Systemic and pituitary pars intermedia antioxidant capacity associated with pars intermedia oxidative stress and dysfunction in horses

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Objective—To determine whether a deficiency in systemic or local (pars intermedia) antioxidant capacity is associated with pituitary pars intermedia oxidative stress and pituitary pars intermedia dysfunction (PPID) in horses.

Sample Population—Blood samples from 20 horses with PPID and 20 healthy client-owned horses, archived paraffin-embedded adrenal gland and substantia nigra tissues from 20 horses, and pituitary gland tissue from 16 horses.

Procedures—Total glutathione, superoxide dismutase, and glutathione peroxidase activities were determined in RBCs. Accumulation of a systemic marker of oxidative stress (3-nitrotyrosine) was assessed in plasma and formalin-fixed, paraffin-embedded adrenal gland and substantia nigra tissues. Local antioxidants (total and manganese superoxide dismutase, glutathione peroxidase, and total glutathione) were measured in pars intermedia tissues.

Results—No significant differences existed in systemic antioxidant enzyme activity or accumulation of 3-nitrotyrosine between horses with PPID and control horses. In pituitary gland tissues, glutathione peroxidase activity was increased in horses with oxidative stress, whereas total glutathione concentration and superoxide dismutase activity remained unchanged. There was an age-associated decrease in manganese superoxide dismutase activity in the pars intermedia.

Conclusions and Clinical Relevance—There was no evidence of systemic accumulation of oxidative stress markers or deficiencies in antioxidant capacity in horses with PPID, suggesting that these are unlikely to be major predisposing factors in the development of PPID. Manganese superoxide dismutase activity in the pars intermedia decreased significantly with increasing age. Role of an age-associated decrease in antioxidant capacity for the pars intermedia in the development of PPID in horses warrants further investigation. (*Am J Vet Res* 2005;66:2065–2072)

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Pituitary pars intermedia dysfunction (PPID) in horses is a naturally developing, progressive disease that affects older horses and ponies. Although all breeds of horses may be affected, ponies and Morgan horses appear to be at greatest risk.[1-5] Horses with PPID have hypertrophy, hyperplasia, and adenomas of the pars intermedia of the pituitary gland, which lead to overexpression of **proopiomelanocortin (POMC)**. The POMC in the pars intermedia is further processed to **α-melanocyte–stimulating hormone (α-MSH)**, β-endorphin, and corticotropinlike intermediate lobe peptide. A small amount of ACTH is also produced. Overexpression of these POMC-derived peptides leads to clinical signs of disease through a poorly defined mechanism.

The cause of PPID is not known, but a loss of dopamine in the pars intermedia appears to be critical to the development of disease.[6] Dopamine inhibits expression of POMC in the pars intermedia, and loss of dopamine results in increased expression and plasma concentrations of POMC-derived peptides. Administration of dopamine or dopamine agonists to affected horses results in a decrease in plasma concentrations of POMC-derived peptides, improvements in clinical signs, and improvements of biochemical variables associated with the disease.[7-9] The concentration of dopamine and dopamine metabolites is 9-fold less than in the pars intermedia of horses with PPID, compared with concentrations for age-matched control horses.[6] In another study[10] conducted by our laboratory group, we reported a loss of the periventricular dopaminergic neurons in affected horses, compared with values for young or age-matched control horses.

The inciting factor or factors that initiate neuronal loss observed in horses with PPID are unknown, but analysis of evidence from our laboratory group suggests that oxidative stress may have a role.[10] Immunohistologic examination of pituitary tissues revealed an increase in 3-nitrotyrosine, a marker of oxidative stress, in horses with PPID.[10] Accumulation of 3-nitrotyrosine has been documented in human patients with neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis.[11-13] Accumulation of 3-nitrotyrosine also has been reported in healthy older people.[14] An age-associated increase in 3-nitrotyrosine has been observed in the pars intermedia of horses.[10]

Several potential factors may predispose an animal to neuronal loss secondary to oxidative stress, including environmental (eg, pesticides or chemical toxins)

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and genetic factors. Free radicals are the product of metabolism and inflammation. Therefore, mitochondrial dysfunction, abnormal systemic metabolism, or chronic inflammation can expose an animal to an increased concentration of free radicals. Another risk factor for oxidative stress is a deficiency in antioxidant capacity. Antioxidant capacity represents a combination of enzymatic and nonenzymatic activities that neutralize free radicals, which prevents them from causing additional cellular damage. A genetic deficiency in enzyme activity or a dietary lack of antioxidants may contribute to oxidative damage. Finally, in some animals, neurons may be more susceptible to damage by free radicals. In humans with familial Parkinson's disease, a mutation in the nerve-terminal protein α-synuclein leads to cytoplasmic accumulation of α-synuclein, which can result in death of nigrostriatial dopaminergic neurons.[15-17] Nitrated α-synuclein accumulates more readily and is more neurotoxic than native unaltered α-synuclein.[18] In another study[10] conducted by our laboratory group, we reported that horses with PPID have an increase in the amount of nitrated α-synuclein in the dopaminergic nerve terminals of the pars intermedia, compared with concentrations in healthy horses. It is likely that oxidant exposure, antioxidant deficiency, and susceptibility of neurons all interact during the development of PPID.

