

Arterial Levels of Oxidized Glutathione (GSSG) Reflect Oxidant Stress *in Vivo*¹

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Neutrophil-related, oxidant-mediated injury to the pulmonary microvasculature appears to follow endotoxemia, cutaneous thermal injury, and ischemia-reperfusion injury to the liver or intestine. Glutathione is an important endogenous intracellular oxygen radical scavenger. Plasma concentrations of oxidized glutathione (GSSG) reflect oxidant injury resulting from an overdose of certain oxidatively metabolized drugs. The purpose of this investigation was to evaluate plasma GSSG as an indicator of oxidant stress resulting from activation of the endogenous inflammatory response. An established model of neutrophil- and oxidant-related acute lung injury following intestinal ischemia and reperfusion in rats was used. Intestinal ischemia was induced by clip occlusion of the superior mesenteric artery (SMA) for 120 min. Reperfusion resulted from SMA clip removal. Following reperfusion for 0, 15, or 120 min, plasma GSSG levels in portal vein, inferior vena cava (IVC), and aorta were obtained. Plasma GSSG was undetectable in sham animals and those with intestinal ischemia alone. Following reperfusion, all plasma samples had significant elevations in GSSG. Aortic plasma GSSG after 15 min of reperfusion was significantly elevated compared to both portal vein and IVC plasma GSSG. These data suggest that oxidant stress after intestinal reperfusion is reflected by elevations in plasma GSSG. The step up in plasma GSSG across the pulmonary vascular bed, a site of known oxidant injury, suggests that plasma GSSG may be a useful marker of oxidant stress *in vivo*, particularly with regard to the pulmonary microvasculature. This simple *in vivo* approach to assessing oxidant stress related to inflammatory tissue injury may have the potential to be of significant use in the clinical setting. © 1990 Academic Press, Inc.

INTRODUCTION

Neutrophil-related, oxidant-mediated injury to the pulmonary microvasculature has been reported following

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endotoxemia [1-3], cutaneous thermal injury [4], and ischemia-reperfusion injury to the liver [5] or intestine [6]. Existing methods of assessing oxygen radical activity *in vivo* are cumbersome, indirect, and have not been clinically applicable [7]. In particular, it has not been feasible to quantify oxidant stress clinically using a plasma marker. A variety of indirect markers of lipid peroxidation including fluorescent products, conjugated dienes, and malondialdehyde have been proposed with limited enthusiasm [7, 8]. However, some information has been accumulated in the toxicology literature suggesting that pharmacologically induced oxidant-mediated tissue injury (using agents such as carbon tetrachloride, acetaminophen, or paraquat) may be assessed more directly by using plasma glutathione determinations [9-13].

Oxygen radical formation in excess of normal physiologic limits appears to be one fundamental mechanism by which tissues are injured. Although highly reactive oxygen radicals result from normal metabolism, efficient endogenous enzymatic systems of removal normally prevent injury. In excess, however, these oxygen products may destroy lipid cell membranes, cause DNA strand breakage, and inhibit important cellular enzymes [14-16]. A critical intracellular component of this endogenous oxygen radical scavenging system is the tripeptide glutathione (GSH). Normally present in its reduced form, intracellular GSH is oxidized in the presence of oxygen radical species and is translocated into the blood in its oxidized form (GSSG) [17, 18]. Arteriovenous differences in plasma GSSG levels across the liver after paraquat administration have been used to evaluate oxidant injury [9]. These data suggest that tissue injured by oxidants may be a source of plasma GSSG. Thus, plasma levels of oxidized glutathione may reflect oxygen radical activity *in vivo*. Systemic plasma GSSG levels might provide an index of whole-body oxidant stress. Arteriovenous differences in GSSG levels across a specific organ might reflect the contribution of that specific organ to plasma GSSG levels [19].

