Antioxidant Enzyme Activities and Mitochondrial Fatty Acids in Pulmonary Hypertension Syndrome (PHS) in Broilers[1]

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ABSTRACT Major objectives of this study were to assess antioxidant protection and fatty acid profile in lung mitochondria and whole liver in broilers with pulmonary hypertension syndrome [(PHS; with and without high dietary vitamin E (VE)] (Experiment 1) and in broilers that did not develop PHS but were genetically selected (S) or not selected (NS) for resistance to PHS (Experiment 2). In Experiment 1, lung mitochondrial glutathione peroxidase (GSH-Px) activity was elevated in broilers with PHS compared to controls, broilers fed high VE, and broilers fed high VE with PHS (VE-PHS), but there were no differences in GSH reductase (GSH-Rd) among groups. In liver tissue, GSH-Px was also elevated by PHS but was lower in VE and VE-PHS groups than in controls. There were no differences in liver GSH-Rd, superoxide dismutase (SOD), or γ-glutamylcysteine synthetase (γ-GCS) activities with the exception that γ-GCS was higher in the VE-PHS group than in the other groups. In Experiment 2, S lung mitochondria exhibited lower GSH-Px and higher GSH-Rd compared to NS broilers. In the liver, there were no differences in GSH-Px, GSH-Rd, or γ-GCS, but SOD was lower in S compared to the NS broilers. High VE increased the percentage of saturated fatty acids and decreased the percentage of unsaturated fatty acids in lung mitochondria in Experiment 1; there were no differences in fatty acid content between S and NS mitochondria in Experiment 2. Thus, it appears that GSH recycling enzyme activities are affected by PHS and high VE presumably in response to differences in oxidative stress and that genetic resistance to PHS is associated with an inherently better capability to metabolize oxidants in lung mitochondria. The increase in saturation of lung mitochondrial fatty acids with high dietary VE would presumably make them more resistant to oxidative stress and, thus, reduce the level of PHS-induced oxidative stress.

(*Key words*: pulmonary hypertension syndrome, antioxidant enzyme, vitamin E mitochondria, liver, lung)

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INTRODUCTION

Oxidative stress occurs when forces that favor oxidation outweigh antioxidant protection within cells (Yu, 1994). The involvement of oxidative stress in pulmonary hypertension syndrome (PHS), also known as ascites, in broilers has now been clearly demonstrated by findings that broilers with PHS exhibit increases in oxidized glutathione (GSSG), or the ratio of oxidized to reduced glutathione (GSSG/GSH) in tissue, increases in plasma lipid peroxides, and decreases in major nonenzymatic antioxidants such as GSH, α-tocopherol, and ascorbic acid in liver and lung (Enkvetchakul et al., 1993; Bottje and Wideman, 1995; Bottje et al., 1995, 1997).

A major cellular source of oxidative stress in cells occurs within mitochondria due to incomplete reduction of oxygen to reactive oxygen species (ROS) (e.g., superoxide) (Chance et al., 1979). Increased mitochondrial ROS production (Maxwell et al., 1996; Cawthon et al., 2001; Iqbal et al., 2001a) may be responsible for much of the oxidative stress observed in PHS. In cells and in mitochondria, several enzymes work in concert to catabolize ROS to prevent oxidation of critical cellular components (e.g., proteins, lipids, DNA). Figure 1 provides an overview of relationships of enzymes investigated in the present study. Superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide that is in turn reduced by GSH peroxidase (GSH-Px) to water. The reduction of hydrogen peroxide by GSH-Px uses reducing equivalents of GSH and results in formation of glutathi-

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Abbreviation Key: γ-GCS = γ-glutamylcysteine synthetase; GSH = reduced glutathione; GSH-Px = glutathione peroxidase; GSH-Rd = glutathione reductase; GSSG = oxidized glutathione; NS = not selected for resistance to PHS; PHS = pulmonary hypertension syndrome; RV:TV = right to total ventricular weight ratio; S = selected for resistance to PHS; SOD = superoxide dismutase; VE = vitamin E.

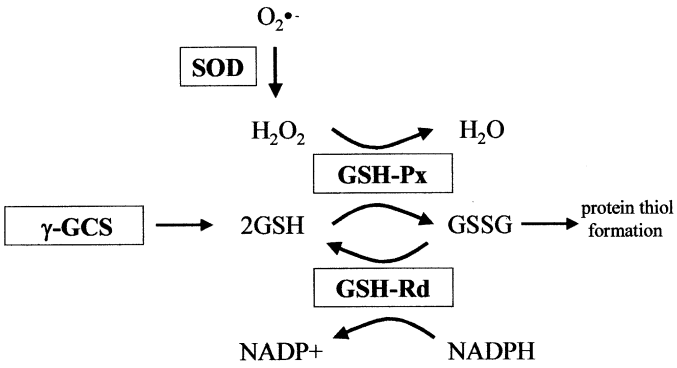

FIGURE 1. Relationships among antioxidant enzymes investigated in this study. Superoxide ($O_2^{\bullet-}$) is dismutated to H_2O_2 by superoxide dismutase (SOD). Glutathione peroxidase (GSH-Px) reduces H_2O_2 (and other peroxides) to water (and lipid alcohols) using reduced glutathione (GSH). Oxidized glutathione (GSSG) is reduced back to GSH by GSH reductase (GSH-Rd) using reducing equivalents from NADPH. The reduction of GSSG helps prevent oxidation of proteins (protein thiol formation). Whereas SOD, GSH-Px, and GSH-Rd are found in mitochondria and cytosol, the γ-glutamylcysteine synthetase (γ-GCS; rate-limiting enzyme in GSH synthesis) is found only in the cytosol. (Adapted from Bottje and Wideman, 1995).

one disulfide (oxidized glutathione, GSSG). The GSSG is reduced back to GSH by GSH reductase (GSH-Rd) with reducing equivalents from NADPH. The activity of GSH-Rd is critical in maintaining low levels of GSSG, thereby preventing protein sulfhydryl formation and oxidative protein modification. Unlike cells, mitochondria lack the ability to export GSSG to prevent protein oxidation, (Olafsdottir and Reed, 1988). Therefore, GSH-Rd activity is particularly important in preventing protein oxidation in mitochondria.