We hypothesized that horses with PPID have a deficiency in systemic antioxidant capacity. To test this hypothesis, we measured total glutathione concentration, glutathione peroxidase activity, and total superoxide dismutase activity in RBCs. In addition, we measured the concentration of 3-nitrotyrosine, an oxidative stress marker, in plasma as well as in adrenal gland and substantia nigra tissues. To assess the role of local antioxidant capacity in oxidative damage to the pars intermedia, total glutathione concentration and total superoxide dismutase, manganese superoxide dismutase, and glutathione peroxidase activities were compared to accumulation of 3-nitrotyrosine in pars intermedia tissues.

Materials and Methods

Sample population—Blood, plasma, and tissue samples were used to conduct 3 experiments. Blood and plasma samples were obtained from 20 horses with PPID and 20 matched control horses. In addition, formalin-fixed, paraffin-embedded adrenal gland and substantia nigra tissues were obtained from another 20 horses. Finally, fresh pituitary pars intermedia tissues were collected from 16 additional horses immediately after the horses were euthanatized. All samples were obtained in accordance with guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee.

Experiment 1—To evaluate peripheral antioxidants, 20 horses with clinical signs of PPID were recruited from the population of horses that were referred to the Atlantic Veterinary College. We confirmed PPID in 19 of the horses on the basis of high endogenous plasma concentrations of α-MSH (> 91 pmol/L)[a] and in 5 horses on the basis of results of postmortem histologic examination. Twenty control horses were selected from the population at the same source farms from which the PPID horses originated, when possible. When a control horse was not available from the same farm, an age-matched control horse was selected from a farm with a similar environment. Control horses had no clinical evidence of PPID and had a plasma α-MSH concentration < 45 pmol/L. This concentration was less than the mean value (59.6 pmol/L) reported for clinically normal horses.[a]

Sample collection—Blood samples were collected by jugular venipuncture into evacuated glass tubes containing EDTA. Samples were maintained on ice until processed; all samples were processed within 4 hours after collection. Samples were centrifuged at 800 × g for 10 minutes at 4°C. Plasma was harvested and separated into multiple aliquots that were placed in polypropylene tubes and frozen at –80°C until assayed. The RBCs that remained after removal of the plasma were diluted 50% in saline (0.9% NaCl) solution and frozen at –80°C until assayed.[19]

Measurement of α-MSH concentration—The α-MSH concentrations were measured by use of a commercially available radioimmunoassay[b] designed for use with human plasma. The radioimmunoassay was validated for use in plasma samples obtained from horses.[20]

Blood antioxidant assays—Hemolysates were prepared by mixing packed equine RBCs with ice-cold distilled water at a dilution of 1:9. The lysate was centrifuged at 800 × g for 20 minutes at 4°C. Total superoxide dismutase activity in RBCs was determined in microtiter plates by use of a method described elsewhere.[21] Briefly, hemolysates were cleared by adding 200 μL of 95% ethanol and 130 μL of chloroform to 800 μL of lysate. Samples were mixed thoroughly, then centrifuged at 10,000 × g for 5 minutes at 4°C. Cleared supernatant was diluted 1:5 in phosphate buffer (pH, 8.0) for assay. Ten microliters of the sample was added to triplicate wells, each of which contained 230 μL of PBS solution, 10 μL of 3mM EDTA, and 10 μL of 3mM xanthine. To start the reaction, 20 μL of xanthine oxidase (58 mU/mL) and 20 μL of 0.75mM water-soluble tetrazolium salt[c] were added to each well. Change in absorbance after incubation for 30 minutes at 25°C was measured at 438 nm. Bovine superoxide dismutase was used as a control sample.

Glutathione peroxidase activity in RBCs was determined by measuring the rate of disappearance of the **reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)** as the change of absorbance at 340 nm, as described elsewhere,[22] and modified for use in a microtiter plate.[23] Hemolysates were diluted 1:10 in a solution consisting of 50mM Tris HCl and 5mM EDTA with 1 mg of bovine serum albumin/mL. Ten microliters of sample was added to triplicate wells, each of which contained 155 μL of 0.4mM NADPH, 2mM glutathione, 0.175 units of glutathione reductase in 50mM Tris HCl, and 5mM EDTA (pH, 7.6). Ten microliters of cumene hyroperoxide (2 mg/mL) was added to each well to start the reaction. Plates were analyzed at 340 nm after incubation for 10 minutes at 25°C. Ovine glutathione peroxidase was used as a control sample.

Total RBC glutathione concentration was determined in microtiter plates by use of the technique of Tietze,[24] which was modified slightly. Briefly, hemolysates were diluted 1:20 in 100mM $NaPO_4$ buffer (pH, 8.0) with 1mM EDTA. Fifty microliters of each sample was pipetted in triplicate into microtiter plate wells. One hundred microliters of reaction mixture (100mM $NaPO_4$ buffer [pH, 8.0] with 1mM EDTA, 0.32mM dinitrothiocyanatebenzene, 0.32mM NADPH, and 1.25 U of glutathione reductase/mL) was added to each well, and change in absorbance was measured at 415 nm for 5 minutes.

Hemoglobin concentration in lysates was determined by adding 20 μL of lysate to 5 mL of Drabkin reagent. Lysates were then incubated for 15 minutes at 25°C, and optical density was measured at 540 nm. Hemoglobin concentration of

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samples was calculated by comparison to known concentrations of ovine methemoglobin.

Experiment 2—Systemic oxidative stress was analyzed in the second experiment. Concentrations of 3-nitrotyrosine were determined in plasma samples obtained from the same 20 pairs of horses used in experiment 1. Concentrations of 3-nitrotyrosine were determined by use of a commercially available ELISA.[d] Plasma 3-nitrotyrosine concentrations were measured on 2 separate days, and the mean concentration was calculated and converted to a categoric variable (detectable or nondetectable concentration) for use in statistical analysis.

