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Alteration of Free Radical Metabolism in the Brain of Mice Infected with Scrapie Agent

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Alteration of free radical metabolism in the mouse brain by scrapie infection was evaluated. The infection of mice with scrapie agent, 87V strain, slightly increased the activities of catalase and glutathione-S-transferase, while it had no effect on glutathione peroxidase, glutathione reductase, and Cu, Zn-superoxide dismutase. Results show that the scrapie infection decreased the activity of mitochondrial Mn-superoxide dismutase by 50% but increased that of monoamine oxidase ($p < 0.05$). Scrapie infection also increased the rate of mitochondrial superoxide generation ($p < 0.05$). Following scrapie infection, the level of free-sulfhydryl compounds in brain homogenates slightly decreased, but the content of thiobarbituric-acid-reactive substances and malondialdehyde increased significantly. Electron microscopy indicated that the ultrastructure of mitochondria was destroyed in the brain of scrapie-infected mice.

These results suggest that elevated oxygen free radical generation and lowered scavenging activity in mitochondria might cause the free radical damage to the brain. Such deleterious changes in mitochondria may contribute to the development of prion disease.

Keywords: Scrapie agent, SOD, free radicals, MDA, mitochondria

INTRODUCTION

Unconventional slow virus infections, also known as prion disease or transmissible spongiform encephalopathy, are central nervous system diseases characterised by dementia.[1] Typically, these diseases show an accumulation of abnormal prion proteins in glial cells.[2] Although the genetic regulation of this altered prion protein expression has been intensively studied by several investigators,[3–5] it is still unclear whether this change in protein conformation is solely responsible for cell death and the development of disease.

Recently, the free radical hypothesis has been suggested as a cause of the onset of neurodegenerative diseases, such as Alzheimer's disease.[6,7] In general, the degeneration of neuronal cells has been linked to various factors such as oxidative stress, impairment of energy metabolism, and calcium cytotoxicity.[8,9] Among these factors,

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oxidative stress caused by free radicals has been suggested as one of the main potential mechanism for neuronal degeneration. The bases of the claim is that various oxidants are produced continuously at a high rate as a by-product of normal aerobic metabolism. Intolerable damage to biomacromolecules by oxidants may contribute to neurodegeneration and aggregated diseases.

The brain is particularly vulnerable to oxidative damage because various toxic metabolites and free radicals are produced during metabolism of neurotransmitters such as catecholamines and excitatory amino acids.[10,11] Also, the brain contains large amounts of easily peroxidizable fatty acids and metals involved in the formation of free radicals.[12] At the same time, protective antioxidants and enzymes are relatively deficient in the brain.[13,14] Schubert and his colleagues[6] reported recent pertinent findings that amyloid peptides including amyloid β increase the accumulation of hydrogen peroxide in cultured cells, and killing cells via oxidative damage.

We postulate that the perturbation of free radical metabolism might also be closely linked to the development of prion diseases. In this study, we report changes in the level of the components related with free radical metabolism and some antioxidant enzymes in the brain of scrapie-infected mice. Our results showed that free radical damage in the brain was significantly higher in scrapie-infected animals than in controls, suggesting that free radical damage may be closely related to the pathological changes seen in these diseases.

MATERIALS AND METHODS

Animal Treatment

Male IM mice (a gift from Dr. Alan Dickison, AFRC & MRC Institute, Edinburg, U.K.) were divided into two groups; control and scrapie infected. Six week old mice were inoculated intracerebrally with 30 µl of 1% (w/v) brain homogenates prepared either from normal mice or from the scrapie-infected mice.[15] All mice were housed at five animals per cage and supplied with water and food *ad libitum* in a clean conventional system. Mice were sacrificed at 273 days after inoculation. Brain was quickly removed and homogenized with 10 mM HEPES buffer (pH 7.4), and cytosolic and mitochondrial fractions were prepared from the homogenates by differential centrifugation.[16]

