Changes in the Expression Pattern of the Nitrergic System of Ovine Cerebellum Affected by Scrapie

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Abstract

The constitutive and inducible isozymes of nitric oxide synthase (NOS) and the end-products of nitration, nitrotyrosine, were analyzed by immunohistochemistry, Western blotting, and enzymatic activity in sheep at different stages of the prion disease, scrapie. Four groups were studied: 1) nonaffected (control), 2) preclinical, 3) clinical, and 4) terminal. Constitutive neuronal NOS (nNOS) was the most abundant isozyme present in cerebellar neurons of the sheep. Expression of nNOS increased in preclinical animals but diminished in the late stages of the disease. The Purkinje cells that usually are not immunoreactive for this protein became immunopositive in the clinical phase. In unaffected sheep, the inducible isozyme (iNOS) was slightly positive in the Purkinje cells. As the disease progressed, the immunoreactivity of Purkinje neurons for iNOS increased. At the final stages, numerous iNOS-positive microglial cells were found in the molecular layer. There was a basal level of protein nitration in the cerebellum of unaffected sheep, especially in the molecular layer. As the disease progressed, the distal prolongations of the Purkinje cells and the astroglia became immunoreactive for nitrotyrosine. Our results suggest that the nitrergic system reacts to the progression of spongiform diseases and may be part of their pathogenesis mechanism.

Key Words: Cerebellum, Nitric oxide synthase, Nitrotyrosine, Prion disease, Sheep, Transmissible spongiform encephalopathy.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of fatal and neurodegenerative diseases that affect both animals and humans. TSEs are the subject of increasing attention because of the growing number of cases that have appeared in recent years. These diseases include bovine spongiform encephalopathy (BSE) (also known as mad cow disease) in cattle, sporadic, familial, and variant Creutzfeldt-Jakob diseases, the Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, kuru, and feline spongiform encephalopathy in cats, and scrapie, which affects sheep and goats (1, 2).

Scrapie was first recognized in Great Britain in 1732 and described as a fatal degenerative disease affecting the central nervous system (3). Historically, scrapie has been considered nonpathogenic for humans (4), but because of the possibility of natural BSE transmission, which has been evidenced in a French goat, the European Union decided that TSE-affected sheep and goats must be kept out of the food chain to avoid possible presence of BSE (5).

Although the true cause of the disease is still debated, it is commonly accepted that scrapie is caused by accumulation of the abnormal form (PrPSc) of a normal (PrPc) prion protein present in the brain of the sheep (6). An affected animal may appear normal if left undisturbed at rest, but when stimulated by a sudden noise, excessive movement, or the stress of handling, the animal may tremble or fall down in a convulsive-like state. Sheep may live 1 to 6 months or longer after the onset of clinical signs, but death is inevitable.

Histologically, 2 types of characteristics have been described: 1) PrPSc deposition in central nervous and lymphoreticular systems and 2) histopathologic lesions in specific areas of the central nervous system, mainly including vacuolation of neuronal perikarya and grey matter neuropils and also neuronal degeneration and neuronal loss (especially “dark” neurons), an increase of reactive glial cells (predominantly astrocytes), and amyloidosis (4, 6, 7).

The nitrergic system plays an interesting role as a neuroprotector agent that may become a neurotoxic substance under pathologic conditions (8). The distribution and expression pattern of its components in the normal central nervous system of different animals has been extensively described, as well as its behavior during some neuropathologic diseases. Nitric oxide (NO) is synthesized from L-arginine in the neural structures of vertebrates and invertebrates (9–11) by the enzyme nitric oxide synthase (NOS) (12). Two biochemical forms of NOS exist; the constitutive (cNOS) and the inducible (iNOS) isozymes (13). The constitutive enzymatic form, found either in vascular...
endothelial cells or in neurons of the central and peripheral nervous system, is recognized as endothelial (eNOS) and neuronal (nNOS) isoforms, respectively.

