Evidence of Mitochondrial Dysfunction in Broilers with Pulmonary Hypertension Syndrome (Ascites): Effect of t-Butyl Hydroperoxide on Hepatic Mitochondrial Function, Glutathione, and Related Thiols

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ABSTRACT The purpose of this study was to assess mitochondrial function and glutathione (a mitochondrial antioxidant) in response to oxidative stress in mitochondria *in vitro* obtained from broilers with and without pulmonary hypertension syndrome (PHS). Liver mitochondria from Control and PHS broilers were incubated with 0, 1, and 5-m*M* tertiary-butyl hydroperoxide (tBH). Indices of mitochondrial function [the respiratory control ratio (RCR) and the adenosine diphosphate to oxygen ratio (ADP:O)], and levels of mitochondrial and extra-mitochondrial reduced (GSH) and oxidized (GSSG) glutathione, cysteine, cystine, glutamate and cysteinyl-glycine were determined following tBH treatment. Lower RCR and ADP:O values were observed in PHS mitochondria than in controls. Whereas control mitochondria remained coupled (RCR > 2.0), only 3 PHS preparations remained coupled after 60 min of incubation with 5 m*M* tBH, indicating a greater susceptibility to oxidative stress in PHS mitochondria. The lower RCR in PHS mitochondria was due to increased oxygen consumption during State IV respiration. Oxidative stress following tBH treatment (decreased GSH and increased GSSG) was observed, but there were no differences in GSH or GSSG between control and PHS mitochondria. The PHS mitochondria did exhibit elevated mitochondrial and extramitochondrial cystine than controls, however. The results indicate that PHS mitochondria do not lack antioxidant protection from GSH, but lower RCR and ADP:O ratios in PHS mitochondria indicate a dysfunction that may contribute to the pathophysiology of this metabolic disease in broilers.

(*Key words*: ascites, broiler, mitochondria, respiratory control ratio, glutathione)

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INTRODUCTION

Mitochondria may contribute to oxidative stress that occurs in ascites (pulmonary hypertension syndrome, PHS) in broilers through generation of reactive oxygen species (Bottje and Wideman, 1995). Pulmonary hypertension syndrome develops in broilers as a consequence of inadequate cardiopulmonary capacity (Wideman and Bottje, 1993; Wideman and Kirby, 1995a; Wideman *et al.*, 1997). The final stage of PHS is congestive heart failure, which leads to substantial mortality in broiler flocks. Broilers with well-advanced symptoms of PHS had lower levels of the nonenzymatic antioxidants vitamins C and E, and reduced glutathione (GSH) in liver and lung tissue, and elevated levels of plasma lipid peroxides compared to age-matched controls (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995). Thus, inadequate antioxidant protection or heightened free radical generation from various sites in the cell including mitochondria may be key factors in the patho-physiology of PHS (Bottje and Wideman, 1995).

Endogenous oxidative stress is unavoidable in eukaryotic organisms and occurs during normal metabolism primarily within mitochondria. Oxidative stress occurs when the generation of reactive compounds, e.g., superoxide, hydroxyl radical, and hydrogen peroxide, that oxidize critical cellular components, such as DNA, proteins, and membranes, overwhelm antioxidant protection (Yu, 1994). As much as 2% of the oxygen utilized by the respiratory chain of mitochondria may be incompletely reduced to reactive oxygen species

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Abbreviation Key: ADP = adenosine diphosphate; ADP:O = ADP:oxygen ratio; ATP = adenosine triphosphate; GSH = reduced glutathione; GSSG = oxidized glutathione; PHS = pulmonary hypertension syndrome; RCR = respiratory control ratio; RV = right ventricle; tBH = tertiary-butyl hydroperoxide; TV = total ventricle.

(Chance *et al.*, 1979). These chemical radicals are normally metabolized by the enzymatic antioxidants superoxide dismutase and GSH peroxidase, as well as by nonenzymatic antioxidants GSH and α-tocopherol (Yu, 1994). Glutathione peroxidase occupies a particularly important role in antioxidant protection of mitochondria in conversion of hydrogen peroxides to water and lipid peroxides to less reactive alcohols. If not metabolized, hydrogen peroxide produced following dismutation of superoxide, can react with metal ions and form the highly reactive hydroxyl radical. During oxidative stress, toxic levels of oxidized glutathione (GSSG) may accumulate within mitochondria (Olafsdottir and Reed, 1988). Radical generation in mitochondria has been hypothesized to be a major contributor to the aging process in animals (Nakahara *et al.*, 1998) and has been associated with metabolic diseases such as diabetes (Kristal *et al.*, 1997).

Due to the great demand placed on mitochondria for energy production to sustain rapid growth in broilers, and the potential of mitochondria to produce large amounts of reactive oxygen species, the mitochondria in broilers may play an important role in contributing to oxidative stress in PHS. Indeed, histological evidence of hydrogen peroxide accumulation within heart mitochondria of birds with PHS (Maxwell *et al.*, 1996) lends support to this hypothesis. Furthermore, as birds with PHS exhibit lower tissue levels of GSH than controls (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995), and mitochondrial GSH levels depend upon movement of GSH into the mitochondria from the cytosol (Griffith and Meister, 1985), mitochondrial GSH, and, therefore, mitochondrial antioxidant capacity, may also be low in birds with PHS.

Mitochondrial function is assessed by monitoring oxygen consumption of isolated mitochondria under various conditions (Estabrook, 1967). State III and State IV respiration rates are determined by the amount of oxygen consumed in the presence of adequate and inadequate adenosmediphosphate (ADP) concentrations, respectively. The respiratory control ratio (RCR), an index of electron transport chain coupling, is calculated by dividing State III by State IV respiration rates. The ADP:O ratio, an index of the ability of mitochondria to carry out oxidative phosphorylation of adenosine triphosphate (ATP), is determined as the amount of ADP utilized per nanomole of oxygen during State III respiration (Estabrook, 1967). A decrease in the ADP:O ratio may represent functional damage to mitochondrial oxidative phosphorylation (Nakahara *et al.*, 1998) as a result of enhanced leakage of electrons that react with oxygen to form toxic reactive oxygen species (Kristal *et al.*, 1997).

One objective of studies outlined below was to assess the effect of oxidative stress on function of mitochondria obtained from broilers with and without PHS. A decrease in either the RCR or ADP:O values in PHS mitochondria in response to oxidative stress would be indicative of poor utilization of oxygen at the cellular level. A lower ADP:O may also indicate enhanced mitochondrial reactive oxygen species generation (Kristal *et al.*, 1997). A second objective was to determine whether antioxidant protection is compromised in PHS mitochondria. Measurement of mitochondrial GSH in response to oxidative stress imposed by tertiary butylhydroperoxide (tBH, a lipid hydroperoxide) has been used as a means of assessing mitochondrial antioxidant protection (Olafsdottir and Reed, 1988). Treatment of isolated mitochondria with tBH resulted in a decrease in GSH and an increase in GSSG (Olafsdottir and Reed, 1988). If overall antioxidant protection is compromised in mitochondria from broilers with PHS, then one would expect to observe an even greater decrease in GSH and increase in GSSG in these mitochondria relative to controls in response to tBH. In addition, mitochondrial and extramitochondrial GSH, GSSG, and related amino acids cysteine, cystine, glutamate, and cysteinylglycine, were monitored to determine whether differences in degradation or mitochondrial efflux of GSH exist in mitochondria isolated from birds with and without PHS during oxidative stress.