Biochemical Assays

The activity of superoxide dismutase (SOD) in cytosolic and mitochondrial fractions were measured by monitoring the inhibition of the superoxide-mediated reaction of cytochrome c reduction at 550 nm by the reaction of xanthine with xanthine oxidase to generate superoxide radicals.[17] Catalase activity was assayed based on the direct measurement of decomposition of hydrogen peroxide at 240 nm spectrophotometrically.[18] Glutathione peroxidase activity was measured with the coupled-enzyme system using cumene hydroperoxide as a substrate[19] and glutathione reductase was assayed by measuring NADPH oxidation at 340 nm.[20] Glutathione-S-transferase activity was determined by the method of Habig *et al.*[21] Contents of sulfhydryl compounds in brain cytosol were measured at 412 nm according to the procedure of Sedlak and Lindsay.[22] The rate of superoxide generation in mitochondria was assayed based on the inhibitory effect of superoxide dismutase on the superoxide-induced oxidation of epinephrine to adrenochrome by the method of Nohl and Hegner.[23]

Thiobarbituric acid reactive substances (TBARS) were measured in brain homogenates without incubation. Briefly, 50 µl of the homogenates were mixed with 200 µl of 8.1% SDS and 1.5 ml of 20% acetic acid. Samples were then boiled for 30 min with 1.0 ml of 1.2% TBA. The absorbency of the solution was measured at 532 nm after centrifugation at $700 \times g$ for 10 min. The content of free malondialdehyde (MDA) in

brain homogenates was determined at 270 nm by HPLC on an aminophase (Lichrospher, 250 × 4 mm) column with acetonitrile/0.03 M Tris buffer, pH 7.4 (1 : 9 v/v).

Total activity of monoamine oxidase (MAO) in brain mitochondria was measured by fluorimetric assay using kynuramine as a substrate.[24] Briefly, aliquots (0.1 ml) of mitochondrial suspension were preincubated at 37°C for 5 min with 0.87 ml of 10 mM phosphate buffer (pH 7.2). The reaction mixture was started by the addition of 30 µl of 3.07 mM kynuramine and was shaken for 15 min before it was stopped by the addition of 0.3 ml of 0.4 M perchloric acid. A 1.0 ml of aliquots of the supernatant after centrifugation of the reaction mixture at 11,600× *g* for 15 s was transferred to test tubes that contained 2 ml of 1.0 M sodium hydroxide. After mixing, the 4-hydroxyquinoline fluorescence was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm in a fluorescence spectrophotometer (Perkin-Elmer LS50B). MAO-B activity was assayed by the procedure of Rajesh *et al.*[25] The reaction mixture contained 0.8 ml of 0.1 M phosphate buffer (pH 7.4), 0.05 ml of 30 mM sodium azide, and 0.1 ml of sample (100–250 µg protein). The reaction was started by the addition of 0.05 ml of 10 mM benzylamine. Incubation took place for 30 min at 37°C, and the reaction was stopped by the addition of 0.5 ml of the hydrogen peroxide measuring solution, which contained 0.5 M phosphate/citrate buffer (pH 4.0), 1.8 mM 2,2′-azino-bis (3-ethylbenzenethiazoline-6-sulfonic acid), and 5 units of horseradish peroxidase. After 5 s, 0.25 ml of 0.75 M hydrochloric acid containing 5% SDS was added, and the colored product was measured spectrophotometrically at 414 nm.

Histopathological Findings

Each animal was anaesthetised with 0.15 ml of 16.5% (w/v) urethane in saline per 30 g body weight and perfused transcardinally with 0.01 M cold PBS (pH 7.7) followed by perfusion fixation with cold 2% paraformaldehyde and 1% glutaraldehyde in PBS. After perfusion fixation, the brain sections were carefully dissected and immersed in the same fixative for 1 h at 4°C, and then washed with PBS. Brain sections were postfixed in 1% osmium teroxide for 1 h at 4°C, dehydrated through a graded series of ethanol and embedded in Poly/Bed 812 Resin (Polyscience Inc., USA). Semithin sections were stained with toluidine blue, and then ultrathin sections were stained with uranyl acetate, and lead citrate was examined using JEM 1200ES electron microscopy (Japan).

Statistical Analysis

Statistical significance was evaluated using the Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Mice infected with scrapie agent did not show any abnormal symptoms until 260 days post-injection of the agent. However, after that time, the body weight of the infected mice gradually declined and their spatial movement diminished remarkably. These results are consistent with those observed previously.[2,26] So mice in all groups were sacrificed at 273 days after scrapie injection and the alteration of biochemical parameters related to free radical metabolism in the brain were investigated.