The nNOS isoform is a soluble homodimer of 155 kDa, the molecular characteristics of which have been described (9, 14). In the rat cerebellum, nNOS immunoreactivity has been found in the following locations: in the basket and stellate cells of the molecular layer; in the basket terminal arborizations that surround the body and the initial portion of the axon of the Purkinje cells; in a very small number of nNOS immunoreactive Purkinje cells of the vermis and the paraffoculus; in some granular cells and their dendritic processes that contribute to form the glomeruli in the granular layer; and, finally, in the deep cerebellar nuclei (15). Recent immunohistochemical studies have demonstrated that nNOS distribution in the sheep cerebellum follows a different pattern to the one described in rats. In the sheep there is immunoreactivity in some neurons (Lugaro cells, Golgi cells, and unipolar brush cells) that are negative in rodents (16).

The iNOS isoform, which has 1,203 amino acids and a molecular mass of 135 kDa, shows 60% homology with the nNOS isoform. The iNOS isoform can be found in occasional Purkinje cells of the sheep cerebellum (16).

NO synthesized by the NOS enzymatic isoforms is a diffusible gas with the properties of a free radical that is highly lipophilic and can diffuse through cell membranes without the aid of specific membrane transporters to act on neighboring cells as a paracrine signal molecule (17). NO is also capable of modulating different brain functions (12, 18) by stimulating soluble guanylate cyclase (19, 20), which in turn raises the levels of cyclic guanosine-5-monophosphate (cGMP) in the cytoplasm of the target cells (9, 18). NO biosynthesis has been established as a key in the pathophysiological response (21). Part of the released NO during or after injury or neuropathologic disease can rapidly react with superoxide produced in excess during reoxygenation, forming peroxynitrite, a potent oxidizing agent with cytotoxic actions (22). Peroxynitrite can act on tyrosine residues in proteins to form the stable end product 3-nitro-1-tyrosine (nitrotyrosine). This compound can thus be used as a marker for the potentially cytotoxic effect of NO production in the presence of superoxide (22, 23).

Immunoreactivity to nitrotyrosine was found as granular deposits in the cytoplasm and perinuclear area of some Purkinje cells, stellate and basket neurons, and in some neurons of the cerebellar nuclei of the rat cerebellum. Nitrotyrosine immunoreactivity was also found in some glial cells of the sheep (16).

Several studies have focused on the distribution of the NOS isoforms and NADPH-diaphorase activity in the brain of mice and hamsters infected with the scrapie protein (24–27), but, surprisingly, no reports have been found in the literature investigating NOS distribution in the brain of scrapie-infected sheep.

This study was designed to investigate the participation of the nitrergic system in the evolution of scrapie in the sheep cerebellum—the part of the brain with higher levels of NOS immunoreaction and capacity of synthesis of NO.

Under normal conditions, the cerebellum is the region of the brain responsible for movement coordination and scrapie courses with locomotor alterations in most cases, thus our interest in this organ.

**MATERIALS AND METHODS**

**Material Collection and Tissue Preparation**

We used a natural model of scrapie. A monitored flock of scrapie-prone ARQ/ARQ sheep is kept at the University of Zaragoza with a prevalence for scrapie of about 10% (28). Twenty-seven sheep, classified into four different groups, were used for this study as follows: 1) Control group (n = 10): healthy animals came from different flocks in which no scrapie cases had ever been observed. 2) Preclinical stage group (n = 5): no clinical signs were detected, but the analysis of nictitating membrane biopsy showed the presence of PrPsc deposits. 3) Clinical stage group (n = 6): in addition to PrPsc deposits in the nictitating membrane, weight loss was evident and different clinical signs varying between animals were also detected. The most frequent signs were postural abnormalities, incoordination, hyperesthesia, tremors, and scratch response. 4) Terminal stage group (n = 6): the signs described for the clinical stage become severe in these animals. They become emaciated, weaker, reluctant to move, and had great difficulty getting up. Sheep were killed for welfare reasons before they become recumbent and unable to rise. PrPsc deposits were also demonstrated in all biopsies assessed.