Immunohistochemical analysis—Formalin-fixed, paraffin-embedded adrenal gland and substantia nigra tissues were obtained from 20 horses. Five of the horses reportedly had clinical signs of PPID. Disease was confirmed during necropsy by the identification of adenomatous hyperplasia or adenoma of the pars intermedia.

Formalin-fixed, paraffin-embedded tissue was mounted on glass slides treated with 3-aminopropyltriethoxysilane diluted in acetone. Slides were placed in xylene to deparaffinize samples and then hydrated by immersion in a graded series of ethanol solutions. Slides were incubated for 5 minutes in 3% hydrogen peroxide to inactivate endogenous peroxidases and digested in trypsin (1 mg/mg) for 10 minutes at 37°C. Slides were incubated in 5% goat serum for 20 minutes at 25°C to reduce nonspecific staining. Incubation with primary antibody was performed by use of 3-nitrotyrosine monoclonal antibody[e] (dilution, 1:100) and incubation for 12 to 24 hours at 4°C, which was followed by incubation with a biotinylated goat anti-mouse IgG secondary antibody (15 µg/mL) for 1 hour at 25°C. Slides were then incubated in an avidin-biotinylated enzyme complex[f] for 30 minutes at 25°C, which was followed by addition of diaminobenzidine and incubation for 8 minutes at 25°C. Slides were dehydrated by immersion in a graded series of ethanol solutions and cleared with xylene, and coverslips were then applied. For sections of pituitary gland tissues, a red chromagen[g] was used instead of diaminobenzadine and slides were counterstained by incubation with hematoxylin for 10 seconds before dehydration and application of a coverslip. This resulted in better visibility of the borders between the lobes of the pituitary gland and other anatomic landmarks, and it aided the ability of investigators to count the fields of view for the pars intermedia. Negative control samples included pituitary sections with positive results but from which the primary antibody was omitted or that were incubated for 24 hours at 4°C with 3-nitrotyrosine[h] before incubation with primary antibody.

After immunohistochemical analysis, the identity of the slides was masked before subsequent evaluation. All fields of view within a section were examined for positively stained cells. The percentage of fields (pars intermedia) or cell bodies (substantia nigra) that had positive staining results for 3-nitrotyrosine was calculated, and results were compared.

Experiment 3—Local antioxidant was evaluated in experiment 3. Fresh pituitary pars intermedia tissues were collected from 16 horses that had been donated to the Atlantic Veterinary College. Two horses had clinical signs of PPID, and the disease was confirmed during necropsy. Pars intermedia tissues were collected and dissected within 15 minutes after the horses were euthanized. Tissues were then washed 3 times in PBS solution and flash-frozen in liquid nitrogen. Samples were stored at –80°C until assayed.

Tissue antioxidant assays—Samples of pituitary pars intermedia tissues were extracted by homogenization in 500 µL of PBS solution, which was followed by centrifugation at 350 X *g* for 20 minutes at 4°C. Protein content of the supernatant was measured[i] and antioxidant assays were performed as described previously. Manganese superoxide dismutase activity was determined by inhibiting copper-zinc superoxide dismutase through the addition of 10 µL of 150mM sodium cyanide to the total superoxide dismutase reaction, as described previously. All results were standardized on the basis of the protein concentration of the sample.

Statistical analysis—All calculations were performed by use of commercial statistical software.[j,k] Normality of the data was confirmed by use of the Kolmogorov-Smirnov test. All results were expressed as mean ± SE. Antioxidant enzyme activities of RBCs were compared by use of a paired *t* test. Plasma concentrations of 3-nitrotyrosine were converted to categoric data (detectable or nondetectable concentrations) and compared by use of χ^2 analysis. The Pearson coefficient of correlation was used to make comparisons between pars intermedia oxidative stress and antioxidant enzyme activities, pars intermedia oxidative stress and substantia nigra oxidative stress, and age and antioxidant activity. Values of $P < 0.05$ were considered significant.

Results

Experiment 1—Breed and sex of the horses from which blood and plasma samples were obtained were summarized. The PPID horses comprised 9 ponies, 6 Quarter Horses, 2 Morgans, 1 Dutch Warmblood, 1 Thoroughbred, and 1 Arabian, whereas the control horses comprised 7 Standardbreds, 4 Quarter Horses, 3 ponies, 1 Morgan, 1 Dutch Warmblood, 1 Arabian, and 3 horses of other breeds. The PPID horses consisted of 9 mares and 11 geldings, whereas the control horses consisted of 13 mares, 6 geldings, and 1 stallion. Mean ± SE age of the control horses (19.4 ± 1.8 years) was significantly ($P = 0.02$) less than that of the horses with PPID (25.8 ± 2 years). This was primarily the result of a wide age difference in 2 pairs of horses because the only available on-farm cohorts were much younger adults (7 vs 34 years and 3 vs 39 years). Results did not differ significantly for statistical comparisons performed for each assay with and without data for the 2

