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GLUTATHIONE STATUS OF ISOLATED RABBIT LUNGS

EFFECTS OF NITROFURANTOIN AND PARAQUAT PERFUSION WITH NORMOXIC AND HYPEROXIC VENTILATION

JACOB R. DUNBAR,* ANTHONY J. DELUCIA and LESTER R. BRYANT

Department of Surgery, Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, TN 37614, U.S.A.

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Abstract—Thirty-minute perfusion of isolated rabbit lungs with a Krebs–Ringer bicarbonate buffer containing 420 μM paraquat (PQ) or nitrofurantoin (NF) resulted in increases in lung oxidized glutathione (GSSG) content of 589 and 2656%, respectively, over control levels. The degree of glutathione efflux was also increased with both agents, i.e. 77 and 238% above control leakage for PQ and NF respectively. The pulmonary toxicity of both compounds is known to be heightened by conditions of hyperoxia(O_2). Ventilation of lungs with 95% O_2–5% CO_2 did not, in itself, significantly alter glutathione efflux, GSH or GSSG levels. However, ventilation with 95% O_2–5% CO_2 increased lung GSSG levels in PQ-perfused lungs 225% over PQ-air-perfused lungs, a combined effect not observed with NF-O_2, wherein mean GSSG levels were only 72% of that observed with NF-air. Glutathione efflux in PQ-O_2-treated lungs declined somewhat (20%) compared to that observed with PQ-air, but a significant increase in the amount of glutathione efflux was seen with NF-O_2-treated lungs, i.e. 120 and 310%, respectively, over that attributable to NF or O_2 alone. Although the biochemical mechanisms of toxicity of these compounds are thought to be very similar, the disparate degree of GSH oxidation observed with equimolar levels of PQ and NF may indicate differences in reactivity towards glutathione and other lung sulfhydryl pools. The stimulation of the oxidative effects of PQ and NF on lung GSH due to hyperoxic ventilation may be related to the reported O_2 enhancement of their toxicity.

There are many biochemical similarities in the pulmonary toxicity of paraquat (PQ)† and nitrofurantoin (NF) [1–3]. Concomitant with NADPH oxidation, both compounds undergo microsomal reduction via NADPH–cytochrome *c* reductase to their free-radical intermediate. Upon reaction with O_2, these radicals are reoxidized to the parent compound with reduction of O_2 to a superoxide free radical ($O_2^{\cdot}$) [2, 4]. The result of this reduction–oxidation cycle is production of reactive oxygen species and increased NADPH oxidation. PQ toxicity has been attributed to peroxidation of lipids by oxygen radicals [5] and metabolic consequences of thiol or NADPH oxidation [6]. It is now evident that similar mechanisms of oxidant-type toxicity may apply to NF [1].

Despite presumptive similarities of NF- and PQ-induced pulmonary toxicity, few comparative studies of their toxic effects have been done. Because of the role of reduced glutathione (GSH) as a primary cellular antioxidant, this investigation was undertaken to compare the oxidant effect of equimolar concentrations of PQ and NF on the isolated, perfused lung through measurements of tissue glutathione status. Oxygen is known to enhance the toxicity of both compounds [1, 7]; hence, possible synergistic effects of 95% O_2 ventilation on lung glutathione were also studied in PQ- and NF-treated preparations.

MATERIALS AND METHODS

Lungs of male, New Zealand white rabbits (2.0 to 2.5 kg) were surgically isolated and perfused at 37° as previously described [8] with 120 ml of a recirculating Krebs–Ringer bicarbonate buffer, pH 7.4, containing 5 mM glucose, 0.01 units insulin/ml, and 4.5% Ficoll 70. Perfusions were conducted with a constant flow rate of 50 ml/min for 30 min. Perfusates for NF and PQ exposures contained a 420 μM concentration of the appropriate compound, equivalent to 50 μmoles/120 ml perfusate. The lungs were ventilated during the 30 min of perfusion with either 95% air: 5% CO_2 or 95% O_2:5% CO_2 at 20 ml tidal volume, 40 breaths/min, with maintenance of a 3 cm H_2O positive end expiratory pressure.