The final diagnoses were determined by postmortem histopathology and immunohistochemistry using monoclonal antibody L42 and the EnVision system (Dako, Carpinteria, CA) in brain tissue, as described previously (29). This methodology is the confirmatory testing methods for TSE used in this laboratory (Fig. 1). All scrapie animals used in this study came from the same flock and presented similar histopathologic and immunohistochemical profiles, suggesting that all were affected by the same prion strain, although we cannot be completely certain.

Ten animals (4 from the control group and 2 from each of the other 3 groups) were used for immunohistochemical studies. After anesthesia with sodium pentobarbital, a fixative solution containing 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) was perfused using a peristaltic pump through a blunt cannula in the carotid artery. After perfusion the brain was removed, trimmed, and placed into embedding cassettes for postfixation for 3 hours in the same fixative at room temperature. The blocks were cryoprotected by immersion in 0.1 mol/L phosphate buffer containing 30% sucrose and kept overnight at 4°C.

Immunoblotting methods were applied on 17 sheep (6, 3, 4, and 4 from the control, preclinical, clinical, and terminal groups, respectively). Animals were killed by pentobarbital injection followed by exsanguination. Immediately after death, the whole brain was removed and a fresh half was frozen at −80°C.

All procedures were carried out in accordance with the European Community Council Directive (86/609/EEC) on animal experiments and reviewed by the Ethics Committees...
Antibodies
Polyclonal antibodies against nNOS, iNOS, and nitrotyrosine were produced in-house and fully characterized (15, 30, 31). They have been previously used successfully in sheep cerebellum (16).

Immunohistochemistry
Immunoreactive sites were identified in the sheep cerebellum following the avidin-biotin peroxidase complex (ABC) procedure as reported (15, 30, 31). Briefly, free-floating sections were prepared with a Vibratome (VT1000S; Leica, Madrid, Spain), washed in PBS for 5 minutes at room temperature, and incubated to quench endogenous peroxidase in PBS containing 3% hydrogen peroxide for 30 minutes. For the prevention of nonspecific staining, sections were preincubated in PBS containing 0.2% Triton X-100 and 3% goat normal serum for 1 hour at room temperature. Sections were separately incubated in primary antibodies: anti-nNOS (1:3000), anti-iNOS (1:5000), and anti-nitrotyrosine (1:1000), diluted in 0.2% Triton X-100 in PBS for 48 hours at 4°C. After several washes in PBS, sections were exposed to biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Sections were finally incubated with peroxidase-linked ABC (Vector Laboratories) for 90 minutes. Peroxidase activity was revealed by nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride as described previously (15).

Immunohistochemical Controls
Control procedures were carried out on sections of the sheep cerebellum. No immunolabeling was detected when the primary antibodies were omitted or replaced with an equivalent concentration of preimmune or normal rabbit sera. The specificity of the nNOS, iNOS, and nitrotyrosine antisera has been thoroughly demonstrated in our laboratory (30–32). Preabsorption of the antibodies with the corresponding antigens resulted in abrogation of the staining.

FIGURE 1. Panoramic views (A, C) and details (B, D) of the cerebellum in preclinical (A, B) and terminal (C, D) sheep stained with antibodies against the prion protein (red). Hematoxylin was used for nuclear counterstaining (blue). Note the spongiform morphology characteristic of this disease in the cerebellum of the terminal specimen (C, D). Scale bars = (A, C) 80 μm; (B, D) 50 μm.
Western Blotting