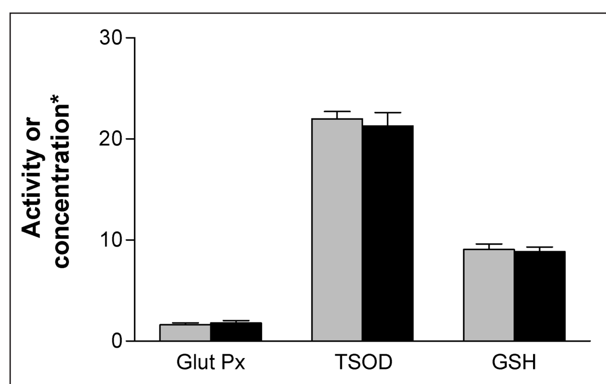

Figure 1—Mean ± SE values for antioxidant enzyme activity in RBCs of 20 horses with pituitary pars intermedia dysfunction (PPID; black bars) and 20 healthy control horses (gray bars). Glutathione peroxidase (Glut Px) activity, total superoxide dismutase (TSOD) activity, and total glutathione (GSH) concentrations were measured per gram of hemoglobin (Hb) in RBC lysates obtained from horses with PPID and control horses. Within each variable, values did not differ significantly ($P < 0.05$) between the 2 groups. *The Glut Px activity is reported as units X 10/g of Hb; TSOD activity is reported as units X 100/g of Hb; and GSH concentration is reported as nmol/g of Hb.

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pairs of horses that differed widely in age; therefore, results for all horses (20 pairs) were reported.

Diagnosis—Mean ± SE α-MSH concentration was markedly higher for the horses with PPID (175.5 ± 21.2 pmol/L), compared with the concentration for the control horses (17.5 ± 2.1 pmol/L). Nine control horses and 7 horses with PPID had their status confirmed during subsequent postmortem histologic examination.

Table 1—Mean plasma 3-nitrotyrosine concentrations* 20 in horses with pituitary pars intermedia dysfunction (PPID) and 20 clinically normal horses.

3-Nitrotyrosine	Clinically normal horses	Horses with PPID
Not detectable	15	13
Detectable		
< 100nM	3	4
100nM–500nM	2	0
> 500nM	0	3
Total†	5	7

*Values represent mean of plasma 3-nitrotyrosine concentrations measured on 2 separate days. †Number of horses with a detectable plasma concentration of 3-nitrotyrosine did not differ significantly (P = 0.49; χ^2 test) between clinically normal horses and horses with PPID.

Results of antioxidant assays—We did not detect significant differences in glutathione peroxidase or superoxide dismutase enzyme activity in RBCs between the control horses and horses with PPID **(Figure 1)**. Similarly, there was no significant difference in total glutathione concentration between the control horses and horses with PPID. Antioxidant activity in RBCs was not correlated with age of horses (data not shown).

Experiment 2—Systemic oxidative stress was evaluated in experiment 2. The number of horses with detectable plasma concentrations of 3-nitrotyrosine did not differ significantly (P = 0.49; χ^2 test) between horses with PPID and control horses **(Table 1)**.

For the immunohistochemical analysis, we did not detect specific staining of 3-nitrotyrosine antibody in the adrenal gland tissues obtained from any of the horses. There was minimal immunoreactivity in the cell bodies of the substantia nigra (≤ 7% of cells had positive results). No correlation was found between the percentage of cell bodies that had positive results for staining with 3-nitrotyrosine in the substantia nigra and oxidative stress in the pars intermedia (r, 0.35; P = 0.11). No correlation was found between the per-