MATERIALS AND METHODS

Animals and Diet

Male broiler chicks (Cobb 500) were randomly assigned to either a thermoneutral or cold temperature environmental chamber and raised on wood shavings litter. Temperatures in the thermoneutral chamber were 32, 30, 28, and 25 C for Weeks 1, 2, 3 and 4 to 7, respectively, whereas temperature in the cold chamber was lowered to 15 C during Week 3 and maintained between 10 and 15 C for the rest of the study to induce a high incidence of PHS (Wideman *et al.*, 1995). Birds were provided *ad libitum* access to a starter diet (23.7% protein, 3,200 kcal ME) and water.

Sampling Procedure

Between 3 and 7 wk, birds were randomly selected from the thermoneutral chamber. Birds from the cold chamber were selected with and without overt symptoms of PHS; i.e., cyanosis of the comb and wattle or ascites fluid accumulation in the abdomen. The birds were killed with an overdose of sodium pentobarbital by i.v. injection into the leg vein and weights of the right ventricle (RV) and total ventricle (TV) determined to calculate the right ventricular weight ratio (RV:TV). The RV:TV is a sensitive indicator of prior exposure of the heart to increased pulmonary arterial pressures (Burton *et al.*, 1968). Birds were classified into one of two groups: control, if RV:TV $<$ 0.27 and abdominal or pericardial fluid were absent; PHS if RV:TV $\geq$ 0.30 and abdominal or pericardial fluid were present. Any bird not falling into one of these two groups was excluded from the study.

Preparation of Mitochondria

Hepatic mitochondria were obtained by differential centrifugation procedures as outlined by Olafsdottir and Reed (1988). Liver mitochondria were utilized due to the relative ease in obtaining sufficient quantities of mitochondria required for the incubation studies described below, and because the liver has a significant contribution to overall oxygen consumption by the animal (Field *et al.*, 1939; Brand *et al.*, 1994). Approximately 20 g of liver tissue were suspended in 50 mL of isolation medium (pH 7.4) containing 220 m*M* d-mannitol, 70 m*M* sucrose, 2 m*M* HEPES, 0.5 mg/mL BSA (fatty acid free), and 1 m*M* EGTA. The tissue was then homogenized with a tissue grinder[2] and diluted to 250 mL in isolation medium. Aliquots were transferred into polycarbonate centrifuge tubes and centrifuged twice for 10 min at 600 × *g*. The pellets containing nuclei and cell debris were discarded. The supernatant was centrifuged for 15 min at 7,750 × *g* to pellet the mitochondria. The mitochondria were resuspended in an isolation buffer (pH 7.0) containing 220 m*M* d-mannitol, 70 m*M* sucrose, 2 m*M* HEPES, 0.5 mg/mL BSA (fatty acid free), and washed twice.

Mitochondrial Incubations

Mitochondrial incubations were carried out with modifications of methods described by Olafsdottir and Reed (1988). Mitochondria were resuspended in incubation buffer containing 210 m*M* d-mannitol, 70 m*M* sucrose, 2 m*M* HEPES, and 10 m*M* succinate. The mitochondrial suspensions, 5 mL in 25 mL Erlenmeyer flasks, were slowly rotated on a Lab-Line® 3-D rotator[3] at 25 C. Tertiary-butyl hydroperoxide[4] was diluted in the mitochondrial incubation buffer and added to the flasks at 1 or 5 m*M* final concentration. Incubation buffer vehicle alone was added to 0 m*M*-treated mitochondria. The least amount of time required to sample the incubation mixture following addition of tBH solution to the mitochondrial preparation was 1 min.

Mitochondrial Function

Mitochondrial function was assessed by monitoring oxygen consumption in isolated mitochondria preparations according to Estabrook (1967). Oxygen consumption of mitochondria was measured polarographically with a Clark-type oxygen electrode[5] in a 1.6-mL thermostatically controlled chamber equipped with magnetic stirring. A pilot study using mitochondria obtained from three control and three PHS broilers, all housed under cold conditions, was conducted at 30 C to help in developing experimental procedures. However, in the tBH studies, the thermostatically controlled chamber was set at 25 C to reduce the efflux of GSH from mitochondria (Olafsdottir and Reed, 1988).

Mitochondrial function measurements were obtained at 1, 30, and 60 min of incubation with tBH. State III and State IV respiration rates of mitochondria were determined in the presence and absence of excess ADP, respectively. Aliquots (0.5 mL)of the mitochondrial incubation were removed and added to the reaction vessel containing 1 mL of reaction buffer (pH 7.0) composed of 220 m*M* d-mannitol, 70 m*M* sucrose, 2 m*M* HEPES, 3 m*M* KH_2PO_4; 5 μL of 1.5 m*M* rotenone, 50 μL of 1 *M* succinate. State III respiration was induced by the addition of 50 μL of 10 m*M* ADP and the RCR calculated by dividing State III by State IV respiration (Estabrook, 1967). Also, the efficiency of ATP synthesis coupled to cell respiration, the ADP:O ratio, was determined by measuring the amount of oxygen consumed following the addition of ADP (Estabrook, 1967). Preparations having an RCR less than 2.0, indicative of uncoupled mitochondria (Estabrook, 1967) at the start of the incubation (1 min with 0 m*M* tBH), were not considered viable and the experiment terminated.

Biochemical Analysis

At 1, 10, 30, and 60 min, an aliquot (0.5 mL) of the mitochondria incubation was removed and centrifuged at 7,000 × *g* for 30 s. Mitochondrial protein was determined using a modified Lowry assay (Simga Kit P5656).[4] The mitochondrial pellet and supernatant were treated with 10% perchloric acid to precipitate proteins. The acid soluble pools of GSH and GSSG were analyzed by HPLC using methods described by Fariss and Reed (1987). Briefly, perchloric acid precipitation of proteins was followed by reaction of iodoacetic acid with thiols to form S-carboxy-methyl derivatives and derivatization of amino groups in the supernatant with 1-fluoro-2,4-dinitrobenzene. Derivatized thiols were separated by ion-exchange column chromatography. The GSH, GSSG, cysteinylglycine, cysteine, cystine, and γ-glutamate were identified by retention times of authentic standards, and concentrations calculated from peak integrated areas. Component concentrations were calculated and presented as nanomoles per milligram of mitochondrial protein. All chemicals were obtained from Sigma Chemical Co.[4]

Statistical Analysis

As there were no differences in any measured variable between mitochondria isolated from birds housed in thermoneutral conditions (n = 3), and those housed under cold conditions that did not exhibit symptoms of PHS (n = 3) (assessed by *t* test), data from these mitochondrial isolates were pooled into a single control group (n = 6). The study was a split-unit experiment with the main unit factor being group (control vs PHS) and the main unit

[2]Thomas Tissue Grinders; Thomas Scientific, Swedesboro, NJ 08085-0099.
[3]LabLine 3-D Rotator, Lab-Line Instruments, Inc., Melrose Park, IL 60160-1491.
[4]Sigma Chemical Co., St. Louis, MO 63178-9916.
[5]Yellow Springs Instrument Co. Inc., Yellow Springs, OH 45387.