Given the large body of recent evidence for the importance of oxidant-mediated tissue injury resulting from activation of the endogenous inflammatory response, this study was designed to evaluate plasma GSSG as a marker

of *in vivo* oxidant stress. The model used was one of adult respiratory distress syndrome (ARDS)-like acute lung injury induced by an ischemia-reperfusion injury to the rat intestine [6]. The data show that tissue injury resulting from activation of the endogenous inflammatory system produces measurable changes in plasma GSSG levels and that selective sampling may allow assessment of specific target organs.

METHODS

Animal Model

Male pathogen-free, Sprague-Dawley rats (150–250 g) (Charles River, Portage, MI) were used for all experiments. Experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals. Anesthesia was administered by intramuscular (im) injection of 100 mg/kg ketamine hydrochloride. Midline laparotomy was performed and intestinal ischemia achieved by occlusion of the superior mesenteric artery (SMA) using a noncrushing Heifetz microvascular clip. Reperfusion was achieved by removal of the Heifetz clip at a second laparotomy following a second anesthetic (50 mg/ml ketamine hydrochloride). The period of ischemia was 120 min, followed by 0, 15, 60, or 120 min of reperfusion. The model is characterized by hemodynamic stability and intestinal reperfusion has been confirmed by blood flow assessment [6]. Following reperfusion, blood samples were obtained from the portal vein, inferior vena cava (IVC), and descending thoracic aorta for analysis as outlined below. Sacrifice was by exsanguination at the designated time points. The heart and lungs were removed *en bloc*. While a spontaneous heart beat persisted, 20 ml of sterile physiologic saline were infused into the right ventricle to wash residual blood from the pulmonary vascular bed. Both lungs were removed from the heart and hilar structures and frozen immediately in liquid nitrogen. Sham-operated control animals underwent identical preparation except that the SMA clip was not applied; therefore, no ischemia resulted.

Glutathione Assay (Plasma)

The method of Adams *et al.* [19] was modified as outlined. Whole blood was collected in heparin-coated syringes. For total GSH, 200 μ l of fresh whole blood was added to 200 μ l of a buffer containing 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 17.5 mM disodium EDTA, and 100 mM potassium phosphate, pH 7.5. Samples were mixed by tilting and then centrifuged 4 min at 2000g. Two hundred fifty microliters of the supernatant was stored on ice for analysis within 3–4 hr or frozen at -70°C for analysis within 48 hr. Freezing does not alter the assay (unpublished data).

To assay for GSSG, 200 μ l fresh whole blood was added to a buffer containing 10 mM *N*-ethylmaleimide (NEM), 17.5 mM disodium EDTA, and 100 mM K_2HPO_4 , pH 6.5. Mixing and centrifugation were as above. NEM de-

derivatizes all reduced GHS, leaving only GSSG for measurement by the enzymatic assay. The sample was then passed through a C18 Sep-pak cartridge (Waters Associates, Framingham, MA) which was prewashed with 1 ml methanol followed by 2 ml distilled water. Each sample was rinsed from the cartridge with 1 ml phosphate-imidazole buffer (0.01 M Na_2HPO_4 and 0.017 M imidazole, pH 7.2). The eluate was placed on ice or frozen for analysis the next day.

A standard curve was generated using known amounts of GSH (Sigma, St. Louis, MO). Determination of GSH equivalents was made in plastic cuvettes containing 700 μ l 0.3 mM DTNB and 700 μ l 0.4 mM NADPH (Sigma) containing 1.2 units/ml glutathione reductase (Sigma, Type III) to which 700 μ l of the experimental sample was added for NEM-treated samples. For total GSH determination, 50–100 μ l of the plasma supernatant was used. Volume differences were corrected with the phosphate-imidazole buffer. The difference in optical density was determined at 412 nm on an LKB Ultraspec II spectrophotometer (Biochrom, Model 4050, Cambridge, England). Concentrations of GSSG and total GSH were then determined by reverse linear regression against the standard curve.