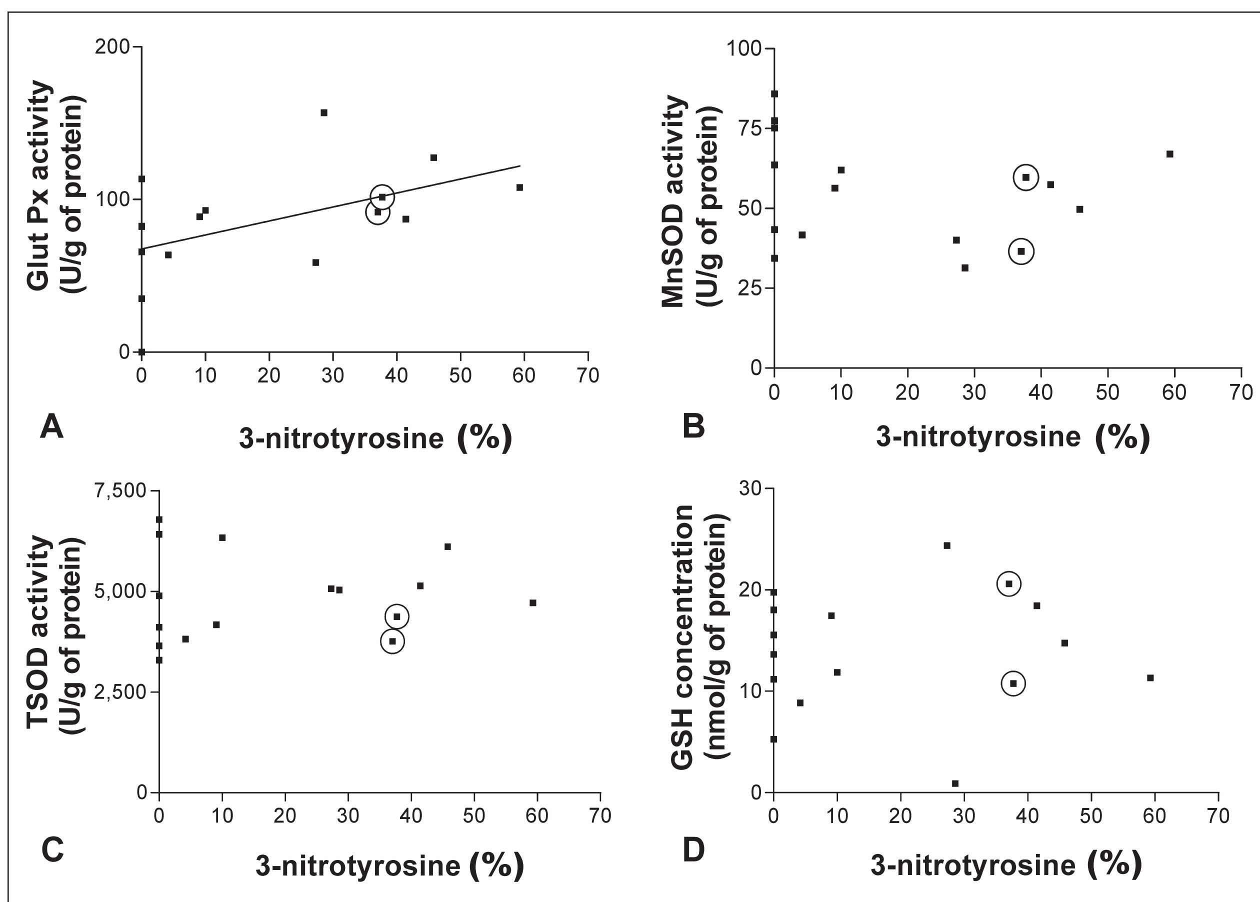

Figure 2—Correlation between Glut Px activity (A), manganese superoxide dismutase (MnSOD) activity (B), TSOD activity (C), or GSH concentration (D) and oxidative stress as measured by the accumulation of 3-nitrotyrosine in the pars intermedia of horses. Each symbol represents results for a clinically normal horse (squares) or a horse with PPID (squares within a circle). Activity for Glut Px increased as the percentage of pars intermedia 3-nitrotyrosine increased (r, 0.51; P = 0.04), whereas MnSOD activity (r, –0.2; P = 0.45), TSOD activity (r, –0.5; P = 0.80), and GSH concentration (r, 0.06; P = 0.84) were not significantly correlated with oxidative stress in the pars intermedia.

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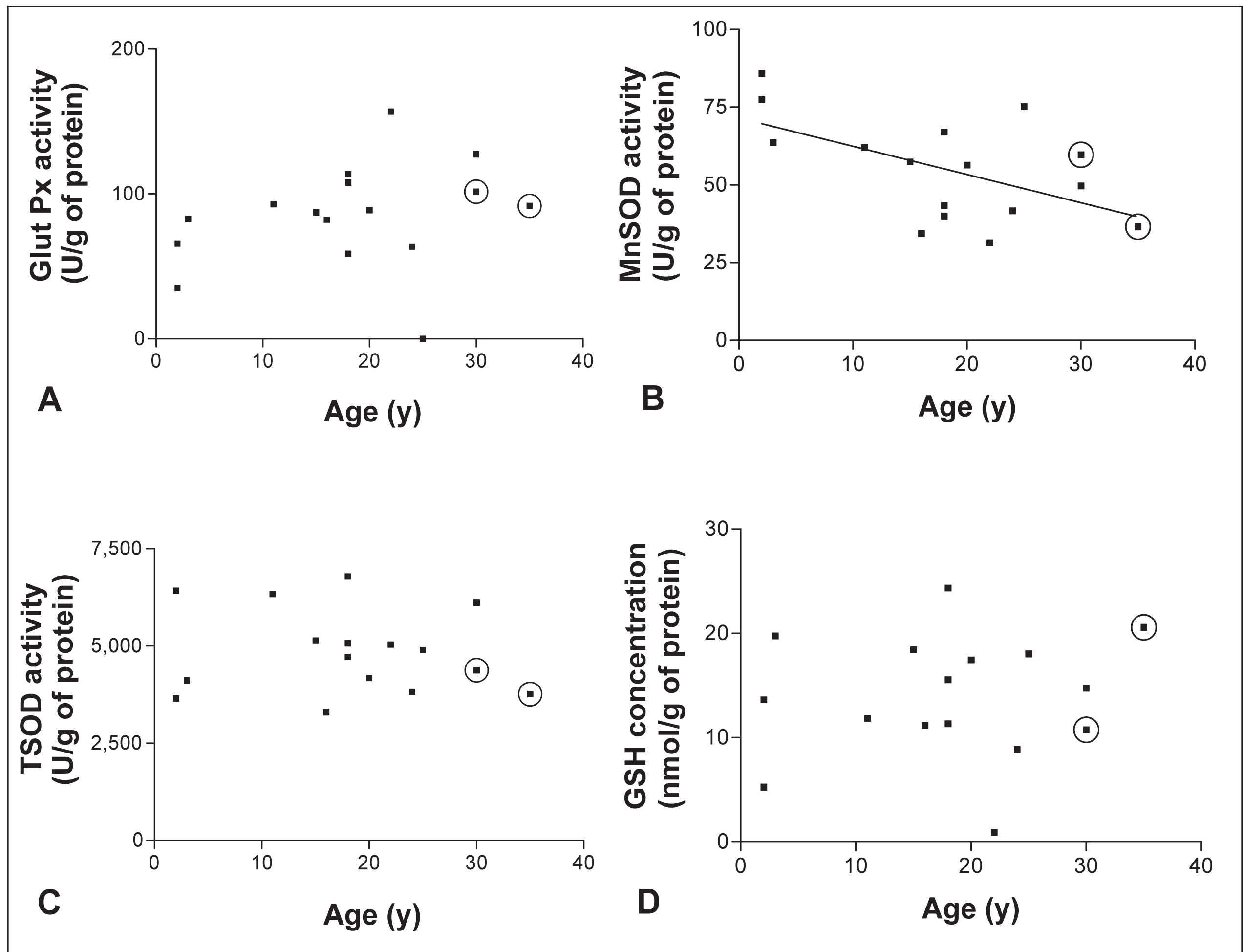

