Effects of *gpx4* Haploid Insufficiency on GPx4 Activity, Selenium Concentration, and Paraquat-Induced Protein Oxidation in Murine Tissues

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Selenium-dependent glutathione peroxidase-4 (GPx4) catalyzes the reduction of phospholipid hydroperoxides. Because a full *gpx4* knockout is embryonic lethal, we examined the effect of deletion of one copy of *gpx4* on the activities of three selenoperoxidases (GPx1, GPx3, and GPx4), selenium concentrations, and pro-oxidant–induced protein oxidation in various tissues of mice. A total of 32 *gpx4* hemizygous ($GPx4^{+/-}$) and wild-type (WT) mice (8- to 10-weeks old; 16 males and 16 females) were fed a selenium-adequate diet and given an intraperitoneal injection of paraquat (PQ; 24 mg/kg body wt) or phosphate-buffered saline (PBS). All mice were euthanized 4 hrs after injection to collect tissues for analyses. In PBS-treated mice, GPx4 activities in lung, liver, kidney, and testes of $GPx4^{+/-}$ mice were 24–39% lower ($P < 0.05$) than in WT mice. Among PQ-treated mice, only testis GPx4 activity in $GPx4^{+/-}$ mice was significantly lower (54% $P < 0.05$) than WT mice. Selenium concentration in testes, but not in other tissues, was reduced (34% $P < 0.05$) in $GPx4^{+/-}$ mice compared with WT mice, irrespective of treatment. Tissue GPx1 activities and plasma GPx3 and alanine aminotransferase (ALT) activities were unaffected by PQ treatment or *gpx4* hemizygosity. Total protein carbonyl was elevated (73% $P < 0.05$) by PQ only in lung, and this effect of PQ was independent of genotypes. In conclusion, *gpx4* haploid insufficiency reduced GPx4 activities and/or selenium concentrations, but had no effect on pro-oxidant–induced protein oxidation in various tissues of mice. Exp Biol Med 230:709–714, 2005

Key words: oxidative stress; paraquat; phospholipid hydroperoxide; glutathione peroxidase; gpx4; hemizygous; selenium

Introduction

Phospholipid hydroperoxide glutathione peroxidase, or GPx4, was discovered by Ursini *et al.* (1) as a new selenoprotein from pig liver extract with the ability to protect cellular lipids against peroxidation and to reduce phosphatidylcholine hydroperoxides. Being distinct from cellular glutathione peroxidase-1 (GPx1; Ref. 2), GPx4 is expressed as three isoforms with alternate start codons and exons: a 23-kDa form (with a 27–amino acid mitochondrial targeting sequence that is later cleaved), a 20-kDa nonmitochondrial form, and a 34-kDa sperm nucleus form (with an alternate first exon; Refs. 3, 4). Compared with other seleno-GPx proteins, GPx4 shares an approximately 30%–40% nucleotide identity (5). The enzyme functions as a monomer rather than a tetramer (as in the case of other GPx proteins), and it is the only GPx that is able to reduce phospholipid hydroperoxides (6). Nutritionally, GPx4 is much more resistant to dietary selenium deficiency than the other GPx enzymes, particularly GPx1. When liver GPx1 activity and protein are reduced to nearly zero in selenium-depleted rodents (7–9), liver GPx4 activity maintains approximately 20% of the selenium adequate levels (7, 10). However, the relative portion of total tissue selenium in the form of GPx4 protein, and the effect of GPx4 expression on the expression of other selenoperoxidases in various tissues are unclear, and could not be determined using conventional selenium-deficient animal models.

Yant *et al.* (11) found that a full knockout of *gpx4* in mice is lethal at embryonic Day 7.5 and postulated an essential function of GPx4 in development. It is well known that GPx4 protein and activity are very high in testes (7, 12). Ursini *et al.* (13) reported that GPx4 is involved in sperm maturation and serves a structural role in the sperm tailpiece in an oxidatively cross-linked state, offering an explanation for the detrimental effects of selenium deficiency on male reproductive function (14). In addition, GPx4 has been proposed as a modulator of various eicosanoids, which serve as inflammatory lipid-signaling molecules (15–19).

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However, GPx4 was initially suggested as an important antioxidant enzyme (6). Although *gpx4* haploid insufficiency was shown to render murine embryonic fibroblasts susceptible to pro-oxidant–induced oxidative stress (11, 20), such *in vivo* impacts of GPx4 have not been well studied.