Glutathione Assay (Tissue)

Frozen lung tissue was lyophilized for 24 hr and stored at -70°C . At the time of assay, tissue was ground under liquid nitrogen with a mortar and pestle and weighed into 50-mg aliquots, mixed quickly with 1 ml of a solution containing 2.5% sulfosalicylic acid in 0.2% Triton X-100, vortexed 15 sec, and centrifuged at 10,000 rpm for 10 sec. The supernatant was removed and placed on ice. For the determination of tissue GSSG, the GSH in a sample was derivatized with 2 μ l 2-vinylpyridine in 10 μ l 1 M Tris at room temperature for 50 min and then frozen at -70°C with the untreated supernatant. Assay of tissue total GSH and GSSG was thereafter identical to the plasma assay.

Statistical Analysis

Statistical evaluation throughout is by an analysis of variance using Tukey's multiple comparisons. Significance is defined as $P < 0.05$ and individual comparisons are indicated in the text.

RESULTS

This model of intestinal ischemia and reperfusion yields a time-dependent, progressive intestinal injury that is associated with an acute pulmonary microvascular injury [6]. This lung injury is characterized by (1) significant reduction in lung tissue ATP levels following 15 min of reperfusion, (2) neutrophil sequestration in the pulmonary microvasculature as indicated by increasing myeloperoxidase concentrations in lung tissue and by quantitative morphometric evaluation, and (3) marked increases in lung microvascular permeability [6]. Both endotoxin and

tumor necrosis factor appear to be involved in the pathogenesis of this injury [20]. The histologic characteristics of this lung injury are illustrated in Fig. 1. These include interstitial edema formation, endothelial cell blebbing with areas of focal necrosis, neutrophil infiltration, and intraalveolar fibrin deposition.

Oxidized Glutathione Determinations in Plasma (Fig. 2) and Lung Tissue (Fig. 3)

GSSG is not detectable in normal rat plasma (Fig. 2) or in normal human plasma (unpublished data). In addition, GSSG does not appear in plasma following intestinal ischemia alone. Following reperfusion of the gut, GSSG appears within 15 min in plasma. At this early time point, a significant ($P = 0.0339$) step up in plasma GSSG occurs between the portal venous blood ($0.64 \pm 0.20 \mu\text{eq/liter}$) and the arterial blood ($2.04 \pm 0.62 \mu\text{eq/liter}$). Following 60 min of reperfusion, GSSG levels are further increased but the differential between venous and arterial plasma compartments no longer exists.

Lung tissue GSSG remained at low levels and was unchanged throughout the experimental periods (Fig. 3).

Total Glutathione in Plasma (Fig. 4) and Lung Tissue (Fig. 5)

Total GSH in plasma was statistically similar in the different vascular compartments in sham (0/0) animals and those subjected to ischemia alone (120/0) (Fig. 4). Following 15 min of intestinal reperfusion, elevations were seen in the aortic plasma ($66.4 \pm 14.7 \mu\text{eq/liter}$) and portal venous plasma ($64.0 \pm 8.2 \mu\text{eq/liter}$) that did not achieve statistical significance, compared to IVC plasma (40.4 ± 5.5 ; $P > 0.05$). These sampling positions reflect effluent blood from the lungs and the intestine, respectively. The IVC, portal vein, and aorta GSH levels were also not significantly increased from those of sham (0/0) or ischemic (120/0) groups at this time. By 60 min of intestinal reperfusion, small elevations at all sites were present but a statistically significant increase was present only in the