Reduced GSH is present in millimolar levels in most cells and is one of the most important cellular antioxidants. Thus, an additional enzyme of importance in determining the antioxidant capacity of cells is γ-glutamyl cysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis (Meister, 1984) (Figure 1). As mitochondria lack γ-GCS, mitochondrial GSH levels depend upon cytosolic GSH levels and on the import of GSH through mitochondrial pores (Griffith and Meister, 1985). Consequently, γ-GCS activity can be profoundly important to the antioxidant capacity of cytosol and mitochondrial compartments of the cell. As the liver is important in interorgan circulation of GSH in mammals and birds (Anderson et al., 1980; Wang et al., 1998), hepatic γ-GCS could be particularly important in overall GSH homeostasis.

Oxidative stress changes activities of antioxidant enzymes. We have recently reported mitochondrial dysfunction in PHS characterized by increased radical production and oxidative stress (Iqbal et al., 2001a,b). High levels of dietary α-tocopherol attenuated dysfunction as well as oxidative stress. Therefore, a major objective of this study was to extend these findings by determining activities of GSH-Px and GSH-Rd in lung mitochondria and γ-GCS and SOD in liver tissue obtained from broilers, with or without PHS, fed normal or elevated levels of α-tocopherol. Genetic resistance to PHS is associated with lower oxidative stress and improved mitochondrial function (Iqbal et al., 2001a,b). Thus, a second objective was to determine activities of antioxidant enzymes in mitochondria and liver tissue obtained from broilers that were genetically selected (S) or not selected (NS) for resistance to PHS. As mitochondrial fatty acids can influence mitochondrial function (Clejan et al., 1980) and can be altered by high dietary VE (Barja et al., 1996), a final objective was to determine the effects of PHS and VE on the fatty acid profile of the lung mitochondria in both experiments.

MATERIALS AND METHODS

Birds and Management

In Experiment 1, 100 male broiler chicks (Cobb 500)[3] obtained from a local hatchery[4] were placed in an environmental chamber (8 m^2 floor space) on wood shavings litter. Birds were provided access ad libitum to water and to a diet (23.7% protein; 3,200 kcal ME) supplemented with 15 (normal) or 100 IU dl-α-tocopherol acetate[5] (vitamin E, VE) per kg. Chamber temperatures were 32 and 30 C during Weeks 1 and 2, respectively, lowered to 15 C during Week 3, and maintained between 10 and 15 C for the rest of the study. The cool temperatures combined with high protein feed to support rapid growth rate have been shown to induce a high incidence of PHS (Wideman et al., 1995). Upon completion, the study was replicated using identical conditions.

In Experiment 2, lung mitochondria and liver tissue were obtained from male broilers[6] that had been genetically selected for resistance to PHS (Wideman and French, 1999, 2000). These birds were third-generation progeny of breeding stock that did not develop PHS following unilateral pulmonary arterial occlusion. The birds were provided the standard dietary VE level (15 IU dl-α-tocopherol acetate/kg) and water ad libitum and maintained under the environmental conditions described above.

Sampling Procedure

In Experiment 1, birds were randomly selected that exhibited overt PHS symptoms (e.g., systemic cyanosis of the comb, wattle, and skin or abdominal fluid accumulation) or appeared clinically healthy (i.e., no cyanosis) as previously described (Cawthon et al., 1999). The birds were weighed and killed with an overdose of sodium pentobarbital by i.v. injection into the wing vein. Both lungs were removed and lung mitochondria were obtained by differential centrifugation according to Iqbal et al. (2001a). A portion of the liver was obtained, frozen in liquid N, and stored at –80 C. The right ventricular weight

[3]Cobb Vantress, Siloam Springs, AR 74364.
[4]Randall Road, Tyson, Springdale, AR 72762.
[5]Roche Vitamins, Inc., Nutley, NJ 07110.
[6]Hubbard Farms, Walpole, NH 03608.

ratio (RV:TV), a sensitive indicator of prior exposure of the heart to elevated pulmonary arterial pressure, was calculated from the right (RV) and total (TV) ventricular weights (Burton et al., 1968). Birds with an RV:TV ≥ 0.30 were classified as having PHS, whereas those with an RV: TV ratio ≤ 0.27 that did not have abdominal or pericardial fluid were classified as non-PHS birds. In the 2 × 2 factorial design, the four treatment groups consisted of the two dietary treatments, control (n = 7), and high dietary vitamin E (VE; n = 8) for healthy non-PHS birds, and PHS (n = 8) or VE-PHS (n = 7), for birds with PHS. The higher RV:TV in PHS and VE-PHS groups confirmed the presence of prolonged pulmonary arterial hypertension in these groups (Burton et al., 1968). Mortality from PHS was not monitored in Experiment 1.

In Experiment 2, birds without PHS were randomly chosen from NS (n = 4) and S (n = 4) for resistance to PHS. These birds were raised under cold conditions and sampled between 49 and 57 d of age after 4 wk of cold temperatures (as opposed to continuous sampling between 21 and 50 d of age in Experiment I). The mortality from PHS in S and NS broilers after 4 wk of cold exposure was 5 and 38%, respectively (Wideman and French, 2000). However, as birds used in Experiment 2 did not exhibit symptoms of PHS, they would likely be the most resistant to PHS within their respective populations.