Table I shows the alteration in the activity of antioxidant enzymes responsible for scavenging reactive oxygen species (ROS). The activities of glutathione peroxidase and glutathione reductase did not change with scrapie infection. On the other hand, catalase activity significantly increased by approximately 30% compared to the control group ($p < 0.05$), glutathione-S-transferase activity also increased by scrapie infection, however, without a significant difference. Interestingly, SOD showed an unique pattern in its

TABLE I Comparison of antioxidant enzyme activities in brains of control and scrapie-infected mice

Enzymes	Control	Infected
Catalase	0.15 ± 0.03	0.20 ± 0.01*
Glutathione peroxidase	0.96 ± 0.08	0.94 ± 0.13
Glutathione reductase	27.7 ± 3.7	25.5 ± 4.9
Glutathione-S-transferase	124 ± 10	159 ± 18

Data are expressed as the mean ± SD for groups of 5–7 mice. Catalase and glutathione-S-transferase activities are depicted as μmoles/mg protein/min, and glutathione peroxidase and glutathione reductase activities as nmoles/mg protein/min. *Significantly different from the control ($p < 0.05$).

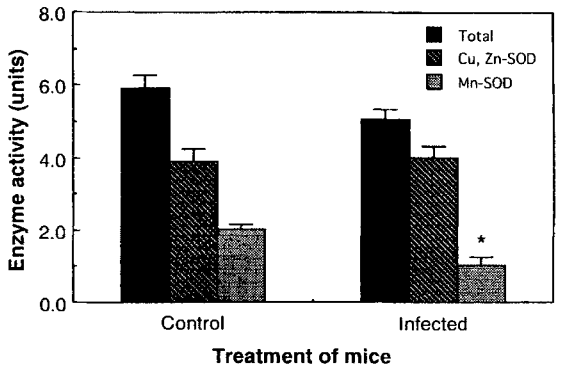

FIGURE 1 SOD activity in the brains of control and scrapie-infected mice. SOD activity was determined by monitoring the inhibition of cytochrome c reduction at 550 nm. Data are expressed as mean ± SD of 5–7 mice per group. *Significantly different from control ($p < 0.05$).

TABLE II Level of sulfhydryl compounds (SH) in brain homogenates of control and scrapie-infected mice

Compounds	Control	Infected
Total-SH	1.61 ± 0.09	1.43 ± 0.18
Protein bound-SH	1.36 ± 0.09	1.24 ± 0.18
Free-SH	0.25 ± 0.04	0.19 ± 0.05

Data are expressed as the mean ± SD of 5–7 mice, and a unit of the content of sulfhydryl compounds was defined as μmoles/g tissue.

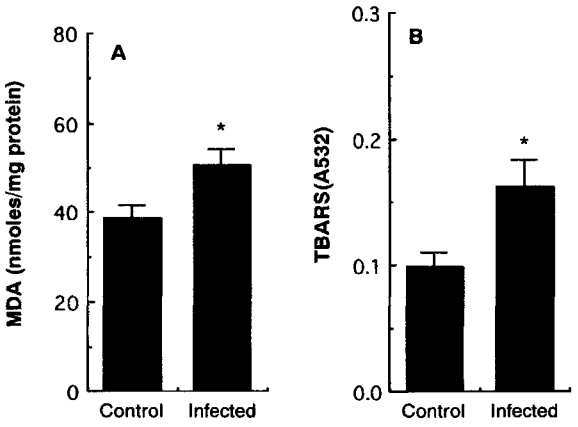

FIGURE 2 Content of MDA (A) and TBARS (B) in brain homogenates of control and scrapie-infected mice. Data are expressed as mean ± SD of 5–7 mice. Details are described in the Materials and Methods section. *Significantly different from control ($p < 0.05$).

activity change as shown in Figure 1. Namely, cytosolic Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity did not change, whereas mitochondrial Mn-superoxide dismutase (Mn-SOD) activity showed a remarkable decrease in scrapie-infected mice compared to the control mice ($p < 0.05$).

We evaluated the *in vivo* damage induced by free radicals by measuring the levels of sulfhydryl compounds and the oxidized cellular macromolecules in cytosol. As shown in Table II, following scrapie infection, the contents of cytosolic free- and total-sulfhydryl compounds reduced slightly with no alteration in protein bound sulfhydryl compounds.