Figure 3—Correlation between Glut Px activity (A), MnSOD activity (B), TSOD activity (C), or GSH concentration (D) in the pars intermedia and age of horses. Each symbol represents results for a clinically normal horse (squares) or a horse with PPID (squares within a circle). Activity of MnSOD was negatively correlated with age (*r*, –0.55; *P* = 0.02), whereas Glut PX activity (*r*, 0.27, *P* = 0.31), TSOD activity (*r*, –0.11; *P* = 0.70), and GSH concentration (*r*, 0.11; *P* = 0.70) were not significantly correlated with age.

centage of cell bodies that had positive results for staining with 3-nitrotyrosine in the substantia nigra and oxidative stress in the pars intermedia when comparing results only for tissues from 5 horses with PPID (*r*, –0.26; *P* = 0.67) or tissues from 15 horses without PPID (*r*, 0.36; *P* = 0.15).

Experiment 3—Local antioxidant was evaluated in the pituitary pars intermedia in experiment 3. Glutathione peroxidase activity increased significantly as 3-nitrotyrosine concentrations (oxidative stress) in the pars intermedia increased (*r*, 0.51; *P* = 0.04; **Figure 2**). There was no correlation between total glutathione, total superoxide dismutase, or manganese superoxide dismutase activity and oxidative stress in the pars intermedia. Manganese superoxide dismutase activity in the pars intermedia decreased significantly with age (*r*, –0.55; *P* = 0.02; **Figure 3**). There was no effect of age on total glutathione concentration or activity of glutathione peroxidase or total superoxide dismutase in the pars intermedia.

Discussion

It is established that environmental and genetic factors may contribute to an individual's risk of disease. The breed-specific increase in the prevalence of PPID in ponies and Morgan horses suggests that there may be genetic factors as well as environmental factors that influence the risk of a horse developing PPID.[1-5] A study[10] conducted by our laboratory group suggested a role for oxidative stress in the pathogenesis of PPID. In that study, 3-nitrotyrosine concentrations were increased in the pars intermedia of affected horses, compared with concentrations for age-matched and young nonaffected horses. A product of nitration of tyrosine by the reactive intermediate peroxynitrite,3-nitrotyrosine is a marker of oxidative stress and can accumulate in tissues of animals with diseases assoc 2-fold increase in 3-nitrotyrosine concentrations, compared with concentrations in nonaffected aged horses, and a 15-fold increase, compared with concentrations in young horses.[10]

Oxidative stress can result from exposure to exogenous or endogenous reactive-oxygen species or from poor antioxidant capacity. Therefore, it is important to consider the impact of environmental factors when assessing risk of developing a disease associated with oxidative damage. In an epidemiologic study,[26]

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investigators reported an increase in the risk of developing Parkinson's disease, a condition associated with oxidative stress and degeneration of the nigrostriatal dopaminergic neurons, with increased exposure to pesticides and herbicides. In studies[27,28] in which animals have been used to investigate Parkinson's disease, exposure to pesticides increases markers of oxidative stress and neurodegeneration. Although it has not been investigated, geographic and farm factors may influence the risk of developing PPID. For example, pesticides, herbicides, and heavy metals in water, soils, or pastures could be a source of exogenous oxidants for grazing animals. Selenium deficiency, which is found in horses fed crops grown in selenium-poor soils, can result in decreased antioxidant capacity in part because of a reduction in glutathione peroxidase activity. A protein-poor diet or heavy parasite burden may also affect an animal's synthesis and homeostasis of glutathione and other antioxidant proteins. Therefore, to minimize the potential impact of environmental factors and their contribution to exposure to free radicals, we prioritized selection of control horses from the same farm from which a horse with PPID originated. When this was not possible, we picked an age-matched horse from a similar environment.

Although horses of all breeds may develop PPID, ponies and Morgan horses are at greater risk.[1-5] We therefore hypothesized that horses with PPID may have a genetic defect leading to a systemic decrease in antioxidant capacity. We chose to measure superoxide dismutase and glutathione peroxidase activities in peripheral blood samples because systemic deficiencies of these 2 enzymes reportedly[29] are associated with dopaminergic neuronal vulnerability and degeneration in animals and in humans with Parkinson's disease. In addition, because catalase is confined to peroxisomes, it is unlikely to have a major role in protecting neurons from exogenous or endogenous exposure to free radicals.[30] In the study reported here, we did not find any difference in superoxide dismutase or glutathione peroxidase activities in peripheral blood samples of PPID-affected horses, compared with activities in clinically normal horses from the same environment (Figure 1). A polymorphism can also occur in the noncoding region of a gene and result in a decrease in the inducible expression of the enzyme or an alteration in tissue specificity of its expression. In this case, in vitro assays of peripheral blood samples would be unlikely to reveal altered enzyme activities. Therefore, we measured antioxidant activity in pituitary pars intermedia tissues.