TABLE 1. Body weight, right ventricular weight ratio (RV:TV), respiratory control ratio (RCR), oxygen consumption during state III (State III) and state IV (State IV) respiration, the adenosine diphosphate (ADP) to oxygen ratio (ADP:O), and protein content (Protein) of mitochondria isolated from individual broilers with an RV:TV < 0.30 (controls) and broilers with an RV:TV > 0.30 exhibiting symptoms of pulmonary hypertension syndrome (PHS) (cyanosis and abdominal fluid accumulation). Mitochondrial variables were obtained after 1 min incubation with vehicle [0 m*M* tertiary butyl-hydroperoxide (tBH)]

Bird	Temperature[1]	BW	RV:TV	RCR (State III/ State IV)	State III	State IV	ADP:O	Protein
(d of age)		(g)			— (nmol O/min/mg Protein) —		(nmol:nmol)	(mg/mL)
		Controls						
27	Tn	1,175	0.20	8.89	44.31	5.10	1.81	3.06
29	Tc	1,200	0.25	10.53	44.49	4.23	1.71	1.94
40	Tn	2,730	0.25	6.52	43.64	6.70	1.71	1.84
48	Tn	3,210	0.22	7.39	37.49	5.08	1.69	1.68
50	Tc	3,316	0.23	8.74	39.12	4.47	1.57	2.05
53	Tc	3,658	0.20	7.84	43.42	5.54	1.70	2.15
Mean		2,548	0.23	8.32	42.25	5.19	1.70	2.12
SE		447	0.01	0.57	1.29	0.36	0.03	0.20
		PHS						
34	Tc	1,440	0.35	4.85	29.37	6.06	1.53	2.95
35	Tc	1,228	0.42	5.76	27.85	4.84	1.73	1.78
42	Tc	1,650	0.38	6.41	33.50	5.23	1.50	2.55
43	Tc	1,850	0.34	8.23	45.92	5.58	1.62	2.60
47	Tc	2,178	0.35	6.62	59.09	8.93	1.61	3.96
49	Tc	3,250	0.30	6.73	60.85	9.04	1.64	1.80
Mean		1,933	0.36	6.43	41.10	6.61	1.61	2.61
SE		296	0.01	0.46	6.03	0.77	0.03	0.33
P value[2]		= 0.278	<0.001	= 0.027	= 0.935	= 0.123	= 0.069	= 0.237

[1]Temperature conditions under which birds were raised. Tn = Thermoneutral, Tc = cold temperature (after 2 wk).

[2]Significance level determined by *t* test between control and PHS means.

being bird. The subunit was the mitochondrial sample that was treated with one level of tBH and for one incubation time. A two-group *t* test using data from mitochondrial samples that were not treated with tBH and had the minimum incubation time (1 min) was carried out to compare treatment groups. The entire data set was then analyzed by mixed model procedures. The mixed linear model that was fit to the data contained main effects and interaction effects of the three fixed factors: group, tBH, and time; the random effects in the model were bird nested within group, the interaction of tBH and bird within group, the interaction of time and bird within group, and the residual. Age of bird, which was considered as a potential covariable, had no significant effect ($P > 0.10$) on any response variable and was omitted from the final analysis. In the results, means and their comparisons are given only for the highest-order factorial effects that are considered significant ($P < 0.05$) in the mixed model analysis. Mean comparisons are done by multiple *t* tests at the 5% significance level. The pooled SE presented in the results includes all four sources of random error; this SE reflects the average error in the mean associated with sampling birds. This SE is appropriate for assessing differences among treatment groups, which have different birds, but is too large for comparisons between levels of tBH or between incubation times because these comparisons involve only within-bird errors. The statistical analysis was accomplished using the MIXED procedure of SAS® software (SAS Institute, 1996).

RESULTS

A pilot study conducted on mitochondria obtained from broilers housed under cold conditions resulted in RCR values of 3.50 ± 0.17 and 2.36 ± 0.15, for control and PHS mitochondria (n = 3 per group), respectively, that were different ($P = 0.019$). The ADP:O ratios for the control and PHS groups were 1.89 ± 0.14 and 1.27 ± 0.12, respectively, which were different ($P = 0.058$). An inverse relationship between incubation temperature and RCR value (unpublished observations) accounts for the lower RCR values in this pilot experiment, conducted at 30 C, than values in the experiment described below conducted at 25 C.

Values for body weight, RV:TV, mitochondrial function variables, and protein content of freshly isolated mitochondria (1 min of 0 m*M* tBH) for individual birds are shown in Table 1. The means for RV:TV were 0.23 in the controls and 0.36 in PHS birds. Liver mitochondria from PHS birds exhibited a lower RCR ($P = 0.027$), and a lower ADP:O ($P = 0.069$) than controls. There were no differences in body weights of controls and PHS birds, or in State III or State IV respiration, and protein content in isolated mitochondria between groups. Regression analysis revealed a significant negative relationship between RV:TV and initial RCR (at 1 min) in vehicle-treated (0 m*M* tBH) mitochondria (data not shown).

Birds in the present study were fed a starter diet throughout the study to reduce fluctuations in variables

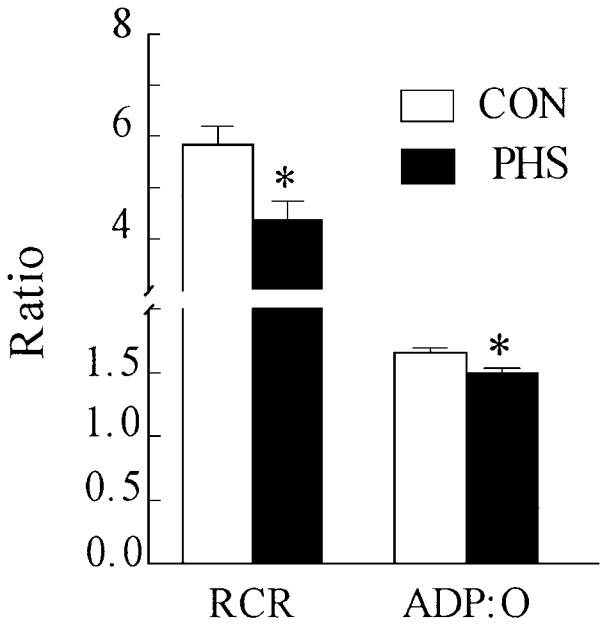

FIGURE 1. The respiratory control ratio (RCR) and the adenosine diphosphate to oxygen (ADP:O) ratio in liver mitochondria isolated from control broilers (CON, open bar) and broilers with pulmonary hypertension syndrome (PHS, closed bar) between 3 and 7 wk of age. Mitochondria were incubated at 25 C for 60 min with tertiary butyl hydroperoxide (tBH, 1 and 5 m*M*) or saline vehicle (0 m*M*) to induce an oxidative stress. Each value represents the mean ± SE from 6 birds and 9 mitochondrial samples per bird. *PHS means differ from control ($P < 0.02$).

that might have been related to a dietary change. Mitochondrial protein concentrations were between 1.8 and 3.9 mg/mL and did not exhibit a tendency to decrease with age (Table 1), as was observed in a preliminary study in which protein levels decreased from 2.1 to 1.2 mg protein/mL when birds were fed a starter diet (21% protein) for 3 wk followed by a grower diet (18% protein) (unpublished observations).

None of the two- and three-factor interactions of treatment group, tBH, or time of incubation were significant ($P > 0.17$) for RCR and ADP:O. A significant main effect of treatment group resulted from both RCR and ADP:O being lower ($P < 0.02$) in PHS than in control mitochondria (Figure 1). Whereas control mitochondria remained well coupled (RCR > 2.0) throughout the 60-min incubation with all levels of tBH, oxidative stress resulted in the uncoupling of mitochondria in three PHS incubations 60 min after treatment with 5 m*M* tBH, and in two PHS incubations 60 min after treatment with 1 m*M* tBH (data not shown).

