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Hypoxia Increases Plasma Glutathione Disulfide in Rats

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Abstract. We tested the hypothesis that hypoxia causes cellular oxidative stress by measuring plasma concentrations of glutathione disulfide (GSSG) in rats exposed to acute and subacute hypoxia. In awake, unanesthetized, catheter-implanted rats, exposure to 8% O_2 for 10 min caused pulmonary vasoconstriction and increased plasma GSSG. This increase in plasma GSSG was reversible upon re-exposure to room air. In another group of rats exposed to 48 hours of hypobaric hypoxia (P_b 450 mmHg, equivalent to about 14,500 feet altitude), plasma GSSG, but not total glutathione, was significantly increased over control values (2.83 ± 0.24 vs 1.84 ± 0.14 nmol/ml, $p < 0.05$). While lung tissue GSSG in high altitude-exposed rats were somewhat higher than in controls (17.4 ± 7.0 vs 11.9 ± 3.6 nmol/g wet lung wt.), the difference was not statistically significant. Treatment of the rats with a radical scavenger, DMSO, before altitude exposure, blocked the increase in plasma GSSG (1.86 ± 0.16 nmol/ml). We conclude that both acute and subacute hypoxia increase plasma GSSG in rats and speculate that hypoxia induces cellular oxidative stress in vivo.

Key words: Hypoxia—Oxidative stress—Glutathione disulfide (GSSG)—Glutathione—High altitude.

Introduction

Subacute hypobaric hypoxia can cause increased-permeability pulmonary edema in susceptible individuals [14, 21] and chronic hypoxia may result in chronic pulmonary hypertension [20]. While the mechanism for these pulmonary circulatory changes are poorly understood, several studies have suggested

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that increased formation of reactive oxygen intermediates occurs during hypoxic exposure. Firstly, in vitro experiments have indicated that hypoxia, but not anoxia, may promote the accumulation of metabolic intermediates that favor the production of free radicals [12]. Secondly, pre-exposure to hypoxia increased lung tissue levels of antioxidant enzymes [13] and protected rats against subsequent radical-mediated lung injury due to hyperoxia and bleomycin [5, 8]. Thirdly, we have recently shown that a hydroxyl radical scavenger, DMSO, prevented increases both in lung transvascular protein escape and wet-to-body weight ratio in rats exposed to simulated high altitude [24].

In this study, we tested the hypothesis that hypoxic exposure in vivo causes increased radical production by measuring changes in plasma levels of glutathione disulfide (GSSG) in rats exposed to acute and subacute hypoxia. Plasma GSSG is known to increase following administration of chemical oxidants [1, 2] and endotoxin [10] and has been suggested as a sensitive indicator of cellular oxidative stress in intact rats [1, 2].

Methods

Animal Preparation

Male Sprague-Dawley rats (B.W. 310–340 g) were purchased from Harlan Industries (Indianapolis, IN) and allowed free access to food and water. Under anesthesia with ketamine (50–100 mg/kg i.m.) and xylazine (5 mg/kg i.m.), sterile catheters were placed in the carotid and pulmonary artery as previously described [9]. Hemodynamic studies were conducted in awake rats, 24 to 48 h after recovery from anesthesia. Blood samples were drawn from the aortic catheter for measurement of plasma total glutathione (GSH + GSSG) and GSSG.

Glutathione Measurement

Blood collection and plasma glutathione measurement were performed as previously described [10]. Briefly, 100 μl of whole blood were added to 100 μl of 3 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in 100 mM potassium phosphate buffer, pH 7.4, which contained 5 mM EDTA (buffer 1). The sample was centrifuged and the supernatant assayed for total glutathione. For measurement of plasma GSSG, 500 μl of whole blood were added to 300 μl of 10 mM N-ethylmaleimide (NEM) in 100 mM potassium phosphate buffer, pH 6.5, with 5 mM EDTA. After centrifugation, 400 μl of the supernatant were loaded onto an octadecyl Sep-Pak cartridge and eluted with 1 ml of buffer 1. Samples of the Sep-Pak eluate and the supernatant from the plasma-DTNB solution were assayed for glutathione using an enzymatic assay initially described by Tietze [26] and recently modified to allow simultaneous assay of multiple samples in a microwell plate [10].