Upon termination of perfusion, the perfusate was collected and centrifuged for 10 min at 1000 *g* for

* Address correspondence to: Jacob R. Dunbar, Department of Surgery, Box 19750A, East Tennessee State University, Johnson City, TN 37614.

† Abbreviations used in text: PQ, paraquat; GSH, reduced glutathione; GSSG, oxidized glutathione; TG, total glutathione; NF, nitrofurantoin; LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GR, glutathione reductase; GP, glutathione peroxidase.

removal of slight red cell contamination. The lung was blotted, and a portion was weighed for dry/wet weight determination. The remainder of the tissue was minced, and a 10% (w/v) lung homogenate was prepared in cold 0.15 M NaCl–0.05 M KH_2PO_4 buffer, pH 7.4. Trichloroacetic acid-deproteinized samples of this homogenate, plus an *n*-ethylmaleimide-treated homogenate aliquot, were prepared as previously described [8] for determination of lung GSH and GSSG by the nanogram-sensitive method of Tietze [9]. This same method was employed for measurement of perfusate GSH + GSSG (glutathione efflux).

The remaining lung homogenate was centrifuged at 10,000 *g* for 20 min at 4°. The following enzyme activities were measured spectrophotometrically at 37° in the resulting post-mitochondrial supernatant fraction by recording changes in 340 nm absorption resulting from the oxidation or reduction of their appropriate pyridine nucleotide cofactors: lactic acid dehydrogenase (EC 1.1.1.27) (LDH) [10], glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PD) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (6PGD) [11], GSSG reductase (EC 1.6.4.2.) (GR) [12], and GSH peroxidase (EC 1.11.1.9) (GP) [13]. Protein measurements were made on whole lung homogenate and the post-mitochondrial fraction by the method of Lowry *et al.* [14].

Data were analyzed by two-way analysis of variance after blocking into two levels of ventilation by three levels of perfusate. If the above analyses indicated significant differences among perfusates, pairwise comparisons of the three perfusates (control, PQ, NF) were made by the Student–Newman–Keuls procedure. The level of statistical significance was chosen at $P < 0.05$.

RESULTS

Tissue and cellular integrity markers. Mean lung tissue dry weight/wet weight ratios for all treatments were not significantly different from that of control perfused lungs (0.17 ± 0.01), indicating minimal fluid accumulation. Lung protein content for all lungs was 79 ± 12 mg/g lung tissue (wet weight; N = 51) with no differences between groups.

LDH leakage was not elevated from the control value of 1.8% of total LDH activity by PQ or NF perfusion. Leakage of LDH from lungs due to O_2 ventilation appeared significantly higher (2.0 ± 0.5% of total LDH) than that due to air ventilation (1.8 ± 0.8%), over all perfusate groups. This apparent increase, however, was the result of a significant decrease ($P < 0.05$) in intracellular LDH activity in O_2-ventilated lungs rather than an actual increase in leakage. LDH activity was 1130 ± 243 and 852 ± 205 moles NADH oxidized·min^{-1}·(mg protein)$^{-1}$ in room air- and O_2-ventilated treatment groups respectively.

Status of lung glutathione pools. Total lung glutathione content (TG), comprised of reduced glutathione (GSH), oxidized glutathione (GSSG), and glutathione efflux, was not significantly different between treatment groups (values for TG ranged from 11.2 to 13.4 μg/mg protein). While TG was comparable in all groups, there were significant treatment effects on individual components constituting TG. Tissue GSH levels were reduced 27% due to NF perfusion. PQ perfusion had no significant effect on GSH (Table 1).

The percentage of intracellular TG accounted for by the GSSG pool (Table 2) was increased significantly over control values following PQ perfusion (589%). NF perfusion increased the GSSG pool 2656 and 300% compared to control and PQ-perfused lungs respectively. There was a significant interaction effect of ventilation gas and perfusate on GSSG. This interaction was attributable to the fact that O_2 ventilation increased GSSG levels 38% in control lungs and 225% in PQ-perfused lungs, while GSSG levels of O_2-ventilated NF-perfused lungs were 28% lower than comparably perfused, air-ventilated lungs.