The lower RCR in PHS mitochondria was due to a significant elevation in State IV respiration rate (Table 2A). Treatment group had significant interactions with tBH and time of incubation for State IV respiration rate. State IV respiration was higher in PHS mitochondria with all levels of tBH and at all time points during the experiment. State III respiration rate decreased in response to time of incubation and level of tBH treatment (Table 2B), but was not affected by treatment group.

There were no differences in concentrations of GSH, GSSG, glutamate, and cysteinyl-glycine in control and PHS liver mitochondria after 1 min of incubation with 0 m*M* tBH (Table 3). A fundamental difference between control and PHS mitochondria was observed in cysteine and cystine concentrations, however (Table 3). Whereas

TABLE 2. Main effects of tertiary butyl hydroperoxide (tBH), time (minutes), or treatment group on State III (A) and State IV (B) respiration rates (nmol O/min/mg protein) in mitochondria treated with 0, 1, and 5 m*M* tertiary butyl hydroperoxide (tBH) in mitochondria obtained from controls and broilers with pulmonary hypertension syndrome (PHS)

A. State IV Respiration Rate (nmol O/min/mg protein)[1]

	tBH			
	0	1	5	
Control	6.2^{b,x}	6.7^{b,y}	7.8^{a,y}	
PHS	7.8^{b,x}	10.5^{a,x}	11.1^{a,y}	pooled SE = 0.62
	Minutes			
	0	30	60	
Control	6.1^{b,y}	6.8^{b,y}	7.8^{a,y}	
PHS	8.1^{b,x}	8.8^{ab,x}	12.5^{a,x}	pooled SE = 0.67

B. State III Respiration Rate (nmol O/min/mg protein)[2]

	Minutes			
	1	30	60	
tBH (m*M*)				
0	42.5^{a,x}	38.7^{b,x}	39.3^{b,x}	
1	43.7^{a,x}	37.7^{b,xy}	33.3^{c,y}	pooled SE = 2.89
5	44.3^{a,x}	36.1^{b,y}	29.7^{c,z}	

^{a,b}Means in the same row with no common superscript differ significantly ($P < 0.05$).

$^{x–z}$Means in a column with no common superscript differ significantly ($P < 0.05$).

[1]Each value represents the mean ± pooled SE from six birds and nine mitochondrial measurements per bird.

[2]Each value represents the mean ± pooled SE from 12 birds.

cysteine and cystine were not detected in control mitochondria, cysteine was detected in two birds with PHS and cystine was detected in all mitochondria obtained from broilers with PHS. It is interesting to note that the birds with the highest mitochondrial cysteine level of 61 nmol/mg of protein in the homogenate, also had the highest RV:TV (Table 1) in this study.

The effects of incubation time and tBH treatment on mitochondrial and extra-mitochondrial GSH and GSSG are shown in Figure 2. For these response variables, treatment group had no effect. A time-dependent decrease in mitochondrial GSH was observed (Figure 2A), due in part to movement of GSH out of mitochondria (Figure 2B). Treatment of isolated mitochondria with tBH produced an oxidative stress as demonstrated by tBH-dependent decreases in mitochondrial GSH (Figure 2A), and a tBH-related increase in GSSG (Figure 2C) and the ratio of GSSG to GSH (data not shown). Lower levels of extramitochondrial GSH following tBH treatment (Figure 2B) were probably due to oxidation of GSH in the media (Figure 2D).

Although there was an interaction between treatment group and incubation time for mitochondrial cystine, PHS birds had greater ($P < 0.05$) amounts than control birds for all incubation times (Figure 3A). The tBH treatments, which had no significant effects ($P > 0.05$), produced detectable cystine in at least one control bird at 1 and 30 min of incubation, but not at 60 min of incubation. Cystine levels declined over time of incubation. Extramitochondrial cystine was elevated in PHS incubations and increased over time in both groups (Figure 3B). No differences in mitochondrial cysteine, glutamate, and cysteinylglycine were observed during the experiment (data not shown). Although there were no effects of treatment group, tBH, or time on mitochondrial glutamate, extramitochondrial glutamate levels increased over time and this increase was attenuated by tBH treatment (Figure 4).

DISCUSSION

Mitochondrial Function

The major indices of mitochondrial function are the RCR and ADP:O ratio, which are determined based on measurements of oxygen utilization by mitochondria *in vitro* (Estabrook, 1967). Ninety percent of the oxygen consumed by a cell occurs within mitochondria as they carry out their primary function of ATP synthesis. The lower RCR and ADP:O ratios (Figure 1) in PHS mitochondria indicate functional impairment of oxidative phosphorylation and less efficient utilization of oxygen than in control mitochondria; i.e., PHS mitochondria consume more oxygen per molecule of ATP synthesized than controls. Because mitochondria in other tissues may respond differently (e.g., Kwong and Sohal, 1998), the results obtained in the liver do not necessarily mean that mitochondria from other tissues will respond similarly. However, hepatic mitochondrial dysfunction alone would represent significant additional metabolic demand for oxygen, as the liver accounts for 15% of the total oxygen use in an entire animal (Field *et al.*, 1939; Brand *et al.*, 1994). The greater oxygen consumption in PHS liver mitochondria would exacerbate inherent deficiencies in cardiopulmonary capacity in broilers (Wideman and Kirby, 1995a;

TABLE 3. Concentrations of reduced (GSH) and oxidized (GSSG) glutathione, GSSG:GSH ratio, cysteine, cystine, glutamate and cysteinylglycine of mitochondria isolated from broilers with an RV:TV < 0.30 (controls) and broilers with an RV:TV > 0.30 exhibiting symptoms of pulmonary hypertension syndrome (PHS) (cyanosis and abdominal fluid accumulation). Mitochondrial variables shown were obtained after 1 min of incubation with vehicle (0 m*M* tBH) tertiary butyl-hydroperoxide

Bird	Temperature[1]	GSH	GSSG	GSSG:GSH	Cysteine	Cystine	Glutamate	Cysteinylglycine
		(nmol/mg protein)						
Controls								
27	Tn	4.87	0.06	0.012	0.00	0.00	0.00	1.68
29	Tc	5.08	0.07	0.013	0.00	0.00	3.15	3.80
40	Tn	6.04	0.05	0.009	0.00	0.00	0.00	0.00
48	Tn	8.89	0.10	0.012	0.00	0.00	2.27	1.71
50	Tc	6.99	0.12	0.017	0.00	0.00	1.17	1.16
53	Tc	5.83	0.13	0.022	0.00	0.00	1.03	0.65
Mean		6.28	0.09	0.013	. . .	. . .	1.27	1.50
SE		0.61	0.01	0.002	. . .	. . .	0.51	0.53
PHS								
34	Tc	5.74	0.07	0.012	8.06	0.36	3.50	1.52
35	Tc	5.17	0.00	0.000	61.16	0.24	2.49	1.19
42	Tc	8.35	0.17	0.020	0.00	0.51	1.25	0.60
43	Tc	3.83	0.06	0.015	0.00	0.14	1.09	0.53
47	Tc	5.82	0.08	0.014	0.00	0.11	1.08	0.64
49	Tc	5.09	0.10	0.019	0.00	0.14	1.21	1.66
Mean		5.67	0.08	0.013	11.54	0.25	1.77	1.01
SE		0.61	0.02	0.003	10.01	0.06	0.41	0.20
P value		= 0.49	= 0.76	= 0.82	= 0.276	<0.003	= 0.46	= 0.40

[1]Temperature conditions under which birds were raised. Tn = Thermoneutral, Tc = cold temperature (after 2 wk).