All chemical reagents were obtained from Sigma-Aldrich (Madrid, Spain) if not otherwise stated. Small pieces of cerebellums that had been kept frozen were homogenized (1:3 w/v) in 20 mmol/L HEPES buffer, pH 7.2, containing 0.2 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L dithiothreitol, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, 2 μg/mL pepstatin, and 0.1 mmol/L phenylmethylsulfonyl fluoride. All procedures were carried out at 4°C. Homogenates were centrifuged at 105,000 g for 1 hour, and the supernatant was collected. Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard. Samples of supernatants were mixed 1:1 with 2× sample buffer (10 mL of Tris-HCl, 0.5 mol/L, pH 6.8, 16 mL of sodium dodecyl sulfate [SDS] 10% [w/v], 8 mL of glycerol, 2 mL of 2-mercaptoethanol, and 0.2 mL of 0.1% bromphenol blue [w/v] plus water to 40 mL) and heated to 95°C for 3 minutes. SDS-polyacrylamide gel electrophoresis was performed in 7.5 or 10% gels with a 3.5% stacking gel and 7.5 or 10% gels with a 3.5% stacking gel and 0.2 mL of 0.1% bromphenol blue (w/v) plus water to 40 mL) and heated to 95°C for 3 minutes. SDS-polyacrylamide gel electrophoresis was performed in 7.5 or 10% gels with a 3.5% stacking gel and 0.2 mL of 0.1% bromphenol blue (w/v) plus water to 40 mL) and heated to 95°C for 3 minutes.

Nitric Oxide Synthase Activity

The activity of NOS in the supernatants of brain extracts prepared as for Western blotting was measured as the rate of conversion of radiolabeled arginine to citrulline in the assay described (33). Briefly, the samples were incubated at 37°C with [U-14C]-L-arginine (Amersham) in buffer containing 50 mmol/L KH2PO4, 1 mmol/L MgCl2, 0.2 mmol/L CaCl2, 50 mmol/L L-Valine, 1 mmol/L L-citrulline, 20 μmol/L L-arginine, 0.1 mmol/L NADPH, 10 μmol/L tetrahydrobiopeterin, and 1.5 mmol/L dithiothreitol, in the presence or absence of N-nitro-L-arginine methyl ester. The addition of 2 mmol/L EGTA was used to differentiate between Ca2+-dependent and Ca2+-independent NOS activities. After a 20-minute incubation, the reaction was terminated by adding AG 50W-X8 resin (Bio-Rad) to remove unreacted arginine and the radioactivity remaining in the supernatant was counted in a liquid scintillation counter (Wallac 1410, Pharmacia).

Statistical Analysis

Quantitative data from Western blotting and NOS activity were analyzed by one-way analysis of variance followed by the Bonferroni multiple comparison test to investigate potential differences between groups. A value of p < 0.05 was considered statistically significant.

RESULTS

Four groups of animals, classified according to the clinical stage of the disease, were analyzed. For each group, immunohistochemistry and Western blotting for nNOS, iNOS, and nitrotyrosine were performed in the cerebellum. In addition, Ca2+-dependent and -independent biochemical determinations of NOS activity were also carried out.

Neuronal Nitric Oxide Synthase Immunoreactivity

The morphologic lamination of the sheep cerebellar cortex is consistent with the pattern described in other mammals with 3 layers (molecular, Purkinje, and granular) in the grey matter and the internal white matter containing the cerebellar nuclei (Fig. 2). In the control animals (Fig. 2A), several types of neurons express nNOS. These include the basket and stellate cells in the molecular layer and the Lugaro cells, Golgi cells, unipolar brush cells (UBCs), and granule cells in the granular layer. In animals belonging to the preclinical group (Fig. 2B), there is a marked increase in nNOS immunoreactivity in all cell types, but this increase is especially noteworthy in the basket and stellate cells of the molecular layer. Interestingly, in animals of the clinical group (Fig. 2C), a general diminution of nNOS immunostaining was evident throughout the whole cerebellum, but the Purkinje cells, which were nonimmunoreactive in all other stages, were positive for nNOS in their perikarya and dendritic processes (inset in Fig. 2C). In terminal animals (Fig. 2D), the Purkinje cells are again nonimmunoreactive for nNOS and most of the cell types that were nNOS-positive in earlier stages of the disease recovered their immunostaining.