Biochemical Analyses

Enzymatic Antioxidants. Antioxidant enzyme activities were determined in lung mitochondria and whole liver tissue. Isolated lung mitochondria were suspended in a buffered mannitol-sucrose solution, pH 7.3 (Iqbal et al., 2001a,b). Liver tissue (200 mg) was homogenized in ice-cold 1% Triton X-100 in 0.01 *M* phosphate-buffered saline solution, pH 7.2, and filtered through double-layered cheesecloth according to Asikainen et al. (1998). Protein levels in lung mitochondria and liver homogenates were measured using a protein detection kit.[7] Enzyme activities were detected spectrophotometerically using a microplate reader (Power Wave X)[8] and a 96-well microplate.[9] Values for each enzyme were corrected for path length of the 96-well microplate and expressed as units of activity per milligram of protein.

Glutathione Peroxidase Activity. The activity of GSH-Px was determined according to Lawrence and Burk (1976) with modifications. The activity was assayed by following the oxidation of NADPH at 340 nm for 5 min (25 C) in the presence of GSH-Rd and GSH. The isolated lung mitochondria or liver homogenate (50 μL) was incubated with 25 m*M* potassium phosphate; 0.5 m*M* EDTA, pH 7.4; 0.5 m*M* $NaNO_3$; 0.3 m*M* NADPH; 0.64 U GSH-Rd; and 1 m*M* GSH[10] (final volume of 140 μL). The reaction was started with 0.1 m*M* hydrogen peroxide. Values were corrected for nonenzymatic oxidation of GSH and NADPH by hydrogen peroxide. The results were expressed in units of GSH-Px activity using a molar extinction coefficient of 6.22×10^{-3} μmol × cm^{-1} for NADPH (Lawrence and Burk, 1976). The unit was defined as the oxidation of 1 μmol of NADPH/min.

GSH-Rd Activity. The activity of GSH-Rd was assayed according to Tabatabaie and Floyd (1994) with modifications. Briefly, lung mitochondrial or liver tissue homogenate samples (50 μL) were incubated for 5 min at room temperature with 36 m*M* potassium phosphate; 2 m*M* EDTA, pH 7.4; 0.1 m*M* NADPH; and 0.3 m*M* GSSG (final volume of 140 μL). The reaction was initiated by adding NADPH (0.1 m*M*). The activity of GSH-Rd, represented as the rate of NADPH disappearance, was determined by following absorbance at 340 nm for 5 min (25 C) based on the molar extinction coefficient of NADPH. One unit of the GSH-Rd was defined as the oxidation of 1 μmol of NADPH/min.

γ-GCS Activity. Liver γ-GCS activity was determined following the methods of Seelig and Meister (1984) with modifications. Briefly, liver homogenates (40 μL) were incubated for 5 min at 25 C with 47 m*M* Tris-HCl, pH 8.2; 23 m*M* KCl; 12 m*M* $MgCl_2$; 2 m*M* L-glutamate; 5 m*M* amino-L-butyrate; 0.6 m*M* NADH; 1 m*M* ATP; 0.1 m*M* EDTA; 2 U pyruvate kinase; and 2 U lactate dehydrogenase (final volume of 160 μL). The reaction was initiated by adding phosphoenol pyruvate (1 m*M*). The decrease in optical density by stochiometric oxidation of NADH due to reduction of pyruvate was measured at 340 nm for 5 min. The lactate dehydrogenase used in this assay was determined to be free of pyruvate kinase activity. The results were expressed in units of activity using a molar extinction coefficient of 6.22×10^{-3} μmol × cm^{-1}. The unit was defined as the oxidation of 1 μmol of NADH/min.

SOD Activity. Total liver SOD activity was determined following the xanthine oxidase-cytochrome c method described by McCord and Fridovich (1969) with modifications. Briefly, liver homogenate (60 μL) was incubated for 5 min at 25 C with 20 m*M* potassium phosphate; 1 m*M* EDTA, pH 7.8; 0.8 m*M* xanthine; and 0.17 m*M* cytochrome c (final volume 150 μL). The reaction was initiated by adding xanthine oxidase (0.16 U) and was assayed by following the reduction of cytochrome c at 550 nm for 5 min (25 C) in the presence or absence of xanthine oxidase and SOD. The results were expressed as units of activity (U/mg protein). One unit of the activity was defined as the amount of SOD that inhibited the rate of cytochrome c reduction by 50% (Iqbal and Whitney, 1991).

Nonenzymatic Antioxidants

Glutathione and Tocopherols. Reduced (GSH) and oxidized (GSSG) glutathione (lung mitochondria only) were measured by HPLC according to Fariss and Reed (1987). Analysis of α- and γ-tocopherol in lung mitochon-

[7]Sigma kit # 610-A, Sigma Diagnostics, St. Louis, MO 63178-9916.
[8]Bio-Tek Instruments, Inc., Wonooski, VT 05404-0998.
[9]Nunc, VWR Scientific Products Corp., West Chester, PA 19380.
[10]Sigma Chemicals Co., St. Louis, MO 63178-9916.

dria and liver tissue was also by HPLC according to Warren and Reed (1991). A complete description of modifications of HPLC methods used in the present study has been previously described (Bottje et al., 1995; Iqbal et al., 2001b).