Over all levels of ventilation, glutathione efflux, as percent of TG, was increased 77% over controls by PQ perfusion (Table 3). NF perfusion increased glutathione efflux 238 and 93% above control and

Table 1. Intracellular reduced glutathione levels of perfused rabbit lungs*

Perfusate	Ventilation: Air	Ventilation: 95% O_2:5% CO_2	Perfusate group means†
Control	11.8 ± 2.1 (15)	11.9 ± 1.6 (6)	11.8 ± 1.9 (21)
420 μM NF	8.1 ± 2.2 (11)	9.3 ± 1.6 (7)	8.6 ± 2.0 (18)
420 μM PQ	10.6 ± 1.4 (7)	11.8 ± 1.9 (5)	11.1 ± 1.7 (12)
Ventilation group means†	10.3 ± 2.6 (33)	10.8 ± 2.0 (18)	

* Expressed in micrograms GSH/milligram lung protein ± standard deviation; numbers in parentheses equal sample size. Perfusate group means were significantly different ($P < 0.05$) by two-way analysis of variance. Pairwise comparisons of perfusate groups by Student–Newman–Keuls indicated: NF < control = PQ ($P < 0.05$).

† Weighted means due to unequal sample size.

Table 2. Oxidized glutathione levels of perfused rabbit lungs as a percentage of intracellular total glutathione*

Perfusate	Ventilation: Air	Ventilation: 95% O_2:5% CO_2	Perfusate group means†
Control	0.8 ± 0.5 (15) [0.7]	1.1 ± 0.6 (6) [1.0]	0.9 ± 0.5 (21) [0.8]
420 μM NF	27.8 ± 12.2 (11) [25.6]	20.0 ± 6.1 (7) [20.0]	24.8 ± 10.8 (18) [22.8]
420 μM PQ	3.2 ± 1.8 (7) [2.7]	10.4 ± 3.9 (5) [10.0]	6.2 ± 4.6 (12) [5.3]
Ventilation group means†	10.3 ± 14.4 (33) [3.1]	11.0 ± 9.2 (18) [5.9]	

* Percent GSSG ± standard deviation; calculated as micrograms intracellular GSSG/micrograms intracellular GSH + GSSG × 100; the numbers in parentheses equal the sample size. At the lowest levels of intracellular GSSG encountered in this study [about 100 ng GSSG/mg protein (equivalent to approximately 15 ng GSSG/1 ml reaction mixture)] recovery of GSSG standard solutions indicated this measurement to be 93 ± 3% accurate. Due to heterogeneity of variance, analysis was performed on log-transformed data; the number in brackets indicates back-transformed means. Two-way ANOVA showed significance ($P < 0.05$) due to perfusate, ventilation, and P × V interaction. Pairwise comparisons of perfusate groups via Student–Newman–Keuls indicated: NF > PQ > control ($P < 0.05$).

† Weighted means due to unequal sample size.

PQ-induced glutathione efflux respectively. Oxygen ventilation increased glutathione efflux in NF-perfused lungs by 120% and in control lungs by 33%. In contrast, glutathione efflux was 20% lower in O_2-ventilated, PQ-perfused lungs compared to air-ventilated, PQ-perfused lungs. Hence, the significant interaction effect of ventilation × perfusion shown in Table 3.

Activities of glutathione-related enzymes. Activities of G6PD, 6PGD, and GP were significantly lower in NF-perfused lungs (Table 4, A, B and C) over all levels of ventilation (29, 19, and 24% less than control respectively). There were no significant changes in the activities of these enzymes due to PQ perfusion. GSSG reductase activity (Table 4D) was 30% higher in O_2-ventilated lungs across all perfusates.

Examination of the data suggested a possible relationship of G6PD and 6PGD activity to glutathione efflux (normalized as a percent of TG—Table 3) in lungs perfused with NF or PQ. Panels A and B of Fig. 1 show that there was, indeed, a significant,

Table 3. Percentage of total lung glutathione leaked to perfusate during 30-min experiments*

Perfusate	Ventilation: Air	Ventilation: 95% O_2:5% CO_2	Perfusate group means†
Control	1.2 ± 0.4 (15)	1.6 ± 0.3 (6)	1.3 ± 0.4 (21)
420 μM NF	3.0 ± 1.4 (11)	6.6 ± 1.6 (7)	4.4 ± 2.3 (18)
420 μM PQ	2.5 ± 0.8 (7)	2.0 ± 0.7 (5)	2.3 ± 0.8 (12)
Ventilation group means†	2.0 ± 1.2 (33)	3.7 ± 2.6 (18)	