[2]Significance level determined by *t* test between control and PHS means.

Wideman *et al.*, 1997) and directly contribute to PHS by accentuating systemic hypoxia (Peacock *et al.*, 1990; Wideman and Kirby, 1995b), a critical step in the sequence of physiological events that lead to pulmonary hypertension in broilers (Wideman and Bottje, 1993).

One mechanism to explain both the lower RCR and lower ADP:O in PHS mitochondria is increased leakage of protons across the inner mitochondrial membrane at sites other than through the f_1f_0ATPase (Brand *et al.*, 1994). The movement of electrons down the electron transport chain is coupled to pumping of protons from the mitochondrial matrix, setting up an electrochemical difference for protons across the mitochondrial membrane (protonmotive force). The protonmotive force in turn drives protons back across the membrane into the matrix through the f_1f_0ATPase (ATP synthase) and, in combination with ADP and phosphate, results in the formation of extramitochondrial ATP. The process is not 100% efficient due to leakage of protons (proton leak) across the membrane at sites other than through the f_1f_0ATPase. The higher State IV respiration rate responsible for the lower RCR in PHS mitochondria (Table 2A) could therefore be a result of increased proton leakage that raises oxygen consumption during State IV respiration (Brand *et al.*, 1994). An increase in proton leakage also decreases the ADP:O because protons that leak into the mitochondria are not involved in ATP synthesis. Thus, one explanation for the lower ADP:O in PHS mitochondria could be attributed to increased proton leakage. The lower ADP:O ratio would in turn mean that PHS mitochondria are less efficient in synthesizing ATP.

Proton leakage is affected by thyroid status of the animal with proton leakage as well as State IV oxygen consumption, being associated with hypothyroid < eu-

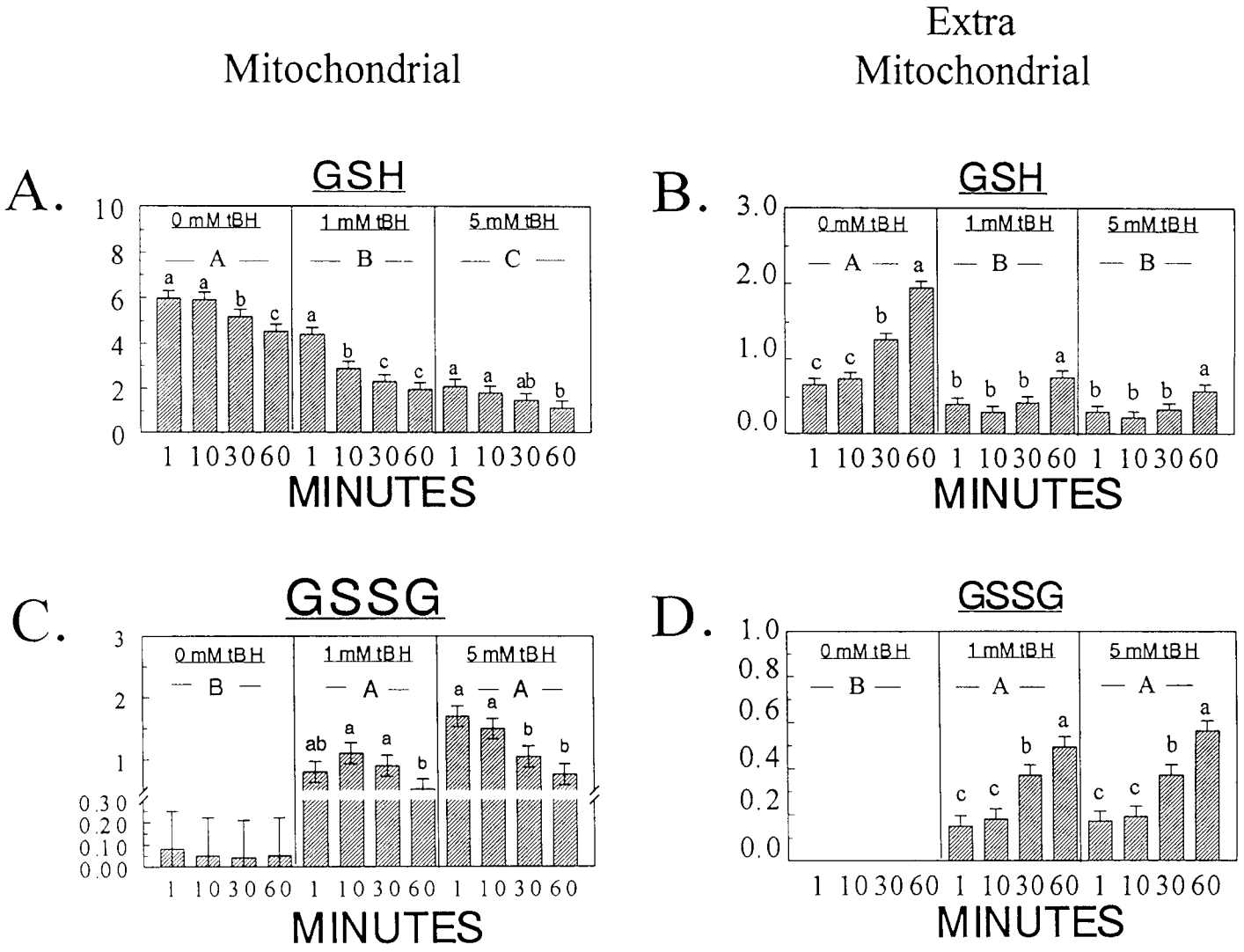

FIGURE 2. Mitochondrial and extra-mitochondrial concentrations (nanomoles per milligrams of protein) of reduced glutathione (GSH) (A and B) and oxidized glutathione (GSSG) (C and D) in incubations of liver mitochondria isolated from broilers with and without pulmonary hypertension syndrome (PHS, closed bar) between 3 and 7 wk of age. Mitochondria were incubated at 25 C for 60 min with tertiary-butyl hydroperoxide (tBH, 1 and 5 m*M*) to induce an oxidative stress or incubation buffer vehicle (0 m*M*). Each value represents the mean ± SE from 12 birds. Means within a tBH treatment that do not share a common lower case letter differ significantly ($P < 0.05$). Tertiary-butyl hydroperoxide means for the same incubation time that do not share a common upper case letter differ significantly ($P < 0.05$).

thyroid < hyper-thyroid animals (Harper and Brand, 1993; Brand *et al.*, 1994). Scheele *et al.* (1992) hypothesized that diets that promote a hypothyroid-like condition would lower PHS mortality, whereas Buys *et al.* (1993) demonstrated that feeding birds triiodothyronine (T_3) increased the incidence of PHS mortality in broilers. A unifying mechanism for the association of thyroid hormone with PHS mortality may be through its stimulation of State IV oxygen consumption in mitochondria.