Antioxidant activity was compared to 3-nitrotyrosine accumulation in pars intermedia tissues obtained from 16 horses. In addition to the antioxidants measured in the peripheral blood samples, we chose to also measure manganese superoxide dismutase activity in the pituitary gland tissue because it is an inducible enzyme whose activity reportedly[30] is decreased in the substantia nigra of patients with Parkinson's disease. Total and manganese superoxide dismutase activities were not correlated with oxidative stress in the pars intermedia (Figure 2). However, glutathione peroxidase activity increased in proportion to the amount of 3-nitrotyrosine in the pars intermedia. Analysis of these data suggested that the accumulation of 3-nitrotyrosine in the pars intermedia was not a function of a deficiency in constitutive superoxide dismutase or glutathione peroxidase expression. In fact, there appears to be local upregulation of glutathione peroxidase activity with oxidative stress. Induction of glutathione peroxidase and manganese superoxide dismutase has been reported[31-33] in cell cultures and in vivo systems in response to oxidative stressors, such as hydrogen peroxide or paraquat. It is possible that the lack of increase in manganese superoxide dismutase activity in association with oxidative damage observed in the study reported here reflects a failure of this enzyme to be appropriately induced by reactive-oxygen species.

Another important cellular antioxidant is the tripeptide, glutathione. Glutathione has the ability to accept electrons and, therefore, to become glutathione disulfide. Together, these 2 comprise the reduction-oxidation system that predominantly determines the antioxidant capacity of cells. Glutathione is manufactured within cells through the action of 2 cytosolic enzymes that use dietary sources of amino acids. Deficiencies in glutathione have been documented[30] in the substantia nigra in patients with Parkinson's disease before there are any clinical manifestations of the disease. Therefore, we measured total glutathione concentration in systemic blood samples as well as in pars intermedia tissues. There was no difference in glutathione concentration in RBCs from horses with PPID, compared with concentrations in clinically normal horses. In addition, glutathione concentration was not correlated with oxidative stress in the pars intermedia.

The antioxidant system is complex and involves a large number of enzymes and nonenzymatic antioxidants beyond those specifically measured in this study. Therefore, we assessed total plasma, adrenal gland, and substantia nigra concentrations of 3-nitrotyrosine as a marker of systemic exposure to free radicals or antioxidant failure. There was no difference in 3-nitrotyrosine accumulation in any tissue examined, except for the pituitary gland, which suggested that PPID is not the result of systemic oxidative stress.

In addition to contributing to the development of disease, oxidative stress is believed to play a role in the physiologic process of aging. Accumulation of oxidative damage is proposed to contribute to age-associated cellular and systemic organ failure.[14] In studies[34-36] of aging in invertebrates and rodents, genetic manipulation that increases antioxidant capacity results in a decrease in incidence of age-related disease as well as clinical manifestations of advanced age, such as frailty. In another study[10] conducted by our laboratory group, we documented an age-associated accumulation of 3-nitrotyrosine in the pars intermedia of horses. In the study reported here, we detected an age-associated decrease in manganese superoxide dismutase activity in the pars intermedia of horses. There was no association between age and systemic blood activity of any antioxidant measured. It is attractive to hypothesize

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that the age-related decrease in manganese superoxide dismutase activity results in chronic accumulation of oxidative damage in the pars intermedia, thereby contributing to increased risk of developing PPID with increases in age. However, we found no association between manganese superoxide dismutase activity and 3-nitrotryosine accumulation in the pars intermedia of horses. Additional studies with larger numbers of horses are necessary to adequately determine the association between age-associated decreases in antioxidant capacity and development of PPID.

The experiments described in this report were designed to address 3 concerns. First, we wanted to determine whether there was a systemic deficiency in antioxidant capacity in horses with PPID. In the study reported here, there was no evidence of systemic accumulation of oxidative stress markers or deficiencies in antioxidant capacity.

Second, we evaluated whether there was a local deficiency in antioxidant capacity that led to oxidative stress in the pars intermedia of horses. For the small number of horses and limited number of antioxidants examined in the study reported here, 3-nitrotyrosine accumulation was not associated with a decrease in antioxidant capacity in the pars intermedia. On the other hand, glutathione peroxidase activity increased with oxidative stress. It is possible that manganese superoxide dismutase should also have been induced, and the lack of response may have contributed to oxidative damage.

Finally, we examined whether there was evidence of an age-related decrease in systemic or local antioxidant capacity in horses. Although systemic antioxidant capacity was maintained during aging in the horses of the study reported here, manganese superoxide dismutase activity in the pars intermedia decreased in an age-dependent manner and therefore may contribute to the increased risk of developing PPID observed with an increase in age.

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a. Horowitz ML, Neal L, Watson JL. Characteristics of plasma adrenocorticotropin, β-endorphin and α-melanocyte stimulating hormone as diagnostic tests for pituitary pars intermedia dysfunction in the horse (abstr). *J Vet Intern Med* 2003;17:386.
b. Euria–α-MSH RIA, American Laboratory Products Co, Windham, NH.
c. WST-1, Dojindo Laboratories, Kumamoto, Japan.
d. Nitrotyrosine ELISA, Hycult Biotechnology b.v., Uden, The Netherlands.
e. Nitrotyrosine monoclonal antibody, Cayman Chemicals, Ann Arbor, Mich.
f. Vectastain Elite ABC kit, Vector Laboratories, Burlington, ON, Canada.
g. Nova Red, Vector Laboratories, Burlington, ON, Canada.
h. Nitrotyrosine, Cayman Chemicals, Ann Arbor, Mich.
i. Biorad DC protein assay, Biorad Laboratories, Hercules, Calif.
j. Minitab, version 13.32, Minitab Inc, State College, Pa.
k. GraphPad Prism, version 3.03, GraphPad Software, San Diego, Calif.