Fatty Acid Analysis

The HPLC mobile phase obtained from tocopherol analysis above was evaporated under N and redissolved in 0.5 mL benzene. The benzene-dissolved samples (150 μL) were placed into autosampler vials with 50 μL of Meth Prep II,[11] and after 2 h, each sample was analyzed for fatty acids by gas chromatography. The fatty acids analyzed were 18:0 (stearic acid), 18:1, (oleic acid, Δ^9), 18:2 (linoleic acid, $\Delta^{9,12}$), 18:3 (γ-linolenic acid, $\Delta^{6,9,12}$), 20:0 (arachidic acid), and 20:3 (eicosatrienoic acid or dihomo- γ linolenic acid, $\Delta^{8,11,14}$). The percentages of saturated fatty acids, unsaturated fatty acids, and the ratio of saturated to unsaturated fatty acids were calculated.

Statistical Analyses

Data are presented as the mean ± SEM. The data of the first experiment were analyzed using a 2 × 2 factorial design. Selection of birds from each treatment group during each week of the study was randomized. There were no main effects due to age or replicate in Experiment 1. The data in Experiment 2 were analyzed using *t*-tests. The above statistical analyses were accomplished using the general linear models procedure of SAS® software (SAS Institute, 1996). A probability level of $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The antioxidant status of a cell or tissue is dependent upon a variety of factors that include the presence of a myriad of nonenzymatic and enzymatic antioxidants as well as forces that favor oxidation (Yu, 1994). Several studies indicate that oxidative stress is involved in the pathophysiology of PHS (Enkvetchakul et al., 1993; Bottje and Wideman, 1995; Bottje et al., 1995, 1997). It is also now apparent that mitochondria are a major source of oxidative stress in PHS due to the enhanced generation of ROS (Maxwell et al., 1996; Cawthon et al., 2001; Iqbal et al., 2001a,b; Tang et al., 2000). The results of the present study extend the findings of these studies by documenting differences in enzymatic antioxidant activities in lung mitochondria and the liver (1) in response to PHS in combination with high levels of dietary VE and (2) in broilers genetically resistant to PHS.

In Experiment 1, broilers were subjected to cold temperatures from 3 to 7 wk of age to induce PHS. The right ventricular weight ratios for control, PHS, VE, and VE-PHS groups were 0.23 ± 0.02, 0.41 ± 0.03, 0.23 ± 0.02, and 0.38 ± 0.02, respectively (n = 6 to 8 per group). In Experiment 2, measurements were obtained from broilers between 7 and 8 wk of age that did not exhibit symptoms of PHS after having been exposed to cold temperatures for 4 wk. The RV:TV for NS and S birds were 0.22 ± 0.02 and 0.23 ± 0.02, respectively (n = 4 per group).

Nonenzymatic and Enzymatic Antioxidants

Oxidative stress was clearly evident in lung mitochondria obtained from broilers with PHS in Experiment 1 as indicated by lower levels of GSH, α- and γ-tocopherol and a higher GSSG/GSH ratio than in controls (Table 1). High dietary VE raised α-tocopherol levels in VE and VE-PHS lung mitochondria and liver tissue by two- to threefold when compared to controls. High dietary VE had no effect on GSH and GSSG in healthy broilers (VE group) but did attenuate oxidative stress in VE-PHS lung mitochondria as indicated by the lower GSSG/GSH compared to PHS lung mitochondria. Levels of γ-tocopherol in lung mitochondria and liver tissue were depressed in PHS and VE-PHS compared to control and VE groups (Table 1). These findings concur with a report by Barja et al. (1996) in which dietary VE raised liver α-tocopherol in a dose-dependent manner but did not eliminate cytosolic oxidative stress. The inability of VE to prevent cytosolic oxidative stress in the VE-PHS group may partially explain why high dietary VE is not always effective in attenuating PHS mortality (Dale and Villacres, 1986; Bottje et al., 1995, 1997). In Experiment 2, despite similar levels of mitochondrial α- and γ-tocopherols, higher oxidative stress (GSSG/GSH) was observed in NS mitochondria (Table 1). Thus, it appears that there is an inherently greater degree of oxidative stress associated with the aqueous component, but not the lipid antioxidant component, in lung mitochondria obtained from NS broilers.

As antioxidant enzyme activity may be important in combating oxidative stress in lung mitochondria of broilers with PHS (Iqbal et al., 2001b), the activities of antioxidant enzymes were measured in lung mitochondria (GSH-Px and GSH-Rd only) and whole liver homogenate (GSH-Px, GSH-Rd, SOD, and γ-GCS). Based on the GSH and GSSG analyses in lung mitochondria outlined above, the determination of GSH-Px and GSH-Rd activities were given first priority. Unfortunately, upon completing analysis of the GSH recycling enzymes, and analysis of GSH, GSSG, and tocopherols (above), an insufficient amount of isolated lung mitochondria remained for the SOD analysis. γ-GCS is a cytosolic enzyme (Meister, 1984) and would not be expected to be present in mitochondrial isolates. However, hepatic γ-GCS activity, the rate-limiting enzyme in GSH synthesis (Meister, 1984), is vitally important in maintaining extra-hepatic GSH levels, as the liver exports GSH into the general circulation for uptake by extra-hepatic tissues (Anderson et al., 1980; Wang et al., 1998).

In Experiment 1, lung mitochondria isolated from broilers with PHS exhibited higher GSH-Px activity, but there were no differences in GSH-Rd activity among treatment

[11]Alltech Associates, Inc, Deerfield, IL 60015-1899.