* Percent GSH + GSSG leaked ± standard deviation; calculated as micrograms GSH + GSSG leaked/micrograms intracellular + perfusate GSH + GSSG × 100; number in parentheses equal sample size. Two-way ANOVA showed significant differences ($P < 0.05$) due to perfusate, ventilation, and P × V interaction. Pairwise comparisons of perfusate group means by Student–Newman–Keuls indicated: NF > PQ > control ($P < 0.05$). GSSG constituted 90 ± 10% of total glutathione effluent and was not significantly different between groups.

† Weighted means due to unequal sample size.

Table 4. Specific activities of enzymes related to glutathione metabolism*

Perfusate	(A) Glucose-6-phosphate dehydrogenase† Ventilation: Air	Ventilation: 95% O_2:5% CO_2	Perfusate group means‡	(B) 6-Phosphogluconate dehydrogenase† Ventilation: Air	Ventilation: 95% O_2:5% CO_2	Perfusate group means‡
Control	105 ± 37 (16)	102 ± 30 (6)	104 ± 34 (22)	86 ± 16 (16)	92 ± 19 (6)	88 ± 17 (22)
420 μM NF	83 ± 26 (11)	59 ± 7 (7)	74 ± 24§ (18)	77 ± 14 (11)	61 ± 9 (7)	71 ± 14§ (18)
420 μM PQ	100 ± 20 (7)	122 ± 31 (5)	109 ± 26 (12)	87 ± 8 (7)	91 ± 18 (5)	89 ± 13 (12)
Ventilation group means‡	97 ± 32 (34)	91 ± 35 (18)		83 ± 14 (34)	80 ± 12 (18)	
	(C) GSH peroxidase†			(D) GSSG reductase‖		
Control	143 ± 33 (16)	129 ± 30 (6)	139 ± 32 (22)	78 ± 35 (16) [74]¶	114 ± 12 (6) [117]	88 ± 34 (22) [81]
420 μM NF	98 ± 40 (11)	117 ± 36 (7)	105 ± 38§ (18)	73 ± 12 (11) [72]	91 ± 7 (7) [93]	80 ± 14 (18) [79]
420 μM PQ	137 ± 38 (7)	132 ± 21 (5)	135 ± 38 (12)	89 ± 33 (7) [81]	107 ± 4 (5) [105]	96 ± 26 (12) [92]
Ventilation group means‡	127 ± 41 (34)	125 ± 29 (18)		79 ± 29 (34) [74]	103 ± 13 (18) [102]	

* Expressed in nmoles NADPH oxidized or NADP reduced per min per mg protein ± standard deviation, measured at 37°; numbers in parentheses equal sample size.

† Two-way ANOVA: $P < 0.05$ due to perfusate.

‡ Weighted means due to unequal sample sizes.

§ Student–Newman–Keuls: NF < control = PQ ($P < 0.05$).

‖ Two-way ANOVA: $P < 0.05$ due to ventilation treatment.

¶ Numbers in brackets indicate back-transformed means. Analysis was performed on log transformed data due to heterogeneity of variance.

inverse relationship of glutathione efflux with G6PD and 6PGD activity. This relationship was not observed in control perfused lungs under either ventilation treatment (Fig. 1, C and D).

DISCUSSION

Generally, several antioxidant mechanisms exist in the mammalian cell, including superoxide dismutase and catalase, vitamin E, ascorbic acid, and GSH and the GSH-peroxidase pathway. Interactions of PQ and NF with the first of these have been investigated [1, 3], but little information is available regarding the relative effects of these compounds on lung status of endogenous GSH and related enzymes. For this reason we chose specifically to limit our study to the GSH system although it is recognized that the former mechanisms may play important roles in protection from the biochemical sequelae of PQ and NF intoxication.

The perfusate concentration of NF used in this study was chosen as one-half the maximal concentration obtainable in aqueous media. Empirically, the latter was found to cause over 50% oxidation of lung GSH. Hence, we selected an equimolar concentration of PQ to provide a common denominator for comparison of the effects of these toxicants on the perfused lung. It is possible that different results may have been seen with doses or measurement times other than the 420 μM, 30-min perfusions reported here.