The evolution of nNOS immunoreactivity can be also followed in details of different cell types (Fig. 3). Cells of the molecular layer (Fig. 3A–D) increase their nNOS immunoreactivity in preclinical animals (Fig. 3B) over healthy controls (Fig. 3A), whereas they lose intensity in the final stages of the disease (Fig. 3C, D). In other neuronal cell types, such as the brush cells (Fig. 3E–H) and the deep cerebellar nuclei (Fig. 3I–L), the progress of the disease can be followed by the number and size of the intracellular vacuoles that are characteristic of all spongiform diseases.

Inducible Nitric Oxide Synthase Immunoreactivity

A few cells in the cerebellum of the normal sheep (Fig. 4A, B) contain immunoreactive material for iNOS.
FIGURE 2. General distribution of neuronal nitric oxide synthase (nNOS)-reactive structures in all layers of the cerebellar cortex
in control (A), preclinical (B), clinical (C), and terminal (D) sheep specimens. The inset in (C) represents a higher magnification
of an nNOS-immunoreactive Purkinje neuron. Scale bars = (A–D) 300 μm; (inset) 20 μm.
These include the perinuclear cytoplasm of the Purkinje cells (Fig. 4A) and some cells of the deep cerebellar nuclei (Fig. 4B). In preclinical animals (Fig. 4C, D) the Purkinje cells showed a marked iNOS immunoreactivity in the apical processes (Fig. 4C), suggesting an activation of this NOS isoform. Neurons of the deep cerebellar nuclei were also positive (Fig. 4D). The animals belonging to the clinical group had higher iNOS immunoreactivity in their Purkinje cells (Fig. 4E) while maintaining immunostaining in the cerebellar nuclei (Fig. 4F). In terminally ill sheep (Fig. 4G–K) the immunoreactivity for iNOS in the Purkinje cells (Fig. 4G) decreased to levels similar to those found in controls (Fig. 4A). A few Golgi neurons in the granular layer showed iNOS immunoreactivity (Fig. 4H, I), but the most

FIGURE 3. Details of neuronal nitric oxide synthase-immunoreactive structures showing the molecular layer (A–D), unipolar brush cells in the granular layer (E–H), and neurons of the deep cerebellar nuclei (I–L) at the different stages of the disease: control (A, E, I), preclinical (B, F, J), clinical (C, G, K), and terminal (D, H, L). Observe the numerous intracellular vacuoles that appear at the final stages of the disease. Scale bars = (A, I) 30 μm; (B, C, E, H, L) 40 μm; (D) 50 μm; (F, G, J, K) 60 μm.
interesting feature of this stage was that intense staining was found in the microglia of the granular layer (Fig. 4G, J). In addition, neurons of the cerebellar nuclei expressed high levels of iNOS (Fig. 4K). The astroglia surrounding these neurons was also intensely stained for iNOS (Fig. 4K).