TABLE 1. Glutathione and tocopherol analyses of lung mitochondria and liver tissue isolated from the broilers fed with or without high dietary vitamin E (Experiment 1) and from broilers with resistance to pulmonary hypertension syndrome (Experiment 2)[1]

	Treatment groups[2]					
	Experiment 1				Experiment 2	
Variables[3]	Control (n = 6 to 7)	PHS (n = 5 to 8)	VE (n = 6 to 8)	VE-PHS (n = 7)	S (n = 4)	NS (n = 4)
Glutathione						
Lung mitochondria	(nmol/mg protein)					
GSH	13.7 ± 1.4[a]	6.8 ± 2.5[b]	12.0 ± 1.1[a]	7.3 ± 1.6[b]	16.8 ± 0.1	16.1 ± 0.6
GSSG	1.2 ± 0.3	1.4 ± 0.2	1.7 ± 0.3	1.5 ± 0.3	2.2 ± 0.5[b]	5.5 ± 0.7[a]
GSSG/GSH	0.09 ± 0.02[c]	0.32 ± 0.10[a]	0.15 ± 0.03[b]	0.18 ± 0.03[b]	0.15 ± 0.04[b]	0.33 ± 0.05[a]
Tocopherols						
Lung mitochondria	(pmol/mg protein)					
α-	6.9 ± 0.4[c]	4.8 ± 0.4[d]	24.4 ± 1.2[a]	14.8 ± 2.0[b]	7.2 ± 0.2	7.2 ± 0.4
γ-	1.8 ± 0.1[a]	0.9 ± 0.1[b]	1.3 ± 0.1[a]	0.5 ± 0.1[b]	2.6 ± 0.2	3.3 ± 0.1
Liver Tissue						
α-	95 ± 6[b]	39 ± 8[c]	319 ± 38[a]	165 ± 54[b]	54 ± 9	74 ± 14
γ-	23 ± 2[a]	6 ± 2[c]	14 ± 3[b]	5 ± 3[c]	17 ± 3	21 ± 5

[a-c]Means in the same row and experiment with no common superscripts differ significantly (P < 0.05).

[1]All values are the means ± SEM.

[2]PHS= pulmonary hypertension syndrome; VE= high dietary vitamin E (no PHS); VE-PHS = high dietary vitamin E with PHS; S = birds selected and NS = birds not selected for resistance to pulmonary hypertension syndrome and fed only with control feed.

[3]GSH = reduced glutathione; GSSG = oxidized glutathione; GSSG/GSH = oxidized to reduced glutathione ratio.

groups (Figure 2A). Higher GSH-Px activity in PHS lung mitochondria could be due to upregulation of the expression of this enzyme and an important adaptive response to greater hydrogen peroxide production as a result of electron leakage from the respiratory chain (Iqbal et al., 2001a). Although enzyme expression was not investigated in the present study, several studies have reported upregulation of GSH-Px in response to oxidative stress in vascular endothelial, tracheobronchial epithelial, and leukemia cells (Shull et al., 1991; Lu et al., 1993; Lee and Um, 1999). In whole liver homogenates, a main effect of dietary VE was evident in that GSH-Px activity was lower in the VE and VE-PHS groups compared to control and PHS values (Figure 2B). As observed in lung mitochondria, there were no differences in liver GSH-Rd activity among groups (Figure 2B). There were no differences in liver SOD activity among the treatment groups (Figure 2C). Liver γ-GCS activity was inexplicably elevated in the VE-PHS group compared to the other treatment groups in Experiment 1 (Figure 2D).

The mechanism by which VE exerts an effect on activities of GSH-Px and γ-GCS is not clearly understood. Variable

TABLE 2. Relative amount of fatty acids in the mitochondria isolated from the lungs of broilers fed with or without high dietary vitamin E (VE), with or without pulmonary hypertension syndrome (PHS), and the main effect of VE in Experiment 1[1]

	Groups[3]				VE Main effect	
Fatty acid[2]	Control (n = 7)	PHS (n = 7)	VE (n = 8)	VE-PHS (n = 7)	VE (n = 15)	No VE (n = 14)
	(%)					
18:0	45.7 ± 1.3[b]	46.9 ± 0.4[ab]	48.1 ± 0.3[a]	48.8 ± 0.7[a]	48.5 ± 0.5	46.3 ± 0.6[*]
20:0	0.60 ± 0.17[b]	0.80 ± 0.04[a]	0.90 ± 0.02[a]	0.90 ± 0.02[a]	0.90 ± 0.05	0.73 ± 0.06[*]
Total saturated	46.4 ± 1.5[b]	47.7 ± 0.5[b]	49.0 ± 0.3[a]	49.7 ± 0.8[a]	49.3 ± 0.6	47.0 ± 0.6[*]
18:1	2.0 ± 0.3[a]	1.2 ± 0.1[b]	1.1 ± 0.1[b]	0.9 ± 0.2[bc]	1.1 ± 0.1	1.6 ± 0.1[*]
18:2	1.0 ± 0.3[a]	0.8 ± 0.1[a]	0.5 ± 0.1[b]	0.5 ± 0.1[b]	0.5 ± 0.1	0.9 ± 0.1[*]
18:3	8.3 ± 0.5[a]	7.9 ± 0.3[a]	6.9 ± 0.3[b]	6.7 ± 0.3[b]	6.9 ± 0.3	8.1 ± 0.3[*]
20:3	6.1 ± 0.4[a]	5.9 ± 0.4[ab]	4.9 ± 0.3[ab]	4.7 ± 0.4[b]	4.9 ± 0.2	6.0 ± 0.3[*]
Total unsaturated	17.4 ± 1.4[a]	15.7 ± 0.7[b]	13.3 ± 0.5[c]	12.8 ± 0.5[c]	13.4 ± 0.4	16.6 ± 0.8[*]
Total saturated/unsaturated	2.7 ± 0.3[b]	3.0 ± 0.2[b]	3.7 ± 0.1[a]	3.9 ± 0.2[a]	3.7 ± 0.2	2.9 ± 0.2[*]

[a-c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[1]All values are the means (number given in parentheses) ± SEM. Total percentage of fatty acid may not be equal 100, as all fatty acids analyzed are not presented.