References

1. Hillyer MH, Taylor FRG, Mair TS, et al. Diagnosis of hyperadrenocortism in the horse. *Equine Vet Educ* 1992;4:131–134.
2. Love S. Equine Cushing's disease. *Br Vet J* 1993;149:139–153.
3. van der Kolk JH, Kalsbveek HC, van Garderen E, et al. Equine pituitary neoplasia: a clinical report of 21 cases (1990–1992). *Vet Rec* 1993;133:594–597.
4. van der Kolk JH, Wensing T, Kalsbeek HC, et al. Laboratory diagnosis of equine pituitary pars intermedia adenoma. *Domest Anim Endocrinol* 1995;12:35–39.
5. Schott HC. Pituitary pars intermedia dysfunction: equine Cushing's disease. *Vet Clin North Am Equine Pract* 2002;18:237–270.
6. Millington WR, Dybdal NO, Dawson R, et al. Equine Cushing's disease: differential regulation of β-endorphin processing in tumors of the intermediate pituitary. *Endocrinology* 1988;123:1598–1604.
7. Donaldson MT, LaMonte BH, Morresey P, et al. Treatment with pergolide or cyproheptadine of pituitary pars intermedia dysfunction (equine Cushing's disease). *J Vet Intern Med* 2002;16:742–746.
8. Orth DN, Holscher MA, Wilson MG, et al. Equine Cushing's disease: plasma immunoreactive proopiolipomelanocortin peptide and cortisol levels basally and in response to diagnostic tests. *Endocrinology* 1982;110:1430–1441.
9. Schott HC, Coursen CL, Eberhart SW, et al. The Michigan Cushing's Project, in *Proceedings*. 47th Annu Conv Am Assoc Equine Pract 2001;47:22–24.
10. McFarlane D, Dybdal N, Donaldson MT, et al. Nitration and increased alpha-synuclein expression associated with dopaminergic neurodegeneration in equine pituitary pars intermedia dysfunction (PPID). *J Neuroendocrinol* 2005;17:73–80.
11. Calabrese V, Bates TE, Stella AM. NO synthase and NO-dependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. *Neurochem Res* 2000;25:1315–1341.
12. Gilgun-Sherki Y, Melamed E, Offen D. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology* 2001;40:959–975.
13. Luo Y, Roth GS. The roles of dopamine oxidative stress and dopamine receptor signaling in aging and age-related neurodegeneration. *Antioxid Redox Signal* 2000;2:449–460.
14. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of aging. *Nature* 2000;408:239–247.
15. Farrer M, Maraganore DM, Lockhart P, et al. α-Synuclein gene haplotypes are associated with Parkinson's disease. *Hum Mol Genet* 2001;10:1847–1851.
16. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* 1997;276:2045–2047.
17. Saha AR, Ninkina NN, Hanger DP, et al. Induction of neuronal death by α-synuclein. *Eur J Neurosci* 2000;12:3073–3077.
18. Giasson BI, Duda JE, Murray IV, et al. Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. *Science* 2000;290:985–989.
19. Marshall MD, Wattam SL, Skinner ND, et al. Feline antioxidant enzyme activity: the effect of sample storage on stability. *J Nutr* 2002;132:1733S–1734S.
20. McFarlane D, Donaldson MT, McDonnell SM, et al. Effects of season and sample handling on measurement of plasma α-melanocyte-stimulating hormone concentrations in horses and ponies. *Am J Vet Res* 2004;65:1463–1468.
21. Peskin AV, Winterbourn CC. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clin Chim Acta* 2000;293:157–166.
22. Tappel AL. Glutathione peroxidase and hydroperoxides. *Methods Enzymol* 1978;52:506–513.
23. Smith AD, Levander OA. High-throughput 96-well microplate assays for determining specific activities of glutathione peroxidase and thioredoxin reductase. *Methods Enzymol* 2002;347:113–121.
24. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502–522.
25. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res* 1999;43:562–571.
26. Priyadarshi A, Khuder SA, Schaub EA, et al. Environmental risk factors and Parkinson's disease: a metaanalysis. *Environ Res* 2001;86:122–127.
27. Gillette JS, Bloomquist JR. Differential up-regulation of striatal dopamine transporter and alpha-synuclein by the pyrethroid insecticide permethrin. *Toxicol Appl Pharmacol* 2003;192:287–293.
28. Sherer TB, Betarbet R, Stout AK, et al. An in vitro model of

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Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci* 2002;22:7006–7015.

29. Kalra J, Rajput AH, Mantha SV, et al. Serum antioxidant enzyme activity in Parkinson's disease. *Mol Cell Biochem* 1992;110:165–168.

30. Schulz JB, Lindenau J, Seyfried J, et al. Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem* 2000;267:4904–4911.

31. Kunikowska G, Jenner P. Alterations in m-RNA expression for Cu,Zn-superoxide dismutase and glutathione peroxidase in the basal ganglia of MPTP-treated marmosets and patients with Parkinson's disease. *Brain Res* 2003;968:206–218.

32. Krall J, Bagley AC, Mullenbach GT, et al. Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *J Biol Chem* 1988;263:1910–1914.

33. Bianchi A, Becuwe P, Franck P, et al. Induction of MnSOD gene by arachidonic acid is mediated by reactive oxygen species and p38 MAPK signaling pathway in human HepG2 hepatoma cells. *Free Radic Biol Med* 2002;32:1132–1142.

34. Martin GM, Austad SN, Johnson TE. Genetic analysis of ageing: role of oxidative damage and environmental stresses. *Nat Genet* 1996;13:25–34.

35. Aigaki T, Seong KH, Matsuo T. Longevity determination genes in *Drosophila melanogaster. Mech Ageing Dev* 2002;123:1531–1541.

36. Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. *Nature* 2000;408:255–262.

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