**Nitrotyrosine Immunoreactivity**

As with iNOS immunoreactivity, some cerebellar structures were positive for nitrotyrosine in normal sheep. These include a low number of stellate and basket neurons of the molecular layer (Fig. 5A), neurons of the deep...
FIGURE 5. Panoramic view of positive structures for nitrotyrosine in the cerebellums of control (A), clinical (B), and terminal (C) sheep. Details show a comparison of deep cerebellar neurons in a control (D) and a clinical specimen (E) and other immunoreactive structures: unipolar brush cell in a control animal (F), and Golgi cells (G), mossy fibers (H), and positive astrocytes (I) in a terminal specimen. Scale bars = (A) 400 µm; (B) 100 µm; (C, E, G, H) 60 µm; (F) 50 µm; (I) 40 µm.
FIGURE 6. Expression of neuronal nitric oxide synthase (NOS) (A), inducible NOS (B), and nitrotyrosine (C) by Western blotting in the cerebella of sheep belonging to the control (a), preclinical (b), clinical (c), and terminal (d) groups. Ca²⁺-dependent (D) and Ca²⁺-independent (E) NOS enzymatic activity in the same samples. No statistically significant differences are found across the groups.
cerebellar nuclei (Fig. 5D), and a few UBCs (Fig. 5F) in the granular layer. Occasionally, some Lugaro cells, medium-sized Golgi cells, and terminals of mossy fibers were also positive for nitrotyrosine (not shown). In preclinical and clinical animals, the number and intensity of the stellate and basket cells that were positive for nitrotyrosine increased steadily (Fig. 5B). Another outstanding difference was the appearance of nitrotyrosine immunoreactivity in the nuclei of the Purkinje cells and a weak staining in their apical prolongations (Fig. 5G). The glia surrounding the neurons of the deep cerebellar nuclei acquired nitrotyrosine staining (Fig. 5E). Terminal animals exhibited the highest levels of nitrotyrosine immunoreactivity. Some stellate and numerous basket neurons were intensely positive for this antibody and were the source of a profuse varicose dendritic arborization that pervaded the whole molecular layer (Fig. 5C). The Purkinje neurons showed a different staining pattern compared with previous stages. The immunostaining disappeared from the soma and the proximal dendrites but was very intense in the apical dendrites (Fig. 5C). Golgi neurons (Fig. 5G) and mossy nerve fibers (Fig. 5H) were also positive at this stage. The astrocytes were nitrotyrosine immunoreactive in all layers of the cerebellar cortex (Fig. 5I).

**Western Blotting and Nitric Oxide Synthase Activity**

nNOS, iNOS, and nitrotyrosine protein expression in the cerebellum of the sheep did not show any difference associated with disease stages as assessed by Western blotting (Fig. 6A–C). Constitutive (Fig. 6D) and inducible (Fig. 6E) NOS enzymatic activity did not show any difference either.

**DISCUSSION**

Our results show that the nitrergic system in the cerebellum of the sheep affected by scrapie varies with the stage of this disease. Previous studies have shown that PrPSc has superoxide dismutase activity and conversion to the prion conformation has important implications on the oxidative balance of the brain (34). In this regard, scrapie-infected sheep present higher levels of NOS and lower superoxide dismutase activity in their brains than uninfected control animals (35).

The distribution of nNOS, iNOS, and nitrotyrosine in the normal sheep cerebellum has been described recently (16). Here we show that scrapie, a neurodegenerative disease of sheep, is accompanied by clear changes in the nitrergic system of the cerebellum. With nNOS, a dramatic initial increase in the immunoreactivity of the molecular layer is followed by a progressive decrease in intensity. Something similar, albeit somewhat more delayed in time, is the expression of nNOS in the Purkinje cells, a cell type that is usually negative for nNOS (8). This expression occurs in clinical specimens but disappears again in the terminal stages of the disease. Expression of nNOS by Purkinje cells has been described, but always in relation with pathologic processes (8, 36). The constitutive isoforms of NOS have a neuroprotective role because they are Ca²⁺-dependent and generate moderate amounts of NO (37). The elevation in nNOS immunoreactivity during the initial stages of scrapie may be interpreted as an attempt of the cerebellum to regain homeostasis against the chronic insult. The final decrease in nNOS may represent the failure of the system as neurodegeneration continues (as shown by the increasing vacuolation of the neurons). On the other hand, iNOS activity is usually considered neurotoxic because it is not regulated by Ca²⁺ and produces high levels of NO, which in turn may react with superoxide to generate peroxynitrite (38). In the cerebellum of sheep affected by scrapie, we have seen 2 main waves of iNOS expression. The first one involves the Purkinje cells that reach its zenith during the clinical stage of the disease to decline in the final phases. When the Purkinje cells stop expressing iNOS, glial cells and especially the microglia in the granular layer begin to express high levels of iNOS. Inflammatory mediators are strong cues for macrophages to produce iNOS (39). Here the microglia, a close relative of the macrophages, may be trying to deal with the local inflammation caused by the accumulation of the aberrant prion protein. Elevations in iNOS have also been described in mice infected with the scrapie prion and in this case the higher expression was mediated through a nuclear factor-κB mechanism (26).