The contents of thiobarbituric-acid-reactive substances (TBARS) and malondialdehyde (MDA) in the brain homogenates of control and scrapie-infected mice are compared in Figure 2. The TBARS level in the brain homogenates showed significant increases in the infected mice compared to control mice ($p < 0.05$). Because TBA analysis can show non-specific responses to many substances other than MDA,[27] we employed HPLC analysis to measure the free MDA level more specifically. This analysis showed that the free MDA content in homogenates was also significantly higher in the infected mice than in the control mice ($p < 0.05$). These results suggest that scrapie-infected mice are more vulnerable to the stress conditions caused by oxidants.

Results showing elevated oxidative damage and reduced Mn-SOD activity encouraged us to analyze the rate of superoxide formation in

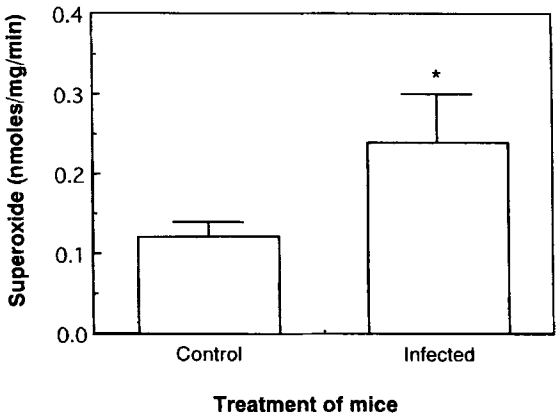

FIGURE 3 Superoxide generation in mitochondria of the brain from control and scrapie-infected mice. Data are expressed as mean ± SD of 5–7 mice. Details are described in the Materials and Method section. *Significantly different from control ($p < 0.05$).

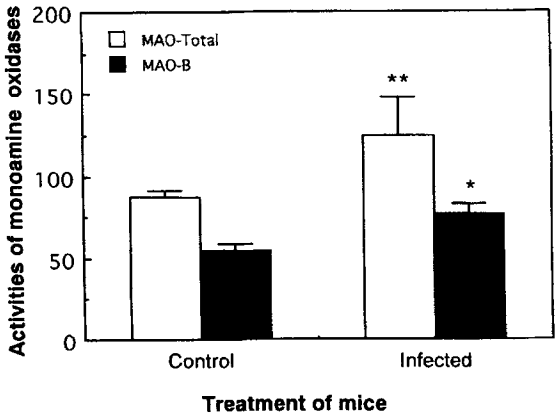

FIGURE 4 Changes in monoamine oxidase activity in brain mitochondria from control and scrapie-infected mice. MAO activity was measured in mitochondrial fractions obtained from the homogenates of whole brain. The data in nmoles hydrogen peroxide produced per mg protein per min for MAO-B and arbitrary units of fluorescence intensity for MAO-T, were mean ± SD of 5–7 mice. *Significantly different from control ($p < 0.05$), **$p < 0.01$.

mitochondria. Thus, we measured the rate of superoxide generation in the mitochondrial fractions of brains from control and scrapie-infected mice. As shown in Figure 3, the infected mice yielded a two-fold higher rate of superoxide generation compared to the control. These results suggest that more ROS are generated in the brain mitochondria of scrapie-infected mice while the mitochondrial antioxidant defence ability is relatively low.

Results in Figure 4 show an analysis of MAO activity, another source of hydrogen peroxide production in brain.[28,29] MAO is located in the outer membranes of mitochondria and exists in two forms, MAO-A and MAO-B, which are based on different inhibitor and substrate specificities. The cellular level of MAO-B, which is responsible for dopamine oxidation is known to increase with age.[30] Therefore, we measured the total activity of MAO (MAO-T) and that of MAO-B in brains of control and scrapie-infected mice. The activities of these enzymes significantly increased following scrapie infection (MAO-B, $p < 0.05$; MAO-T, $p < 0.01$). These results suggest that infected mice can generate more ROS than their uninfected control counterparts via the oxidation of biogenic amines.

Figure 5 indicates the morphological change of brain mitochondria of scrapie-infected mice. The structure of mitochondria cristae was almost completely disrupted in the scrapie infected mice (C and D in Figure 5), while uninfected control mice showed no change (Figure 5A and B).