[2]18:0 = stearic acid; 18:1 (Δ^9) = oleic acid; 18:2 ($\Delta^{9,12}$) = linoleic acid; 18:3 ($\Delta^{6,9,12}$) = γ-linolenic acid; 20:0 = arachidic acid; 20:3 ($\Delta^{8,11,14}$) = eicosatrienoic acid (dihomo-γ-linolenic acid); total saturated/unsaturated = ratio of total saturated to unsaturation fatty acids.

[3]PHS= pulmonary hypertension syndrome; VE= high dietary vitamin E (no PHS); VE-PHS = high dietary vitamin E with PHS; S = birds selected and NS = birds not selected for resistance to pulmonary hypertension syndrome birds fed only with control feed.

[*]Means of VE main effect (VE, VE-PHS Vs C, PHS differ significantly ($P < 0.05$).

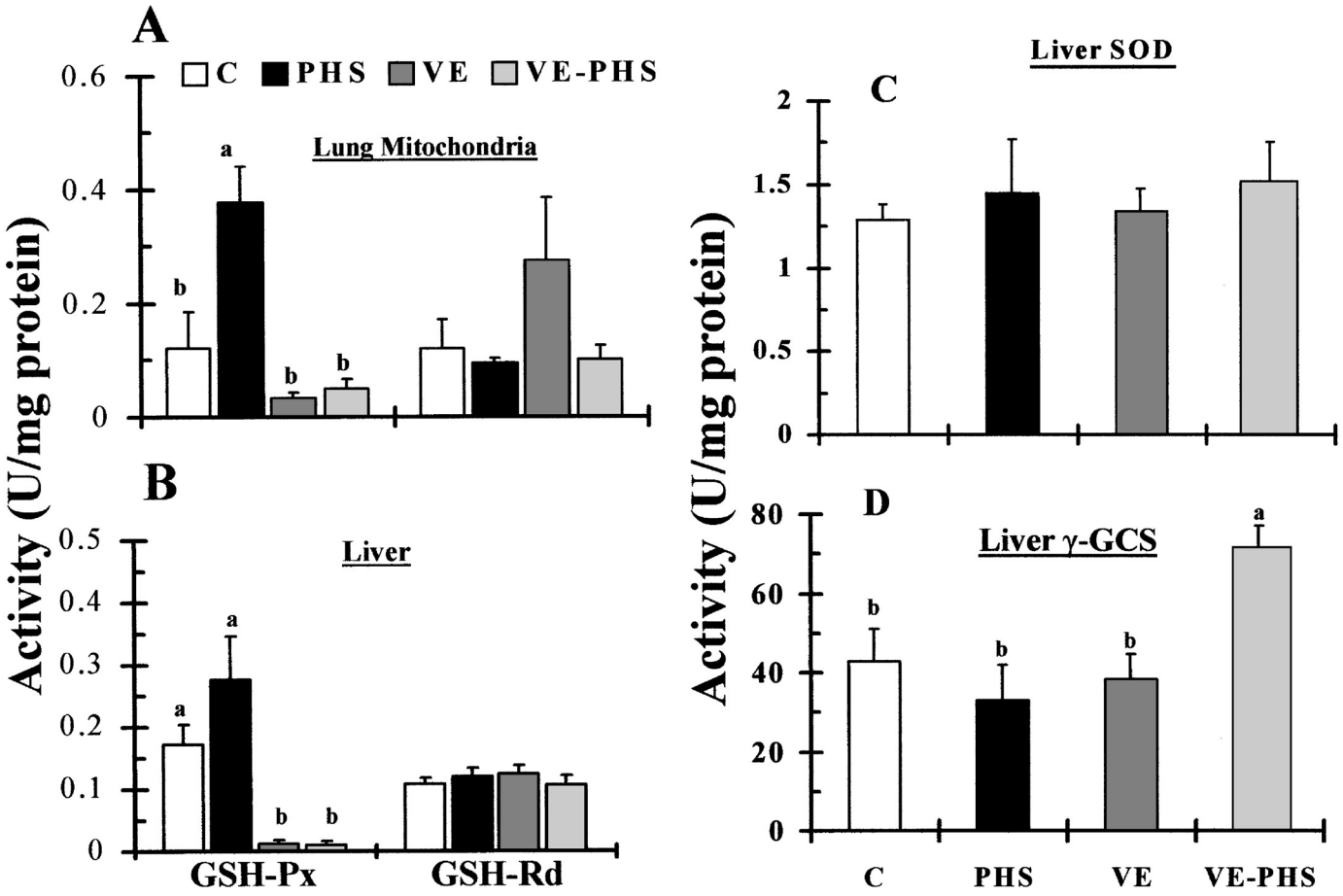

FIGURE 2. Activities of glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-Rd) in A) lung mitochondria and B) liver, and activities of C) superoxide dismutase (SOD), and D) γ-glutamylcysteine synthetase (γ-GCS) in liver obtained from broilers with and without pulmonary hypertension syndrome (PHS) fed diets with standard or high vitamin E (VE) supplementation in Experiment 1. Each bar represents the mean ± SE for broilers without PHS fed the standard diet (C, n = 6 to 7), broilers with PHS (PHS; n = 5 to 8), for broilers without PHS fed the diet with high levels of VE (VE, n = 4 to 8), and for broilers fed high dietary vitamin E that developed PHS (VE-PHS; n = 4 to 7).[a,b]Means with no common letters are significantly different ($P < 0.05$).

effects of VE have been reported for these antioxidant enzymes; for example, Li et al. (1996) reported a twofold increase in GSH-Px activity in human cardiomyocytes incubated with 200 μmol of α-tocopherol, whereas dietary VE supplementation had no effect on heart SOD, GSH-Px, and GSH-Rd and catalase in guinea pigs (Cadenas et al., 1995; Rojas et al., 1996). Thus, further studies may be warranted to investigate the effects of PHS, VE, and other antioxidants and on expression of antioxidant enzymes in broilers.