Production of peroxynitrite in tissues results in drastic biochemical changes in the surrounding molecules. Nitrination of exposed tyrosine residues is the main modification in proteins exposed to peroxynitrite (40). Because iNOS produces higher levels of NO than the other isoforms, usually protein nitration closely follows iNOS expression (41), although it may be also found in regions expressing the constitutive isoforms (16). In our study, we found a basal level of nitration in the cerebellum of unaffected sheep, suggesting the existence of high levels of NO under physiologic conditions and the fact that nitration may constitute a normal mechanism for protein turnover. This basal nitration has been previously shown in the sheep cerebellum (16). As the disease progresses, there is an increase in the number of neural structures that stain for nitrotyrosine. There is also a curious intracellular migration of the immunostained structures going from the nucleus to the more distal prolongations. This centrifugal movement may represent the directional transport along the microtubules of proteins that were nitrated in or near the nucleus and are translocated toward the periphery with time.

Glia cells, and especially astrocytes, experienced severe nitration in the final stages of the disease. Astrocytes have been described in other pathologic models as nitration targets (42, 43) and glial fibrillar acidic protein may be one of the structural proteins that experience nitration. This increase in reactive astrogia has been also described in mice infected with the scrapie protein (25).

Western blotting and biochemical analysis of NOS activity did not show any global change when scrapie-infected sheep were compared with control animals. These results suggest that the modulation of the nitrergic system during scrapie occurs at the cellular level without an overall repercussion. In other words, there is a redistribution of the antigen without a major change in the general amount of protein present in the cerebellum. A clear example is provided by the evolution of the nitrotyrosine pattern that goes from
nuclear/perinuclear localization to a more peripheral distribution. In addition, other facts may contribute to the apparent discrepancy between immunohistochemistry and biochemical techniques. There are some suggestions that nNOS may colocalize with PrPSc in lipid rafts of the neuronal membrane (44) and that it may leave this subcellular localization upon scrapie infection, thus losing part of its activity (45). Other authors have pointed out that NOS activity diminished in mice and hamsters infected with scrapie due to aberrant folding of the nNOS protein (27). Still other studies have found a decrease in iNOS activity after experimental scrapie infection (46).

The nitrergic system has been involved in other neurodegenerative diseases, such as Alzheimer (42, 47), Huntington (48), and Parkinson (49), and in situations of schizophrenia (50) and brain ischemia (31, 51). In all these cases, a sequential activation pattern, beginning with an increase on nNOS, followed by induction of iNOS, and ending with an intense protein nitration, has been described, coinciding with our findings in scrapie infection. It seems that the nitrergic system is a reliable defensive mechanism elicited by the brain in emergency situations. During the course of the disease, there is an early attempt to reestablish homeostasis by overexpressing NOS, which ultimately fails in the final steps of this fatal disease.

Other authors use experimental infection models to study scrapie (52, 53). In our case, we prefer to study spontaneously occurring cases of the disease as they represent a more natural environment. Further experiments are required to investigate whether boosting the nitrergic system early on the disease process may prevent or delay the fatal outcome of scrapie.

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REFERENCES

3. Williams ES, Young S. Neuropathology of chronic wasting disease of mule deer (Odocoileus hemionus) and elk (Cervus elaphus nelsonii). Vet Pathol 1993;30:36–45