Because homozygous *gpx4* knockout is lethal (11), we used the newly developed *gpx4* hemizygous ($GPx4^{+/-}$) mice (11) in the present study to examine the effect of deletion of a single copy of *gpx4* on the activities of three selenoperoxidases (GPx1, GPx3, and GPx4), selenium concentrations, and susceptibilities to pro-oxidant–induced protein oxidation in various tissues. Paraquat (methyl viologen, PQ) was chosen as the inducer of acute oxidative stress. This pro-oxidant compound primarily targets lung, a site of relatively high GPx4 activity (7, 21, 22). Because it is believed that PQ promotes the formation of superoxide radicals causing oxidative damage to important biomolecules, we chose protein carbonyl formation as an indicator of protein oxidation (23).

Materials and Methods

Mice. The generation of $GPx4^{+/-}$ mice was previously described (11). All mice were bred and housed in the mouse facility at Cornell University and were given free access to a selenium-adequate (0.26 mg of Se/kg body wt) rodent diet (#8604; Harlan-Teklad Madison, WI) and distilled water. Genotypes of mice were verified by a polymerase chain reaction (PCR) method modified from the one previously described (11), using a different reverse knockout primer located within the *hprt* cassette: 5′-GCACACTGGCAAAACAATGC-3′. Our study was approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with the National Institutes for Health guidelines for animal care.

Experimental Procedure. A total of 32 $GPx4^{+/-}$ and WT mice (half male, half female; 8- to 10-weeks old) were assigned to treatments with PQ or phosphate-buffered saline (PBS) in a 2 × 2 factorial design (n = 6–10 per genotype by treatment). Mice were given an intraperitoneal injection of 24 mg of PQ/kg body wt (PQ was dissolved in PBS at 2.4 mg/ml and passed through a 0.2-μm sterile filter) or an equivalent volume of sterile-filtered PBS. All mice were euthanized at 4 hrs after injection by carbon dioxide asphyxiation followed by exsanguination with a heparinized syringe. The PQ dose and treatment interval were chosen based on responses of mice with the same genetic background to various doses of pro-oxidants and different lengths of exposure time (24–26).

Two plasma samples were prepared from the blood: one was used fresh for plasma alanine aminotransferase (ALT) activity assay and the other was snap frozen in liquid nitrogen for later GPx3 activity analysis. Lung, liver, kidney, and testes were collected, rinsed in 0.9% saline and snap frozen in liquid nitrogen. All snap-frozen samples were stored at –80°C before analysis.

Biochemical Analyses. All chemicals and materials were purchased from Sigma Chemical (St. Louis, MO), unless otherwise indicated. Plasma ALT activity was assayed using the Infinity ALT reagent (Thermo Electron Corp., Waltham, MA) at 30°C, according to manufacturer's instructions. Tissue homogenates were prepared as previously described (27). Activities of GPx1 and GPx4 in tissue homogenates were measured by the NADPH–coupled assay, using hydrogen peroxide and phosphatidylcholine hydroperoxide as the substrate, respectively (27). The GPx3 was measured in plasma using the same assay as GPx1. Protein was measured by the Lowry method (28). Tissue selenium concentration was determined using the improved fluorometric method of Olson *et al.* (29) and expressed as microgram per gram of wet tissue weight. As a hallmark of protein oxidation, total protein carbonyl was determined by a spectrophotometric method (based on Refs. 23 and 30) and expressed as nanomoles of protein carbonyl per milligram of protein.

Statistics. Data were analyzed using the general linear model procedure in SAS (release 9.1; SAS Institute, Cary, NC) as a 2 × 2 factorial analysis of variance (ANOVA) for main effects. A Tukey test was used for mean comparisons. Significance was defined as $P < 0.05$. Results are expressed as mean ± SEM.

Results

All animals survived the PQ injection and were apparently healthy throughout the 4-hr treatment. After euthanasia, no gross abnormalities of internal organs were observed.

Tissue GPx4 and GPx1 Activities. Testes GPx4 activity was 36% ($P < 0.05$) and 54% ($P < 0.01$) lower in the PBS- and PQ-treated $GPx4^{+/-}$ mice than in the WT mice, respectively (Fig. 1). Paraquat treatment enhanced testes GPx4 activity in the WT mice ($P < 0.05$), but not in the $GPx4^{+/-}$ mice. Liver, lung, and kidney GPx4 activities in the PBS-treated $GPx4^{+/-}$ mice were 24%, 27%, and 39% lower ($P < 0.05$) than the PBS-treated WT mice, respectively. The PQ treatment did not cause significant difference in GPx4 activities between the two genotypes in these three tissues. There was no PQ or genotype effect on GPx1 activities in any of these tissues (Table 1).