A second explanation for the lower ADP:O ratio in PHS mitochondria may involve a site-specific defect (electron leak) in the respiratory chain and heightened reactive oxygen species generation (Kristal *et al.*, 1997). A consequence of defective electron transport within PHS mitochondria, would be a self perpetuating cycle of oxidant damage due to the formation of toxic compounds such as malondialdehyde and hydroxyalkenals (Esterbauer *et al.*, 1991) that contribute to further mitochondrial dysfunction (Esterbauer *et al.*, 1991; Chen *et al.*, 1995; Kristal *et al.*, 1994, 1996). Increased reactive oxygen species generation within PHS mitochondria would, in turn, increase oxidant load, and may account in part for the compromised antioxidant status observed in birds with PHS (Envetchakul *et al.*, 1993; Bottje *et al.*, 1995). The likelihood that PHS mitochondria do indeed produce increased amounts of reactive oxygen species is supported by histological evidence of increased hydrogen peroxide accumulation in PHS mitochondria (Maxwell *et al.*, 1996). Hypoxia increases reactive oxygen species generation as a result of a reductive stress in which electron transport chain proteins are in a reduced state and unable to accept electrons (Dawson *et al.*, 1993). Therefore, conditions conducive to increased radical production appear to be present in PHS mitochondria. Heightened radical production in PHS mitochondria, could reduce the ability mitochondria to replace radical-damaged respiratory complexes by inhibiting mitochondrial DNA transcription (Kristal *et al.*, 1994) causing an imbalance between respiratory chain components (Kristal *et al.*, 1997) and free radical production (Bandy and Davison, 1990).

The dysfunction in PHS mitochondria may result in part from the systemic hypoxia that occurs with PHS. Hypoxia develops as a result of a marginal pulmonary capacity in broilers that has been elegantly demonstrated by Wideman and co-workers (Wideman and Kirby, 1995a; Wideman *et al.*, 1997). The marginal pulmonary capacity leads to a ventilation-perfusion mismatch and a rapid decrease in blood oxygen levels (Wideman and Kirby, 1995b). Although all control mitochondrial incubations

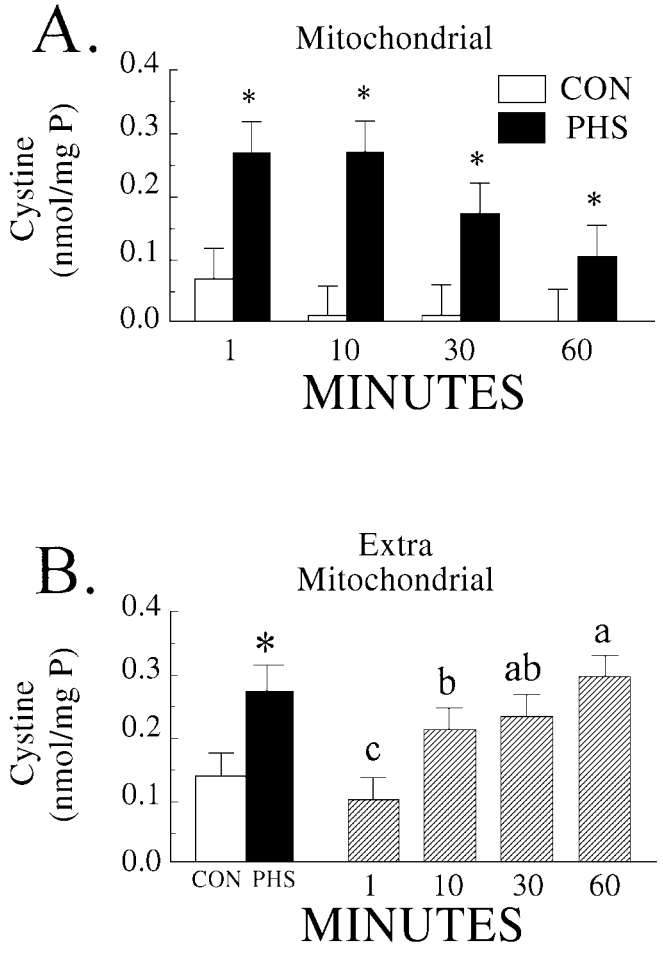

FIGURE 3. Mitochondrial (A) and extra-mitochondrial (B) concentrations of cystine in incubations of liver mitochondria isolated from control (CON, open bar) broilers and broilers with pulmonary hypertension syndrome (PHS, closed bar) between 3 and 7 wk of age. Mitochondria were incubated at 25 C for 60 min with tertiary-butyl hydroperoxide (tBH, 1 and 5 m*M*) to induce an oxidative stress, or saline vehicle (0 m*M* tBH). Each value in A represents the mean ± SE of 6 birds and 3 mitochondrial samples per bird. In B, treatment group means ± SE are derived from 12 mitochondrial samples on each of 6 birds, and incubation time means ± SE are from 3 mitochondrial samples on each of 12 birds. *PHS means differ from control ($P < 0.05$).

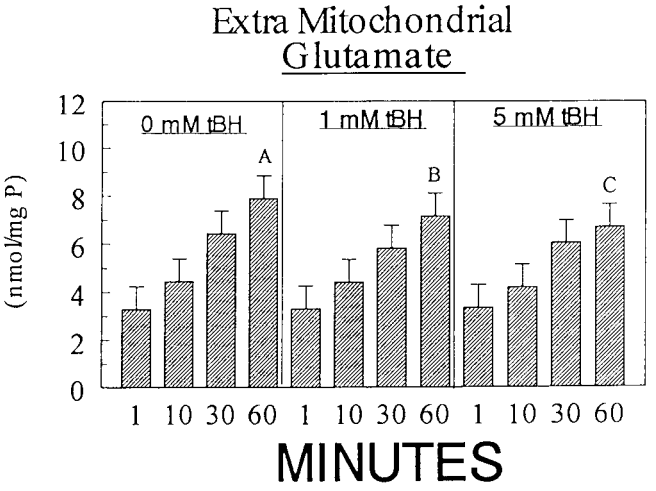

FIGURE 4. Extra-mitochondrial concentrations of glutamate of mitochondria isolated from broilers between 3 and 7 wk of age averaged over control and Pulmonary Hypertension Syndrome groups. Mitochondria were incubated at 25 C for 60 min with tertiary butyl hydroperoxide (tBH, 1 and 5 m*M*) to induce an oxidative stress, or saline vehicle (0 m*M* tBH). Each value represents the mean ± SE of 12 birds. Means within a tBH treatment that do not share a common lower case letter differ significantly ($P < 0.05$). Means within each incubation time that do not share a common upper case letter are differ significantly ($P < 0.05$).

remained coupled (RCR > 2.0) throughout the experiment, only three PHS mitochondria exhibited RCR greater than 2.0 at the end of the 60-min incubation period with 5 m*M* tBH. These mitochondrial preparations came from birds with the highest RV:TV (0.35, 0.38, and 0.42) observed in this study. These observations indicate that mitochondria from birds with PHS may be less able to cope with oxidative stress than are mitochondria obtained from birds without PHS.

At this point, it is not known whether the dysfunction observed in PHS mitochondria is secondary to conditions that develop during PHS, or whether the dysfunction is actually a primary defect that contributes to PHS susceptibility. The known detrimental effects of hypoxia on mitochondrial function (Dawson *et al.*, 1993), would suggest that the dysfunction could be secondary following the development of hypoxia, as birds with PHS were selected based on exhibiting symptoms (cyanosis of comb and wattles). However, prolonged hypoxia was associated with a decrease in State III respiration rate in rat hepatocytes (Chandel *et al.*, 1995), whereas State III respiration was not different between PHS and Control mitochondria. Therefore, it is possible that the dysfunction observed in PHS mitochondria may actually contribute to the onset of PHS.