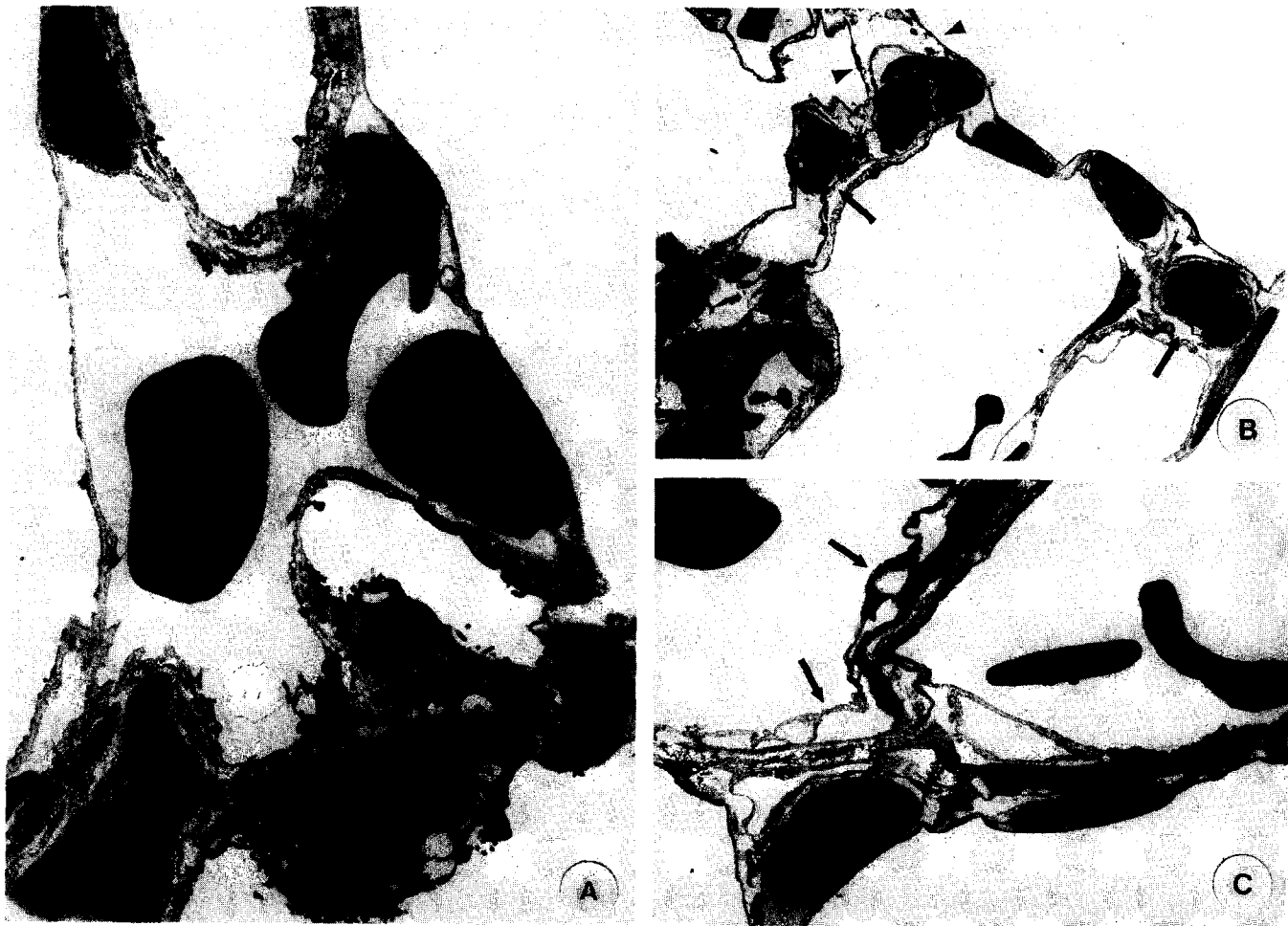


FIG. 1. The histological features of this acute lung injury are illustrated here. Normal (sham) lung histology is demonstrated in A (5600 \times). B (2000 \times) and C (4400 \times) are representative of lung histology after an intestinal injury of 120 min of ischemia and 120 min of reperfusion. Note the interstitial edema (\blacktriangleright), endothelial cell blebbing (\blacktriangleright), sequestration of neutrophils (PMN) in the microvasculature, and intraalveolar hemorrhage (rbc).