Plasma ALT and GPx3 Activities. The PQ injection caused no changes in plasma ALT or GPx3 activities in either genotype (Table 1). There were no genotype differences in plasma ALT or GPx3 activities within either treatment.

Tissue Selenium Concentrations. Testis selenium concentration in the $GPx4^{+/-}$ mice was 34% lower ($P < 0.01$) than in WT mice, regardless of the treatment (Fig. 2). There was no treatment or genotype effect on selenium concentrations of other tissues (Table 1).

Protein Carbonyl Concentrations. Total protein carbonyl in lung of the PQ-treated mice was 73% higher (P

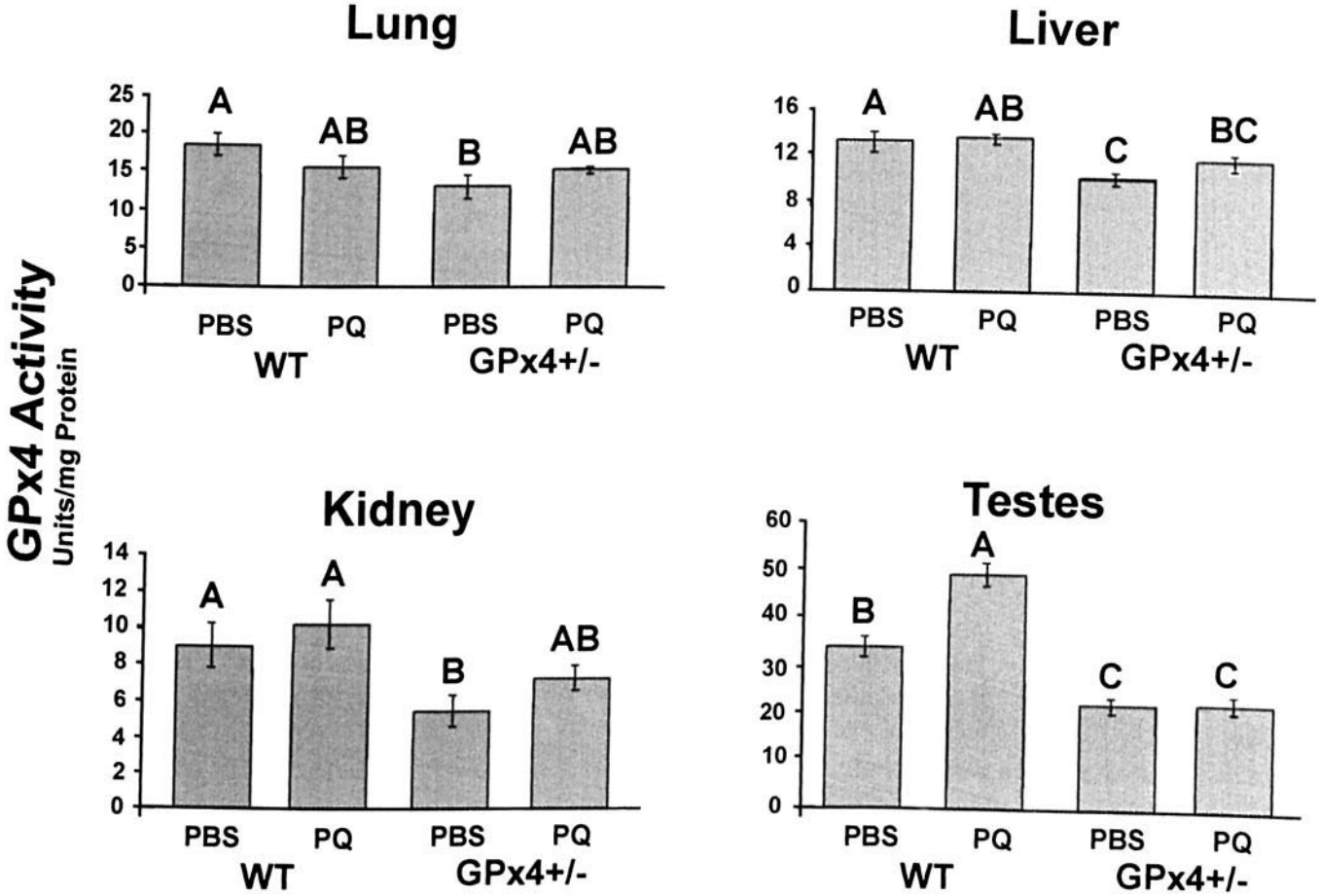

Figure 1. Effect of *gpx4* hemizygosity and PQ treatment on GPx4 activity. The PBS-treated GPx4$^{+/-}$ mice showed 24%, 27%, 39%, and 36% lower activity in the lung, liver, kidney, and testes than WT mice, respectively ($P < 0.05$). Values are expressed as means ± SEM ($n = 3$ for testes; $n = 6$ for other tissues). Values not sharing a common superscript are significantly different at $P < 0.05$.

< 0.05) than in the PBS-treated mice, irrespective of genotypes (Fig. 3). No significant effects of genotype or treatment were shown on total protein carbonyl in liver, kidney, or testes.

Discussion

Our results provide us with three major novel findings. First, deletion of one copy of *gpx4* resulted in a 24%–39% baseline reduction in GPx4 activities in liver, lung, kidney, and testes, compared with the WT mice. Although Yant *et al.* (11) and Ran *et al.* (20, 31) determined GPx4 mRNA and protein changes in various tissues and cells of GPx4$^{+/-}$ and GPx4-overexpressing mice, respectively, our study represents the first evidence for the effect of *gpx4* haploid insufficiency on its enzymatic activity *in vivo*. Because changes in GPx4 mRNA or protein do not necessarily predict the actual activity of GPx4, it is necessary to know GPx4 activities in different tissues of GPx4$^{+/-}$ mice, if the model is to be used for metabolic functional studies. For example, Yant *et al.