GSH and Related Peptides and Amino Acids

Mitochondrial GSH plays a vital role in maintaining function in this important organelle (Meredith and Reed, 1988; Mårtensson *et al.*, 1989). Reduced GSH levels during oxidative stress are dictated primarily by the combined activities of GSH peroxidase, that uses the sulfhydryl group of GSH to reduce peroxides with the formation of GSSG, and GSH reductase, that uses reducing equivalents of NADPH to reduce GSSG back to GSH (Meister, 1984). Tertiary-butyl hydroperoxide treatment induced an oxidative stress in broiler mitochondria, but there were no differences in GSH and GSSG between control and PHS mitochondria at any time or dosage level of tBH (Figure 2).

It was assumed that GSH levels would be lower in PHS mitochondria because mitochondria GSH is derived from cytosolic GSH (Olafsdottir and Reed, 1988), and lower tissue levels of GSH in birds with PHS have been observed (Enkvetchakul *et al.*, 1993; Bottje *et al.*, 1995). However, no differences in mitochondrial GSH levels were found between controls and PHS birds. The results may indicate a preservation of mitochondrial GSH at the expense of cytosolic GSH during PHS similar to that observed following chemical depletion of cytosolic GSH with diethyl maleate (e.g., Rodriguez *et al.*, 1987).

Olafsdottir and Reed (1988), observed a rebound in GSH after 10 min in mitochondria treated with 1 m*M* tBH to levels identical to vehicle-treated mitochondria. This rebound in mitochondrial GSH was attributed to GSH reductase activity. A similar rebound in mitochondrial GSH was not observed in the present study in mitochondria treated with 1 m*M* tBH. These results may indicate that broiler mitochondria lack sufficient GSH reductase activity to cope with oxidative stress similar to that imposed on rat liver mitochondria. Elevations in mitochondrial GSSG could quickly lead to thiol toxicity as mitochondria lack the ability to export GSSG into the cytosol (Olafsdottir and Reed, 1988). The inability to export GSSG may explain why loss of mitochondrial GSH, rather than cytosolic GSH, is critical in some types of cell injury (Meredith and Reed, 1988). The appearance of GSSG in the media of tBH-treated mitochondria was due to oxidation of GSH, and not to GSSG export, as no GSSG was exported from mitochondria treated with 0 m*M* tBH (Figure 3D).

Cystine was detected in PHS mitochondria, but not in controls treated with 0 m*M* tBH (Table 3). However, cystine was observed in one or more controls following treatment with 1 or 5 m*M* tBH (Figure 4A). Loss of cysteine and other amino acids from adenine nucleotide translocase in tBH treated mitochondria was observed in mitochondria as a result of peroxidative modification (Girón-Calle and Schmid, 1996). Thus, some type of radical-mediated oxidation combined with release of cysteine moieties from mitochondrial membrane and matrix proteins may be occurring in PHS, but not in control, vehicle-treated mitochondria. Only two birds with PHS exhibited detectable levels of cysteine, whereas cysteine was not detected in the other four PHS birds or in controls (Table 1). Whether the very high levels of cysteine (60 nmol/mg protein) detected in liver mitochondria obtained from the bird with the highest right ventricular weight ratio in this study (0.42) is merely an anomaly or truly characteristic of advanced PHS remains to be determined.

Efflux of GSH was detected in broiler mitochondria (Figure 3). Olafsdottir and Reed (1988) reported a similar efflux of mitochondrial GSH that was decreased by lowering the incubation temperature from 30 to 25 C and abolished when mitochondria were maintained at 4 C. This movement of GSH has been hypothesized to be an important mechanism *in vivo* that allows migration of GSH between cytosol and mitochondria, necessitated by the inability of mitochondria to synthesize GSH. This homeostatic mechanism of GSH movement differs from that associated with mitochondrial permeability transition that occurs during abnormal conditions (e.g., Lemasters *et al.*, 1997). When mitochondrial permeability transition is attained, there is an increased permeability to small molecular weight compounds, release of ions, and large amplitude mitochondrial swelling (Hunter *et al.*, 1976; Hunter and Haworth, 1979). Conductance of material is high enough during mitochondrial permeability transition, that the term “megachannel” has been coined for this phenomenon, and the opening of one megachannel may be sufficient to uncouple oxidative phosphorylation (Zoratti and Szabo, 1995). It is unlikely that mitochondrial permeability transition was reached in this study as EGTA was used in the isolation buffer to

remove excess calcium that is involved in precipitating this condition. Additionally, the rate of GSH loss from broiler mitochondria was similar to a previous report (Olafsdottir and Reed, 1988), and permeability transition was probably not reached because all mitochondria incubated with media alone (0 m*M* tBH) remained viable (RCR > 2.0) throughout the 60-min incubation period.

In addition to GSH efflux, there was an obvious loss of cystine (Figure 3B), glutamate (Figure 4), and cysteinylglycine (not shown) from mitochondria into the media. As these compounds have a smaller molecular weight than GSH, they may be able to move out of the mitochondria by the same mechanism as does GSH. The loss for glutamate was much greater (final concentration of 7 to 8 nmol / mg protein) than for either GSH or cystine, which attained final concentrations of approximately 2 and 0.4 nmol / mg protein, respectively. There were no differences between control and PHS mitochondria in the efflux of any of these compounds.

In summary, liver mitochondria obtained from broilers with PHS exhibit a functional defect characterized by a decrease in the RCR and in the ADP:O. The lower RCR was due to an increase in State IV respiration rate in PHS mitochondria. The decrease in RCR and ADP:O indicate that these mitochondria would consume more oxygen to sustain oxidative phosphorylation and potentially produce higher amounts of chemical radicals than in control mitochondria, both of which could contribute to the pathophysiology of PHS by accentuating the development of systemic hypoxia and oxidative stress. The uncoupling of PHS mitochondria during incubation with tBH appears to indicate a greater susceptibility of PHS mitochondria than controls to oxidative stress. There is an indication of oxidation of cysteine moieties in PHS mitochondria due to the presence of cystine, whereas this amino acid was not detected in control mitochondria. The mitochondrial dysfunction observed in broilers with PHS does not appear to be due to a lack of antioxidant protection from glutathione, as neither GSH nor GSSG levels differed between mitochondria obtained from controls or broilers with pulmonary hypertension syndrome during exposure to oxidative stress. Further research is required to determine the exact nature of this mitochondrial dysfunction and its specific contribution to the pathophysiology of PHS.

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REFERENCES

Bottje, W. G., and R. F., Wideman, Jr., 1995. Potential role of free radicals in the etiology of pulmonary hypertension syndrome. Poult. Avian Biol. Rev. 6:211–231.

Bottje, W., B. Enkvetchakul, R. Moore, and R. McNew, 1995. Effect of α-tocopherol on antioxidants, lipid peroxidation, and the incidence of pulmonary hypertension syndrome (ascites) in broilers. Poultry Sci. 74:1356–1369.

Bandy, B., and A. J. Davison, 1990. Mitochondrial mutations may increase oxidative stress. Implications for carcinogenesis and aging. Free Radic. Biol. Med. 8:523–529.

Brand, M. D., L. F. Chien, E. K. Ainscow, D.F.S. Rolfe, and R. K. Porter, 1994. The causes and function of mitochondrial proton leak. Biochim. Biophys. Acta 1187:132–139.