Acute Hypoxic Exposure Protocol

Five unanesthetized, catheter-implanted rats were studied. Heart rate, mean aortic and pulmonary artery pressures were monitored throughout the study. After collection of the baseline arterial plasma glutathione samples with the rats breathing room air, the study chamber was flooded with 8% O_2 and blood samples were obtained for glutathione measurement 10 min later. In 3 rats the hypoxic exposure was extended to 30 min to allow blood sampling at this later time point. Ten minutes after return to room air breathing, a second normoxic blood sample was obtained.

High Altitude-Exposure Protocol

In the first experiment, 14 male Sprague-Dawley rats (B.W. 250–270 gm) were divided into 2 groups (n = 7 each). One group (HA) was exposed to hypobaric hypoxia (P_b 450 mmHg, equivalent to approximately 14,500 ft.) in an altitude chamber for 48 h, and the other group was left at Denver altitude (5,200 ft, mean P_b of 635 mmHg) and served as controls. At the end of the 48-h exposure period, the rats were anesthetized with pentobarbital (60–80 mg/kg i.p.). One milliliter of whole blood was removed from the right ventricle for glutathione measurement as described above. The lungs were quickly removed into liquid N_2, weighed and homogenized in 4 volumes of buffer 1. Five hundred microliters of the homogenate was added to either 500 μl DTNB (for total glutathione measurement) or 500 μl NEM (for GSSG measurement) and the tissue precipitated with 500 μl of 4% sulfosalicylic acid. After centrifugation (at 2000 x g for 5 min), 500 μl of the supernatant of the homogenate-NEM mixture was loaded onto a Sep-Pak cartridge and eluted with 2 ml of buffer 1 [10]. Glutathione assay on plasma and tissue samples were performed as described above.

In a second experiment, 5 rats were pretreated with 50 mmole/kg of dimethylsulfoxide (DMSO) intraperitoneally before exposure to 48 h of high altitude and 5 additional rats (altitude control) were given saline injection before altitude exposure. Plasma and lung tissue samples were collected and assayed for total glutathione and GSSG. Since the data for the altitude-exposed groups in the 2 experiments were similar, the data for the 2 experiments were combined for analysis.

Statistical Analysis

Data are expressed as means ±SE. Student's t-test is used to compare the means of hypoxic and normoxic values. Means of the high altitude experiment are compared using 1-way analysis of variance and Scheffe's multiple comparison test. Differences are considered significant when $P < 0.05$.

Results

Exposure to 8% O_2 in awake, catheter-implanted rats decreased arterial PO_2 from mean (±SE) of 71.8 (±2.0) torr to 29.5 (±0.9) torr. Figure 1 shows the changes in mean pulmonary artery pressure and plasma GSSG in 1 rat during exposure to hypoxia. A brisk hypoxic vasoconstriction was initially observed with a roll-off during the 30 min of exposure to 8% O_2. Plasma GSSG increased at 10 min and 30 min of hypoxia and returned to baseline value within 10 min after termination of hypoxia. With reexposure to 8% O_2, both the pulmonary arterial pressure and plasma GSSG increased.

As a group, plasma GSSG significantly increased after 10 min of hypoxia (Fig. 2). In the group of 5 rats, the mean increase in plasma GSSG value was 15%. In 3 rats in which hypoxic exposure was extended to 30 min, plasma GSSG remained elevated (mean increase of 14% over normoxic values), but was not significantly higher than the level after 10 min of hypoxia. Plasma reduced glutathione (GSH) was more variable during hypoxia and was not significantly different from normoxic values.

Forty eight hours of hypobaric hypoxia significantly increased plasma GSSG, but not total glutathione (Table 1). This increase in plasma GSSG was blocked by pretreatment with DMSO. Similarly, although lung tissue GSSG in