In Experiment 2, lung mitochondria obtained from S broilers (selected) for PHS resistance exhibited lower GSH-Px activity and higher GSH-Rd activity compared to lung mitochondria from broilers that were NS (not selected) for PHS resistance (Figure 3A). These findings are compatible with the GSSG/GSH ratio in Experiment 2, i.e., the higher GSH-Px activity in NS mitochondria, combined with lower GSH-Rd activity, would be expected to produce higher GSSG levels and a higher GSSG/GSH (Table 2, right hand side). Greater hydrogen peroxide production was observed in NS than in S lung mitochondria as a consequence of greater electron leak from the respiratory chain (Iqbal et al., 2001a). These findings, therefore, indicate that NS lung mitochondria experience an inherently greater degree of oxidative stress than do S lung mitochondria that would potentiate hydrogen peroxide formation. Lung mitochondria from NS birds exhibited higher GSH-Px that would help in catabolizing the greater hydrogen peroxide load, but the lower activity of GSH-Rd present in NS lung mitochondria may not be sufficient to reduce GSSG back to GSH (sSee Figure 1). Higher GSSG levels could in turn potentiate protein thiol formation that could be responsible in part for functional inefficiencies previously observed in NS compared to S lung mitochondria (Iqbal et al., 2001a,b).

There were no differences in GSH recycling enzyme activities in S and NS liver tissue in Experiment 2 (Figure 3B). Liver tissue from NS broilers did exhibit higher SOD activity (Figure 3C), but there were no differences in γ-GCS activity (Figure 3D). Whether higher SOD activity in NS liver tissue is again due to oxidative stress-induced upregulation of enzyme expression remains to be determined.

Fatty Acids

The types of fatty acids present in mitochondria can be important with regard to mitochondrial function. For

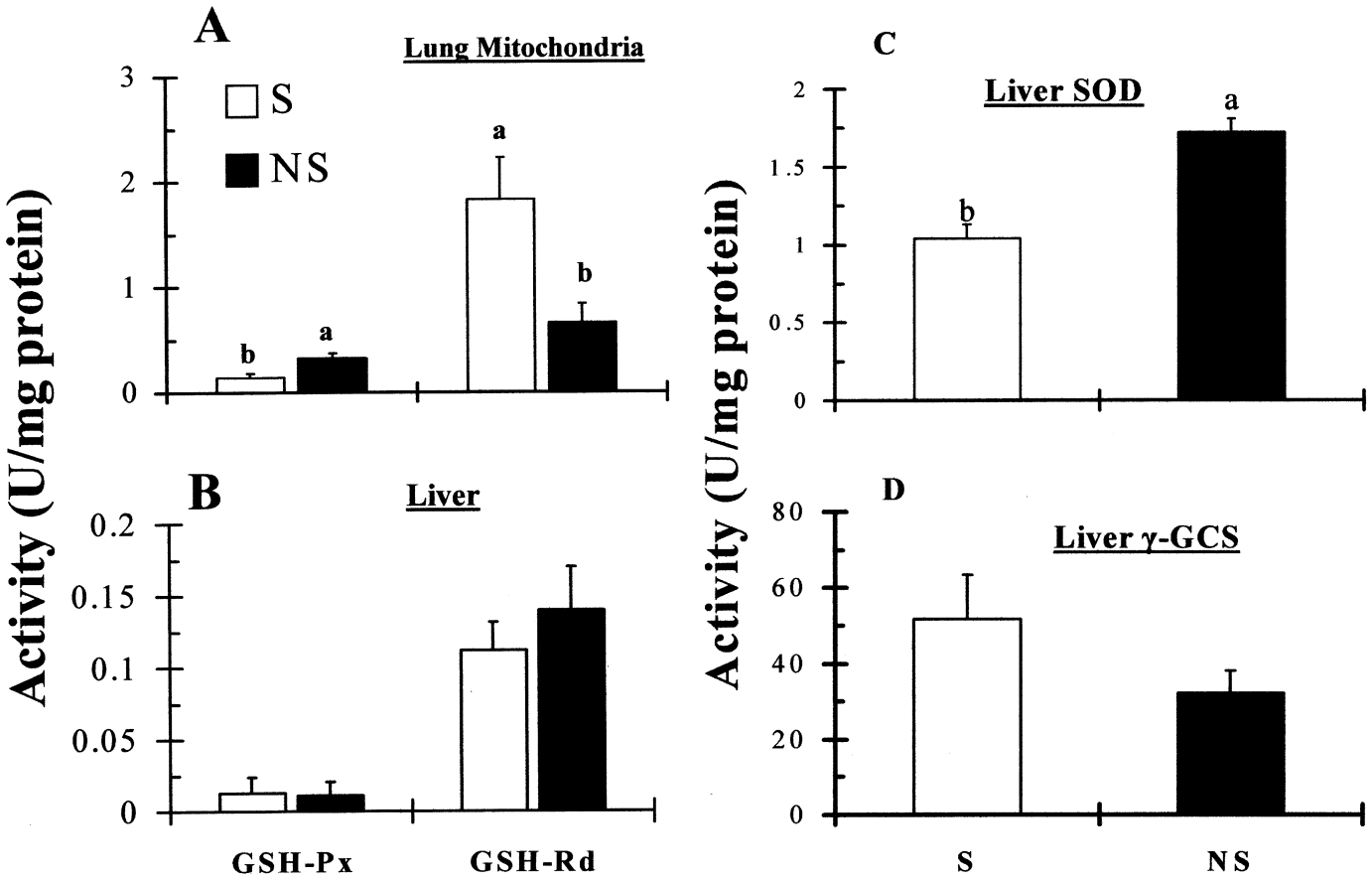

FIGURE 3. Glutathione peroxidase (GSH-Px) and reductase (GSH-Rd) enzyme activity in, A) lung mitochondrial and B) liver, and activities of C) superoxide dismutase (SOD), and D) γ-glutamylcysteine synthetase (γ-GCS) in liver obtained from broilers that were selected (S) or not selected (NS) for resistance to pulmonary hypertension syndrome (PHS) in Experiment 2. Each bar represents the mean ± SE for broilers that were selected (S; n = 4) or not selected (NS, n = 3 to 4) for resistance to pulmonary hypertension syndrome in Experiment 2. [a,b]Means with no common letters are significantly different ($P < 0.05$).