Burton, R. R., A. Joyce, and K. U. Ingold, 1968. Effect of chronic hypoxia on the pulmonary arterial blood pressure of the chicken. Am. J. Physiol. 214:1438–1442.

Buys, N., J. Buyse, and E. Decuypere, 1993. Ascites syndrome mortality and growth in two commercial broiler strains subjected to different early temperature programs. Poultry Sci. 72:135. (Abstr.)

Chance, B., H. Sies, and A. Boveris, 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59: 527–605.

Chandel, N., G.R.S. Budinger, R. A. Kemp, and P. T. Schumacher, 1995. Inhibition of cytochrome-c oxidase activity during prolonged hypoxia. Am. J. Physiol. 268: L918–L925.

Chen, J. J., H. Bertrand, and B. P. Yu, 1995. Inhibition of adenine nucleotide translocator by lipid peroxidation products. Free Rad. Biol. Med. 19:583–590.

Dawson, T. L., G. J. Gores, A. Nieminen, B. Herman, and J. J. Lemasters, 1993. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. Am. J. Physiol. 264:C961–967.

Enkvetchakul, B., W. Bottje, N. Anthony, and R. Moore, 1993. Compromised antioxidant status associated with ascites in broilers. Poultry Sci. 72:2272–2280.

Esterbauer, H., R. J. Schaur, and H. Zollner, 1991. Chemistry and biochemistry of 4-hydroxy-nonrenal, and related aldehydes. Free Rad. Biol. Med. 11:81–128.

Estabrook, R. W., 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol. 10:41–47.

Farris, M. W., and D. J. Reed, 1987. High performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. Methods Enzymol. 143:101–109.

Field, J., H. S. Belding, and A. W. Martin, 1939. Evaluation of wet and dry weight bases for expression of respiratory rate *in vitro*. Proc. Soc. Exp. Biol. Med. 40:565–568.

Girón-Calle, J., and H.H.O. Schmid, 1996. Peroxidative modification of a membrane protein. Conformation-dependent chemical modification of adenine nucleotide translocase in Cu^{2+} / tert-butyl hydroperoxide treated mitochondria. Biochem. 35:15440–15446.

Griffith, O. W., and A. Meister, 1985. Origin and turnover of mitochondrial glutathione in the isolated hepatocyte. Proc. Natl. Acad. Sci. (USA) 82:4668–4672.

Harper, M. E., and M. D. Brand, 1993. The quantitative contributions of mitochondrial proton leak and ATP turnover reactions to the changes respiration rates of hepatocytes from rats of different thyroid status. J. Biol. Chem. 268:14850–14860.

Hunter, D. R., R. A. Haworth, and J. H. Southard, 1976. Relationship between configuration, function and permeability in calcium-treated mitochondria. J. Biol. Chem. 251: 5069–5077.

Hunter, D. R., and R. A. Haworth, 1979. The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. Arch. Biochem. Biophys. 195:453–459.

Kristal, B. S., J. J. Chen, and B. P. Yu, 1994. Sensitivity of mitochondrial transcription to different free radical species. Free Radical Biol. Med. 16:323–329.

Kristal, B. S., B. K. Park, and B. P. Yu, 1996. 4-Hydroxyhexenal is a potent inducer of the mitochondria permeability transition. J. Biol. Chem. 271:6033–6038.

Kristal, B. S., C. T. Jackson, H. Y. Chung, M. Matsuda, H. D. Nguyen, and B. P. Yu, 1997. Defects at center P underlie diabetes-associated mitochondrial dysfunction. Free Rad. Biol. Med. 22:823–833.

Kwong, L. K., and R. S. Sohal, 1998. Substrate and site-specificity of hydrogen peroxide generation in mouse mitochondria. Arch. Biochem. Biophys. 350:118–126.

Lemasters, J. J., A. L. Nieminen, T. Qian, L. C. Trost, and B. Herman, 1997. The mitochondrial permeability transition in toxic, hypoxic, and reperfusion injury. Mol. Cell. Biochem. 174:159–165.

Mårtensson, J., A. Jain, W. Frayer, and A. Meister, 1989. Glutathione metabolism in the lung: inhibition of its synthesis leads to lamellar body and mitochondrial defects. Proc. Nat. Acad. Sci. USA 86:5296–5300.

Maxwell, M., G. W. Robertson, and C. Farquharson, 1996. Evidence of ultracytochemical mitochondria-derived hydrogen peroxide activity in myocardial cells from broiler chickens with an ascites syndrome. Res. Vet. Sci. 61:7–12.

Meister, A., 1984. New aspects of glutathione biochemistry and transport: Selective alteration of glutathione metabolism. Fed. Proc. 43:3031–3042.

Meredith, M., and D. J. Reed, 1988. Depletion *in vitro* of mitochondrial GSH in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and BCNU. Biochem. Pharmacol. 32:1383–1388.

Nakahara, H., T. Kanno, Y. Inal, K. Utsumi, M. Hiramatsu, A. Mori, and L. Packer, 1998. Mitochondrial dysfunction in the senescence accelerated mouse (SAM). Free Rad. Biol. Med. 24:85–92.

Olafsdottir, K., and D. J. Reed, 1988. Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment. Biochim. Biophys. Acta 964: 377–382.

Peacock, A. J., C. Pickett, K. Morris, and J. T. Reeves, 1990. Spontaneous hypoxaemia and right ventricular hypertrophy in fast growing broiler chickens reared at sea level. Comp. Biochem. Physiol. 97A:537–541.

Rodriguez, J. V., A. M. Torres, and M. M. Elias, 1987. Renal and hepatic glutathione pool modifications in response to depletion treatments. Can. J. Physiol. Pharmacol. 65:84–86.

SAS Institute, 1996. SAS® User's Guide. Version 6.12 Edition. SAS Institute Inc., Cary, NC.

Scheele, C. W., E. Decuypere, P.F.G. Vereijken, and F.J.G. Schreurs, 1992. Ascites in broilers II. Disturbances in the hormonal regulation and fat metabolism. Poultry Sci. 71: 1971–1984.

Wideman, R. F., Jr., and W. Bottje, 1993. Current understanding of the ascites syndrome and future directions. Pages 2–20 *in*: Proceedings of the Nutritional and Technical Symposium. Novus International, St. Louis, MO.

Wideman, R. F., Jr., and Y. K. Kirby, 1995a. A pulmonary artery clamp model for inducing pulmonary hypertension syndrome (ascites) in broilers. Poultry Sci. 74:805–812.

Wideman, R. F., Jr., and Y. K. Kirby, 1995b. Evidence of a ventilation-perfusion mismatch during acute unilateral pulmonary artery occlusion in broilers. Poultry Sci. 74: 1209–1217.

Wideman, R. F., Jr., Y. K. Kirby, M. Ismail, W. G. Bottje, R. W. Moore, and R. C. Vardeman, 1995. Supplemental l-arginine attenuates pulmonary hypertension syndrome (ascites) in broilers. Poultry Sci. 74:323–330.

Wideman, R. F., Jr., Y. K. Kirby, R. L. Owen, and H. French, 1997. Chronic unilateral occlusion of an extrapulmonary primary bronchus induces pulmonary hypertension syndrome (ascites) in male and female broilers. Poultry Sci. 76:400–404.

Yu, B. P., 1994. Cellular defenses against damage from reactive oxygen species. Physiol. Rev. 74:139–162.

Zoratti, M., and I. Szabo, 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241:139–176.