity as we found in the case of NOS in ScN2a cells.

It could be argued that in scrapie-infected brains there is a nondiscriminating process of neuron loss and that NOS-posi-
which results in the accumulation of PrPSc in a chemical change caused in scrapie brain and in the protein decrease in NOS activity could be the result of a specific bio-merely loss of neurons during scrapie infection. Rather, the findings were not in vivo paired by such mechanism, directly causes the pathology of other as for yet unidentified protein, whose function was im-

NOS is totally abolished in scrapie-infected neuroblastoma cells. N2a cells and ScN2a cells were extracted and amplified after reverse transcription with either NOS- or PrP-specific primers as described under "Experimental Procedures." A, N2a cells reacted with monoclonal anti-nNOS antibodies. B, ScN2a cells reacted with monoclonal anti-NOS antibodies. C, N2a cells reacted with polyonal anti-NOS antiserum. D, ScN2a reacted with polyonal anti-NOS antiserum. Amplification: $\times 20$.

protein conformation, which probably results in impaired function, may be caused by a translational or post-translational process. This is further supported by the finding that these two peculiar bands of aberrant nNOS occurred also in brains of scrapie-infected hamsters. Since the tissue contains infected cells as well as normal cells, it was expected to find also the normal nNOS (see Fig. 6).

In addition to PrP and nNOS, heat shock proteins, known to function as chaperons (49, 50), were also shown to be aberrantly processed in ScN2a cells (51). In ScN2a cells, Hsp73 in particular, is localized to intense stained cytoplasmic regions and is not translocated to the nucleus after heat shock. Molecules like Hsp73, which bind to unfolded proteins until their degradation (52), may recognize PrPSc as an aberrantly folded protein and bind to it. However, since PrPSc is not readily degraded and accumulates in the cell, molecular chaperons may remain bound to it until their own degradation. As a result, chaperons will be unavailable to fold other newly synthesized proteins. This may be the reason for the increased expression of molecular chaperons in scrapie infected brains (53). Similar results were also reported for Alzheimer's disease brains (54, 55). We speculate that nNOS may be affected by such a mechanism, resulting in an insoluble and inactive polypeptide that accumulates in the cells.

There may be other proteins in scrapie-infected cells and brains that were rendered inactive by lack of proper folding, resulting in aberrant conformation. While it is possible that the accumulation of aberrantly folded proteins like PrPSc and nNOS causes brain degeneration, it is also possible that another as for yet unidentified protein, whose function was impaired by such mechanism, directly causes the pathology of prion diseases.

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