DISCUSSION

The progression of the scrapie-like diseases is associated with the accumulation of abnormal brain prion proteins in glial cells, the formation of amyloid plaques and vacuoles, and finally, the death of surrounding cells. The consequence of these histopathological alterations in relation to the development of disease is unclear. We have been interested in the involvement of the perturbation of brain free radical metabolism as a possible mechanism involved in the development of prion disease. In this study, scrapie agent 87V strain in IM mice showed similar histopathological changes and latency to those reported previously.[2,26]

We developed the following conclusions concerning the involvement of free radical damage

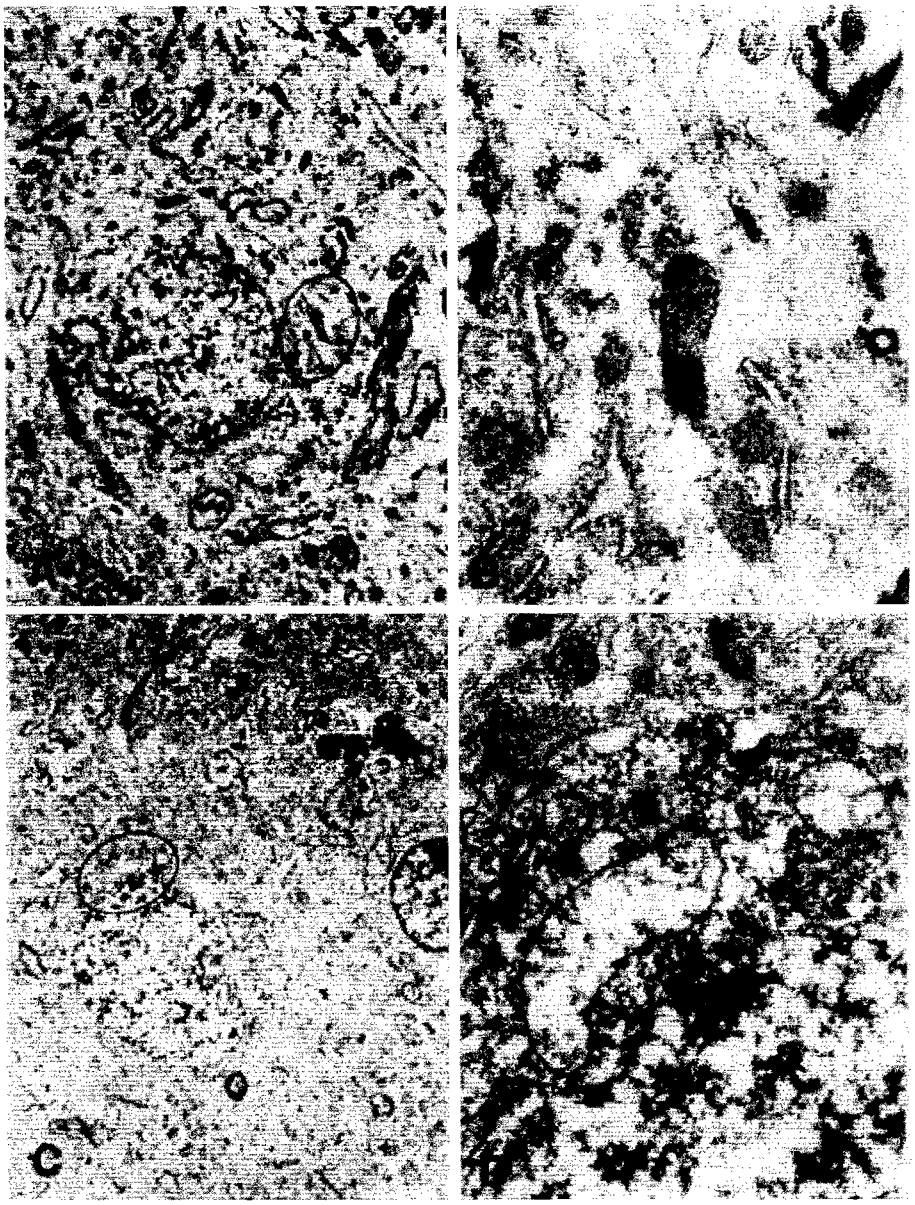

FIGURE 5 Histopathological observation of brain cerebral cortex from control (A and B) and scrapie-infected mice (C and D) by electron microscope. Mitochondria represented in B and D show a magnification of 20,000 fold of represented in A and C; 3–4 mice in each group. A complete disruption in mitochondria ultrastructure was found in brains of scrapie-infected mice (D).