* (11) found an approximately 50% reduction in GPx4 mRNA and protein in the liver of GPx4$^{+/-}$ mice. However, we have found only a 24% reduction in GPx4 activity. Similarly, Yant *et al.* (11) showed the lowest percentage reduction of GPx4 mRNA and protein in testes of GPx4$^{+/-}$ mice, whereas we have demonstrated that the *gpx4* allelic deletion produced the highest percentage change in overall GPx4 activity (average 40% decrease in PBS- and PQ-treated groups combined) in the testes. Overall, the deletion of one allele of *gpx4* did not lead to a 50% activity reduction in any tissue, contrary to some other cases of gene knockout (32–34). Thus, GPx4 activity or expression of the remaining allele of *gpx4* might be upregulated somewhat in these tissues to compensate for the deletion.

Our second interesting finding was that *gpx4* haploid insufficiency resulted in the reduction of selenium concentration in testes, but not in other tissues. This implies that a relatively small amount of tissue selenium was associated with the reduction of GPx4 activities in lung, liver, and kidneys. In contrast, selenium concentration in testes of GPx4$^{+/-}$ was reduced in proportion to the activity reduction. Overall, the PBS- and PQ-treated animals had an average of 40% reduction in GPx4 activity and a 34% reduction of

Table 1. Effects of *gpx4* Hemizygosity and PQ Treatment on GPx1, GPx3, and ALT Activity and Tissue Selenium Concentrations[a]

	WT mice		$GPx4^{+/-}$ mice	
	PBS	PQ	PBS	PQ
Cellular GPx1 (nmol GSH oxidized/min/mg protein)				
Lung	123.4 ± 10.1	127.4 ±12.5	113.4 ± 11.4	131.8 ± 13.2
Liver	1042.0 ± 32.6	1157.4 ± 30.4	1078.5 ± 47.1	1114.9 ± 42.7
Kidney	733.1 ± 70.2	744.0 ± 25.7	803.6 ± 74.3	757.2 ± 35.3
Testes	76.0 ± 8.3	61.7 ± 8.8	86.3 ± 5.5	67.4 ± 8.6
Plasma GPx1 (nmol GSH oxidized/min/mg protein)				
	61.5 ± 4.1	71.8 ± 7.6	78.1 ± 9.3	59.4 ± 3.9
Plasma ALT (U/L)				
	99.5 ± 28.3	52.7 ± 19.6	82.7 ± 21.4	91.5 ± 34.3
Tissue selenium concentration (μg/g wet tissue weight)				
Lung	0.47 ± 0.02	0.46 ± 0.02	0.46 ± 0.03	0.45 ± 0.02
Liver	1.49 ± 0.06	1.47 ± 0.07	1.49 ± 0.07	1.42 ± 0.04
Kidney	1.34 ± 0.05	1.35 ± 0.06	1.33 ± 0.06	1.25 ± 0.04