Alterations of intracellular glutathione redox status. The PQ- and NF-induced increases of intracellular GSSG levels and glutathione efflux further suggest that oxidant mechanisms are involved in the acute pulmonary toxicity of both compounds. This GSH oxidation and increased efflux of glutathione may have occurred in the course of the aerobic biotransformation of PQ and NF secondary to: (1) the increased rate of NADPH consumption following the cyclic reduction of these compounds via an NADPH-dependent process; (2) direct oxidation of GSH by oxygen free-radicals and/or H_2O_2 formed consequent to the reoxidation of the free-radical intermediates of NF and PQ; or (3) H_2O_2 reduction via GP enzyme.

While lungs perfused with either PQ or NF showed perturbations in GSH:GSSG redox, it is notable that NF perfusion resulted in markedly greater GSH oxidation than did equimolar concentrations of PQ. This difference in potency may be due to different intracellular availability of PQ and NF at sites of biotransformation or relative differences in their rates of redox cycling. As an argument against the latter, however, Mason and Holtzman [2] reported that *in vitro* stimulation of rat pulmonary microsomal

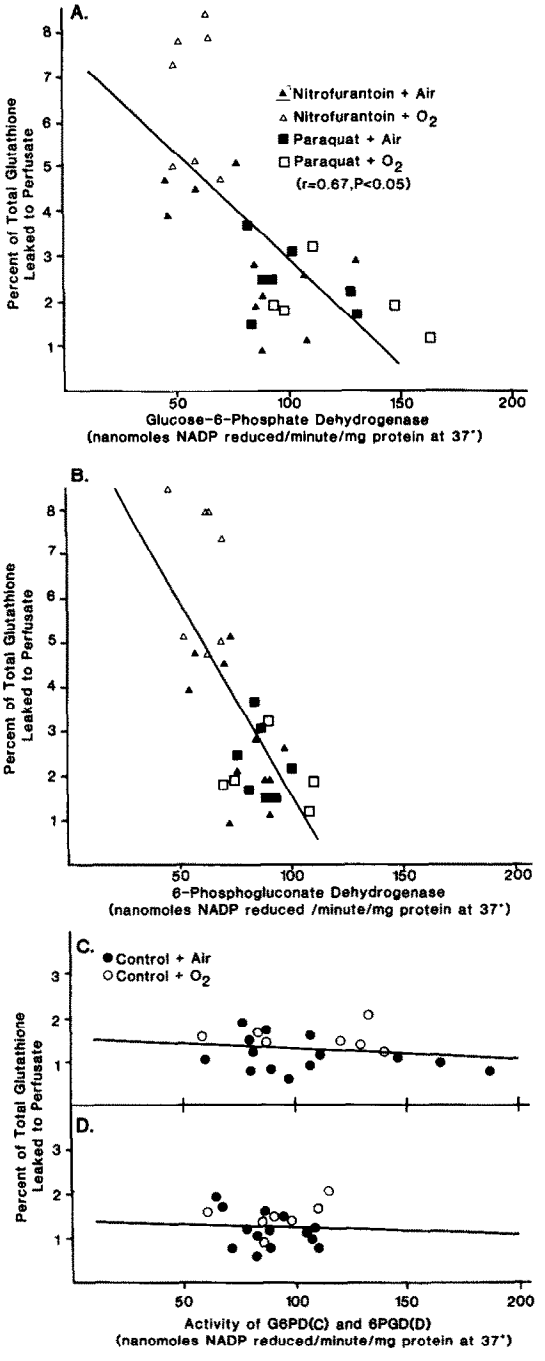

Fig. 1. Activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase vs glutathione efflux from perfused lungs. Significant correlation coefficients were found for G6PD (A: $r = 0.67$) and 6PGD (B: $r = 0.67$) on glutathione efflux. There was also a significant multiple correlation coefficient for both G6PD and 6PGD on glutathione efflux ($r = 0.602$). There was no significant correlation of these enzymes with glutathione efflux in control/air or control/O_2 lungs (C and D: G6PD vs glutathione efflux, $r = 0.22$; 6PGD vs glutathione efflux, $r = 0.03$).