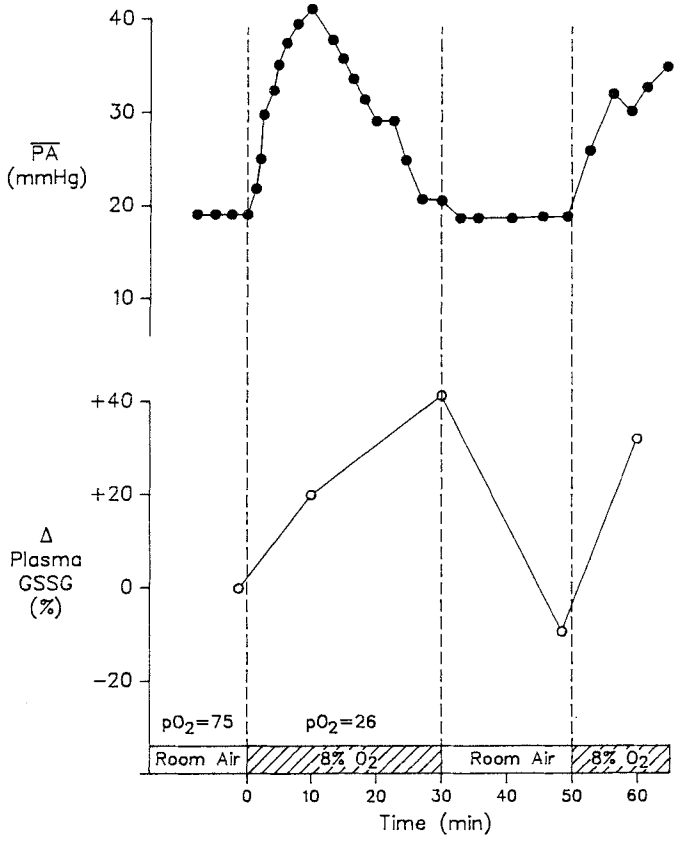

Fig. 1. Pulmonary artery pressure and plasma GSSG changes in an unanesthetized rat exposed to 8% O_2.

altitude-exposed rats was not significantly different from that in controls, lung GSSG was lowest in DMSO-treated rats exposed to altitude (Table 1).

Discussion

In the presence of oxidants such as hydrogen peroxide or lipid peroxides, and catalyzed by the glutathione peroxidase enzyme, 2 molecules of GSH are converted to 1 molecule of GSSG. Because even a small increase in intracellular GSSG is toxic to the cells [18], GSSG is either rapidly converted back to GSH by glutathione reductase enzyme or is actively transported out of the cells. An efficient mechanism for the extracellular transport of GSSG has been shown for erythrocytes and various tissues including liver, heart, and lungs [3, 6, 15, 16, 23]. As extracellular GSSG is not reduced back to GSH, plasma GSSG levels can be a sensitive marker of intracellular oxidative stress. In intact rats, plasma GSSG has been shown to increase following administration of chemical oxidants such as paraquat and nitrofurantoin [1, 2], and agents that stimulate the production of endogenous oxidants such as endotoxin [10].

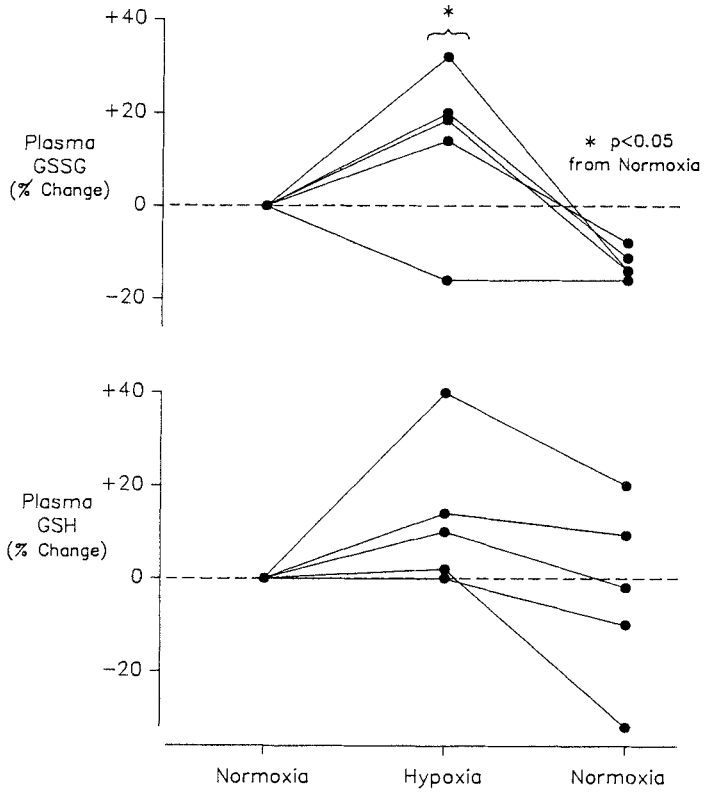

Fig. 2. Effect of acute hypoxic exposure on plasma glutathione in unanesthetized rats. Values for plasma glutathione disulfide (GSSG) and reduced glutathione (GSH), are expressed as % change from the baseline value, and shown for each rat. For the whole group, mean (±SEM) plasma GSSG values (in nmol/ml) for normoxia, hypoxia and normoxia periods were 1.58 (±0.13), 1.78 (±0.14) and 1.39 (±0.11), respectively. The GSH value during hypoxia was not significantly different from that during normoxia.