[a] Values are means ± SEM; $n = 6$ for lung, liver, kidney, and plasma; $n = 3$ for testes. GSH, glutathione.

testes selenium concentration. Accordingly, full expression of GPx4 should account for 85% of total selenium in testes. Because the PQ-associated GPx4 increase in activity in the testes of WT mice did not alter selenium concentration, post-translational regulation of the enzyme might be responsible for these changes under the present experimental conditions. A similar GPx4 response to oxidative stress has been reported in rat polymorphonuclear neutrophils, in which GPx4 activity was upregulated in response to increased oxidative stress during an inflammatory challenge (35). Apparently, these upregulations of GPx4 activity by pro-oxidant challenge depend on both genotype and tissue or cell type (35). Because tissue GPx1 activities and plasma GPx3 activity were unaffected by $GPx4^{+/-}$, the expression of these two selenoperoxidases is independent of that of GPx4. Our data extend a similar observation by Ran *et al.* (20) in embryonic fibroblasts of $GPx4^{+/-}$, and are consistent with the impact of GPx4 overexpression on tissue GPx1 activity in mice (31). Conversely, Cheng and colleagues showed unchanged GPx3 and GPx4 activities in $GPx1^{-/-}$ mice (36).

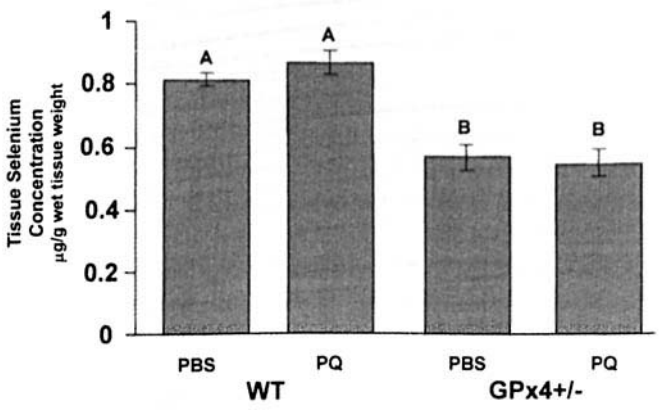

Figure 2. Effect of *gpx4* hemizygosity and PQ treatment on testes selenium concentration. $GPx4^{+/-}$ testes exhibited a 31%–37% lower selenium concentration than WT mice ($P < 0.05$). Values are expressed as means ± SEM ($n = 3$). Values not sharing a common superscript are significantly different at $P < 0.05$.

Our third interesting finding is that reduction of GPx4 activities in liver, lung, kidney, and testes of $GPx4^{+/-}$ mice did not sensitize these tissues to the PQ-mediated protein oxidation. This is contrary to the greater susceptibility of embryonic fibroblasts derived from $GPx4^{+/-}$ mice than those from WT mice to oxidative stress inducers of λ-irradiation, PQ, t-butyl hydroperoxide, hydrogen peroxide, and normoxic versus hypoxic conditions (11, 20). Obviously, results from cultured cells do not necessarily reflect the metabolic role of GPx4 under physiologic conditions, and the level of oxidative stress used in these cell studies may be higher than in the present study. Although the dose of PQ used in the present study did not affect survival or cause changes in plasma ALT activity and gross pathology, it did cause a significant increase in protein carbonyl formation in the target tissue of lung in both WT and $GPx4^{+/-}$ mice. This induction of protein oxidation in lung was comparable to that by other doses of PQ in liver of selenium-deficient mice in our previous studies (24, 26) and in the lung of rat in studies by Winter *et al.* (37). Thus, the 24%–39% reduction in GPx4 activity in different tissues of $GPx4^{+/-}$ mice was not sufficient to compromise the defense against the pro-oxidant–induced protein oxidation. Because measurements were made at only one time after only one level of PQ dosage in the present study, future research with various doses of PQ or other pro-oxidants targeting different tissues should be tested to assess the role of GPx4 in *in vivo* antioxidation. Paraquat doses >50 mg/kg body wt may produce sufficient oxidative stress to detect differences between