example, various hormones can alter fatty acid composition and respiration of mitochondria (Clejan et al., 1980). Increased unsaturation of fatty acids following thyroid hormone treatment enhances the susceptibility of these mitochondria to lipid peroxidation (Maddaiah, 1990). High dietary VE has also been shown to decrease fatty acid unsaturation in mitochondrial membranes (Barja et al., 1996) that in turn would make these lipids less susceptible to lipid peroxidation. The inner mitochondrial membrane is particularly prone to oxidation due to the close proximity of a major site of oxygen radical generation from the electron transport chain (e.g. Chance et al., 1979). Increased mitochondrial oxygen radical generation has been observed in broilers with PHS (Cawthon et al., 2001; Iqbal et al., 2001a). Thus, differences in mitochondrial function in broilers with PHS and high dietary VE (Cawthon et al., 1999; Iqbal et al., 2001b) could be due in part to differences in mitochondrial fatty acid content or shifts in the ratio of saturated to unsaturated fatty acids.

Fatty acid analysis revealed that that the percentage of C18:1 and total unsaturated fatty acids were higher in control than that in PHS lung mitochondria (Table 2, left side). Whereas there were no differences between VE and VE-PHS mitochondria, a main effect of VE was evident; i.e., high dietary VE increased saturation and decreased unsaturation leading to a higher saturated to unsaturated fatty acid ratio (Table 2, right side). A similar decrease in percentage of unsaturated fatty acids has been reported in liver membrane phospholipids in guinea pig following dietary VE supplementation (Barja et al., 1996). Pamplona et al. (1996) reported that the sensitivity of mitochondria to oxidative stress depended on unsaturated fatty acid contents of their membranes in rats and pigeons. The VE-induced shift in fatty acid content would affect the lipid component of cells, making the lipids more resistant to peroxidation but not the aqueous compartment of mitochondria where GSH would primarily exert its antioxidant protection (Barja et al., 1996). Cadenas et al. (1995) reported that VE supplementation at six times the minimum daily requirement increased protection against hepatic lipid peroxidation in guinea pigs. Lipid peroxides were also lowered by VE supplementation in broilers (Bottje et al., 1995). There were no main effects of PHS on lung mitochondrial fatty acid profile (data not shown).

In Experiment 2, there were no differences in percentages of fatty acids in lung mitochondria from S and NS broilers (Table 3). Furthermore, the ratio of saturated to

TABLE 3. Relative amount of fatty acids in the mitochondria isolated from the lungs of broilers selected (S) and not selected (NS) for resistance to pulmonary hypertension syndrome fed only with control feed in Experiment 2[1]

	Fatty acids[2]								
	Saturated			Unsaturated					Total saturated/ unsaturated
Groups[3]	18:0	20:0	Total	18:1	18:2	18:3	20:0	Total	
	(%)								
S (n = 4)	44 ± 1.0	1.00 ± 0.02	45 ± 1	3.1 ± 0.4	2.6 ± 0.2	4.5 ± 0.2	3.5 ± 0.1	14.2 ± 1.7	3.3 ± 0.2
NS (n = 4)	45 ± 1.0	1.00 ± 0.01	46 ± 1	2.8 ± 1.5	2.3 ± 0.6	5.1 ± 0.2	3.9 ± 0.2	16.0 ± 2.5	3.2 ± 0.3

[1]All values are the means ± SEM. Total percentage of fatty acid may not be equal to 100 as all fatty acids analyzed are not presented.

[2]18:0 = stearic acid; 18:1 (Δ^9)= oleic acid; 18:2 ($\Delta^{9,12}$) = linoleic acid; 18:3 ($\Delta^{6,9,12}$) = γ-linolenic acid; 20:0 = arachidic acid; 20:3 ($\Delta^{8,11,14}$) = eicosatrienoic acid (dihomo-γ-linolenic acid); saturated/unsaturated = ratio of total saturated to unsaturated fatty acids.

[3]S = birds selected and NS = birds not selected for resistance to pulmonary hypertension syndrome.

unsaturated fatty acids was nearly identical between the two groups of birds.

The results of this study provide insight into lung mitochondrial fatty acid proportions and antioxidant enzyme activity in lung mitochondria and liver tissue associated with PHS and high dietary VE in broilers. High dietary VE was associated with a decrease in lung mitochondrial fatty acid unsaturation that would make the mitochondria more resistant to lipid peroxidation. Higher GSH peroxidase activity observed in lung mitochondria of broilers with PHS may be a response to increased oxidative stress. Genetic resistance to PHS was associated with lower oxidative stress and higher lung mitochondrial GSH reductase activity compared to broilers that were not selected for genetic resistance. Higher GSH peroxidase activity in lung mitochondria and higher superoxide dismutase activity in liver tissue of broilers not selected for resistance to PHS may be a response to an inherently greater degree of oxidative stress than in birds that are genetically resistant to pulmonary hypertension syndrome. Further studies are planned to examine expression of these antioxidant enzymes in PHS.

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