Table 1. Plasma and lung tissue glutathione status in rats exposed to 48 hours of hypobaric hypoxia

	(n)	Plasma		Lung		
		GSSG (nmol/ml)	GSH + GSSG (nmol/ml)	GSSG (nmol/g)	GSH + GSSG (umol/g)	GSSG/ GSH + GSSG (%)
Control	(7)	1.84 ± 0.14†	27.8 ± 1.2	11.9 ± 3.6	1.40 ± 0.02	0.85 ± 0.26
HA	(12)	2.83 ± 0.24*	30.7 ± 1.3	17.4 ± 7.0	1.40 ± 0.03	1.20 ± 0.45
DMSO + HA	(5)	1.86 ± 0.16†	25.2 ± 1.1	3.5 ± 0.7	1.15 ± 0.08*†	0.32 ± 0.06

Mean ± SEM of plasma and lung tissue glutathione disulfide (GSSG) and total glutathione (GSH + GSSG) are shown for control, high altitude (HA) and DMSO + HA rats. The calculated plasma reduced glutathione (GSH) values did not differ among the 3 groups of rats. *$p < 0.05$ from controls, †$p < 0.05$ from HA rats

In this study, we found a significant increase in plasma GSSG in rats exposed to acute and subacute hypoxia. This change in plasma glutathione is selective for GSSG since no significant difference was observed for plasma GSH between control and hypoxia-exposed rats (Table 1, Fig. 2). This increase in GSSG is not likely to be due to artifactual oxidation of GSH to GSSG during sampling for two reasons: 1) our method of adding blood directly to DTNB or NEM solutions followed by rapid separation of plasma from erythrocytes has been shown to minimize the in vitro oxidation of GSH [1], and 2) Joshi et al. have shown that hypoxia retards the rate of in vitro autooxidation of GSH to GSSG [17]. In addition, the increase in plasma GSSG is not the result of a nonspecific increase in the leakiness of plasma membrane, because the intracellular concentration of GSSG is low and any increase in cell membrane permeability should lead to a greater rise in plasma GSH and not GSSG. More likely, the increased plasma GSSG represents enhanced intracellular redox cycling of glutathione followed by increased extracellular release of GSSG. Our findings that DMSO, a radical scavenger, blocked the increase in plasma GSSG in altitude-exposed rats is consistent with this hypothesis. However, we can not exclude the possibility that DMSO may exert its effect through the inhibition of neutrophils' oxidative metabolism [4], or through other less well-characterized biologic actions.

The cellular/tissue source for the increase in plasma GSSG is not clear from this in vivo study. A recent in vitro study suggested that hypoxia did not increase the hepatic release of GSH or GSSG [11]. Lung is the organ first exposed to the hypoxic gas and subacute hypoxia may lead to lung injury manifested by increased transvascular escape of albumin [25]. While oxidant-induced lung injury has been shown to increase lung tissue GSSG in rats [27], the lack of a significant increase in lung GSSG following high altitude exposure does not rule out a significant degree of oxidative stress to lung cells because: 1) increased tissue GSSG is probably a late finding, signifying the inability of cells to adequately reduce or export GSSG [3, 6, 15, 16], and 2) increased intracellular GSSG may have occurred in a subpopulation of lung cells without affecting total lung GSSG level. It is of interest that lung GSSG level was lowest in the high altitude animals pretreated with DMSO, although the difference from rats exposed to high altitude alone was not statistically significant.

The result of our study is consistent with the hypothesis that hypoxia induces oxidative stress in intact rats. Other studies have come to a similar conclusion. In rats exposed to 10% O_2 for 7 days, Sjostrom and Crapo reported increased cyanide-insensitive oxygen uptake in lung tissue, suggesting an enhanced rate of production of partially reduced oxygen species [22]. More recently, Block and Patel reported that exposure of pulmonary endothelial cells to hypoxia in vitro is associated with increased formation of lipid peroxidative products such as malondialdehyde and conjugated dienes [7]. Potential mechanisms for increased formation of oxidants during hypoxia include enhanced rate of autooxidation of electron transport components [12, 22], and univalent reduction of oxygen by reduced flavin or semiquinone [19]. However, it is important to acknowledge that this hypothesis is far from being proven. All of

the evidence to date is indirect and the challenge remains for a direct demonstration of increased free radical concentration in hypoxic cells or tissues.

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