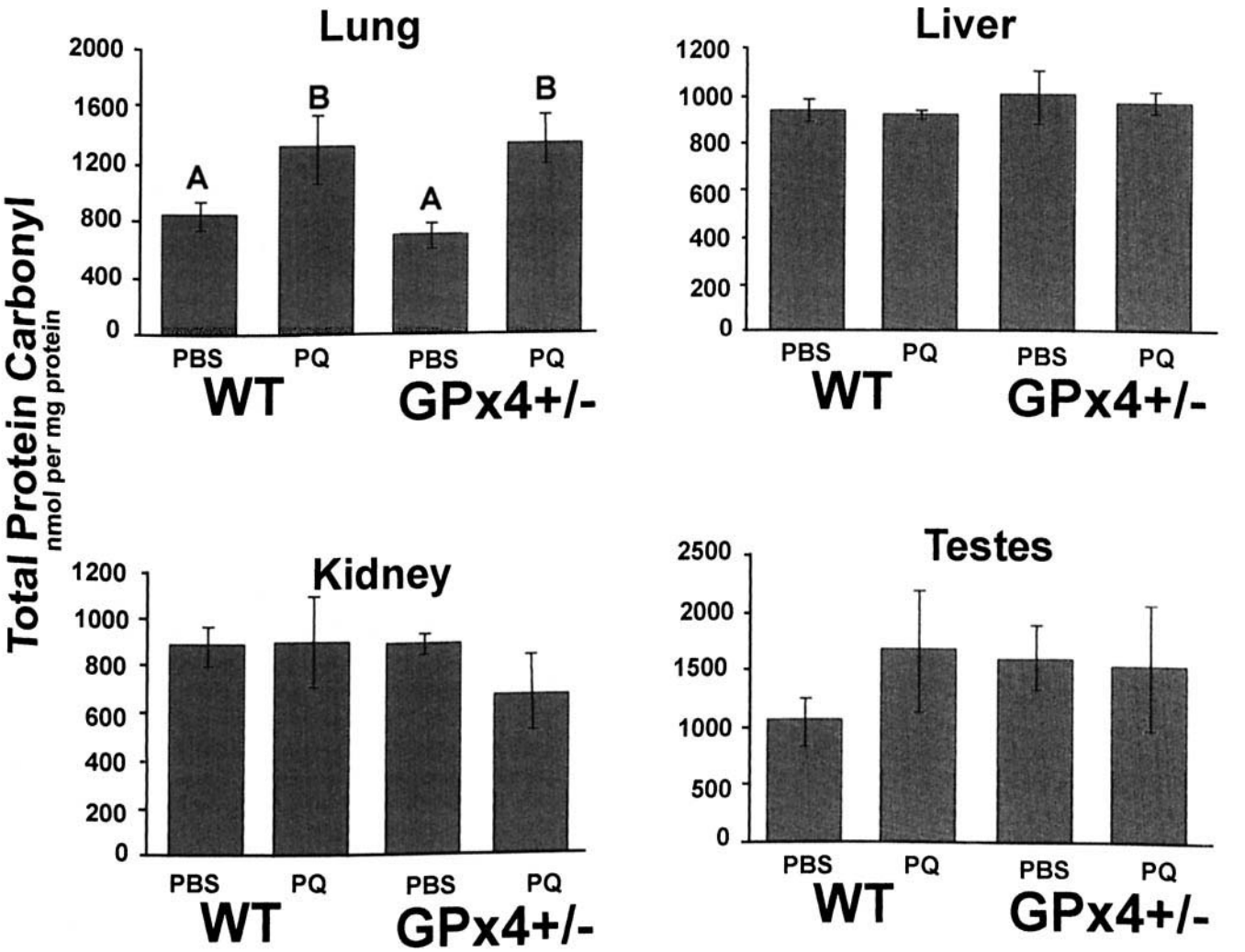

Figure 3. Effect of *gpx4* hemizygosity and PQ treatment on total tissue protein carbonyl. Overall, PQ-treated mice had a 73% higher carbonyl concentration in the lung than PBS-treated controls ($P < 0.05$). Values are expressed as means ± SEM ($n = 3$ for testes; $n = 6$–10 for other tissues). Values not sharing a common superscript are significantly different at $P < 0.05$.

WT and GPx4$^{+/-}$ mice (23). Diquat is structurally and functionally similar to PQ and targets the liver rather than the lung. We have found doses of diquat in the range of 24–48 mg/kg body wt to be useful for detecting changes in antioxidation (38). Cadmium is believed to damage the testes by oxidative stress (39, 40); therefore, this heavy metal presents an opportunity to target an important site of GPx4 function with oxidative stress *in vivo*.

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