O_2 uptake was comparable with equimolar concentrations of PQ and NF.

Differences in PQ- and NF-stimulated GSH oxidation suggest that PQ and NF may be detoxified by different mechanisms in the perfused lung. Bus *et al.* [5] found that *in vivo* administration of PQ to mice caused depletion of liver GSH but not lung GSH. Instead, a depletion of lipid-soluble antioxidants (mainly vitamin E) was observed in the lung. Adding our findings of greater GSH oxidation in NF-perfused vs PQ-perfused lungs to data on organ speciificity of PQ-induced biochemical lesions, it is possible that the pulmonary detoxification of PQ-induced oxidants occurs primarily at the expense of lipid-soluble antioxidants, while NF-produced oxidants are detoxified predominantly via GSH-related mechanisms. Future comparative studies of both TG and vitamin E in PQ- and NF-perfused lungs should prove of interest in this regard.

NF-induced inhibition of GR has been demonstrated *in vitro* [15, 16], which may have contributed to the disparate effects of PQ and NF on GSSG levels. While we did not detect inhibition of GR at the end of perfusion, we cannot rule out the occurrence of GR inhibition during the 30-min treatment since this inhibition is readibly reversible upon removal of NF.

Differences in the degree of oxidation of GSH by PQ and NF perfusion observed in the present study may be offset *in vivo* by the active uptake of PQ by the lung [17, 18]. Active uptake would ensure longer exposure of lung tissue to PQ than would result from an equivalent dose of NF, a drug which is not actively accumulated by the lung [19]. Our findings suggest, however, that given equivalent exposure, NF may have greater potential than PQ to generate oxidative damage involving lung GSH. The latter statement is further supported by our findings of enzyme inhibition with NF, but not PQ, perfusion.

Hyperoxic potentiation of GSH:GSSG changes. Hyperoxia has been demonstrated to increase toxicity of both PQ and NF [1, 7, 20, 21], possibly by maximizing the rate of redox cycling. The increased levels of GSSG seen in PQ-perfused lungs with concomitant O_2 ventilation may, indeed, have been due to stimulated redox cycling of PQ, resulting in increased formation of superoxide radical. The absence of hyperoxia-stimulated GSH oxidation in NF-perfused lungs might suggest that the redox cycling of 420 μM NF was already maximal with normoxic ventilation of perfused lungs. However, as discussed below, O_2 ventilation did increase NF-induced glutathione efflux, which might indicate that hyperoxia is capable of enhancing the pulmonary toxicity of NF, as has been reported by Boyd *et al.* [1].

Glutathione efflux. Cellular glutathione efflux may be a sensitive monitor of oxidant stress in isolated cells and perfused organs [22]. In our test system, both NF and PQ perfusion resulted in increased glutathione efflux. Hyperoxia significantly increased glutathione efflux in NF-perfused lungs only.

While glutathione efflux is a marker of oxidant stress, it may be, more specifically, a reflection of GSSG production beyond the capacity of the GR system to convert it to GSH [23]. Under conditions of NADPH depletion, NADPH-producing pathways would require stimulation to ensure optimal function of NADPH-dependent processes, such as the activity of GR. Therefore, as a consequence of PQ or NF

depletion of the NADPH pool, biosynthetic and antioxidant cellular processes could be directly dependent upon the rate of NADPH production through the pentose phosphate pathway. The significant correlation of G6PD and 6PGD activity with glutathione efflux observed in this study may reflect, indirectly, a dependence of lung GR activity on immediate provision of NADPH via G6PD or 6PGD activities.

In conclusion, in our model the oxidant toxicity of equimolar concentrations of PQ and NF appeared to differ quantitatively. While these disparate effects on GSH oxidation in the lung may reflect differences in level of toxicity, other interpretations possibly remain hidden due to limitations in the above model and testing procedures employed. The relative potential of PQ and NF to mediate such forms of cellular injury as lipid peroxidation and DNA damage should be considered in light of the variable effects on glutathione that we observed. Other investigation is warranted to include measurement of glutathione simultaneously with studies of relative availability of PQ and NF at sites of activation, functional activity of GR, and involvement of other cellular antioxidant systems.

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