in the pathogenesis of prion diseases from our study: (i) Infection of the scrapie agent causes an increase in free radical damage, as evidenced by increased MDA and superoxide generation; (ii) The infection perturbates mitochondrial free radical metabolism, as shown by changes in mitochondrial radical clearing enzymes. Although we observed no significance differences in the content of sulfhydryl compounds between two groups, the consistent decline of the compounds caused by the scrapie infection provides additional evidence of free radical damage, as

cellular sulfhydryl compounds play an important role in protecting membranes and other biomacromolecules from oxidative damage. Among the cellular sulfhydryl compounds, reduced glutathione (GSH) is an essential tripeptide present in virtually all animal cells, and contributes to the detoxification of toxic metabolites by a conjugation with endogenous substances or lipid peroxidation products.[31] Therefore, we compared the activity of the enzymes related to GSH. Glutathione reductase did not show any significantly changes, while glutathione-S-transferase, known to remove toxic metabolites *in vivo*, increased slightly by scrapie infection. Catalase, which catalyzes the conversion of hydrogen peroxide to water to suppress the formation of the hydroxyl radical, also increased. The depletion of the sulfhydryl compounds and the increased activities of these enzymes suggest that infected animals were in a state of oxidative stress. Increased levels of MDA and TBARS were much more obvious in the brain of infected mice and could be direct evidence of the free radical damage.

The next step of our study was to determine the potential source of free radical generation in the brain of mice during scrapie infection. There are several sources for the generation of ROS in brain, which include the uncoupling of the mitochondrial respiratory chain, the MAO system, and microsomal mixed function oxidase (MFO).[32–34] The uncoupling of the microsomal MFO system is probably not a main source of ROS generation in the brain because the amount of brain cytochrome P-450, the major component of microsomal MFO, is no more than 1% of that of liver.[34] In contrast, up to 95% of molecular oxygen is metabolized within mitochondria via the electron transport chain, therefore the perturbation of the mitochondrial respiratory chain may generate a significant amount of ROS. The results showing an increase in the rate of superoxide generation and a decrease in the activity of mitochondrial Mn-SOD can be argued, because Mn-SOD, which scavenges superoxide, is known to be increased by the superoxide. Future investigation will be aimed at assessing these paradoxical findings.

Electron microscopy showed a complete disruption in ultrastructure of mitochondria in the brain of the scrapie-infected mice. These findings provide a potential explanation for the changes observed in the Mn-SOD. Increased superoxide generation and reduced Mn-SOD activity in the mitochondria of scrapie-infected mice suggest that the infection may disturb mitochondrial free radical metabolism. The brain predominately depends on mitochondrial energy supply, therefore mitochondrial dysfunction may severely affect the nervous system.[35]

Alteration in catecholamine metabolism in brain can be another source of the ROS generation. In mouse brain, most biogenic amines, including dopamine, are preferentially deaminated by MAO.[30,36] The enhancement of MAO activity can cause an increase in ROS formation and lead to dopamine depletion and dopaminergic cell death. To test this possibility, we compared the activity of MAOs in the brains of control and scrapie-infected mice. The activity of MAOs showed significant increases in the brain of scrapie-infected mice as well as aged mice.[30] The enhanced MAO activity in brains of infected mice seems to be derived from astrocytes, because prion disease accompanied astrocytosis, and astrocytes contain abundant MAO activity.[37] Therefore, the induction of this enzyme in the brain by scrapie infection may cause a decrease in dopamine level and an increase in hydrogen peroxide formation. If this occurs, the oxidative stress caused by unusual dopamine metabolism could be related to the development of the illness. The increased of catalase activity without SOD induction in the brain of scrapie-infected mice supports this possibility because MAO generates hydrogen peroxide but does not form superoxide.

In summary, the results of this study indicate that free radicals, especially those ROS generated by damaged mitochondria in the brain cells of scrapie-infected mice perform a key function in the pathogenesis of scrapie-like diseases. Further

studies of the biochemical changes in brain caused by the infection of the scrapie agent are required.

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