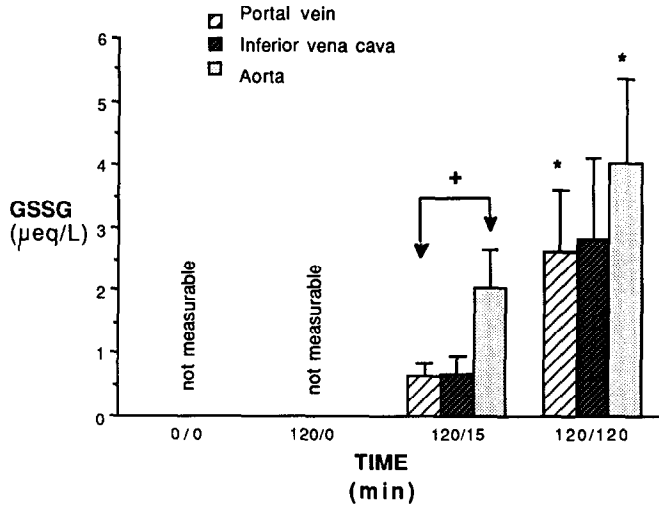


FIG. 2. Plasma GSSG levels from portal vein, inferior vena cava (IVC), and aorta are shown. Time points on the x-axis represent minutes of intestinal ischemia/minutes of intestinal reperfusion (e.g., 120/15 indicates 120 min of ischemia, 15 min of reperfusion). Following intestinal reperfusion, plasma GSSG is elevated. At 15 min of reperfusion a step up in GSSG occurs across the pulmonary vascular bed (between venous and arterial plasma). By 120 min of reperfusion, plasma GSSG is higher still, but differences among vascular compartments are no longer present. (* $P = 0.0009$ compared to 0/0 or 120/0, + $P = 0.0339$ as shown.)

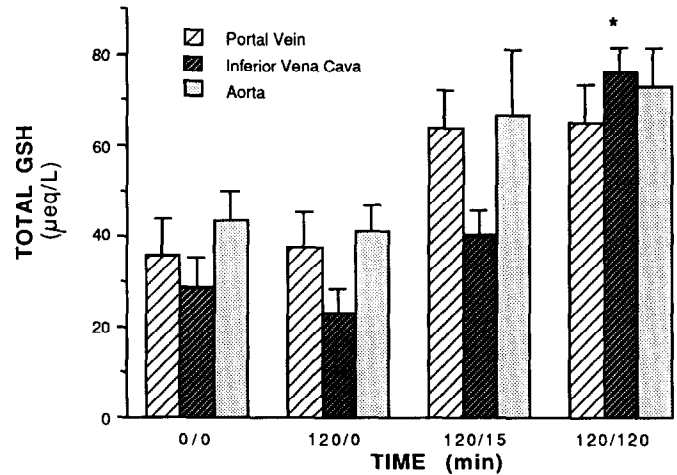


FIG. 4. Total plasma GSH in portal vein, inferior vena cava, and aorta is shown. Following intestinal reperfusion, elevations in total GSH occur that do not achieve statistical significance at the 120/15 time point. These portal vein and aorta sampling sites reflect effluent blood from the intestine and lungs, respectively. By 60 min of reperfusion, total GSH in all compartments is slightly elevated but only the IVC elevation achieves statistical significance compared to the 0/0 group. [Time points on the x-axis represent minutes of intestinal ischemia/minutes of intestinal reperfusion (e.g., 120/15 indicates 120 min of ischemia, 15 min of reperfusion)]. (* $P = 0.0001$ compared to 0/0 or 120/0.)

IVC plasma. No intercompartmental differences were present.

Total GSH in lung tissue remained constant throughout the experimental periods (Fig. 5).

DISCUSSION

Normal cellular respiration involves the generation of toxic oxygen radicals. Ordinarily, they are quickly re-

moved from cells and from the circulation before they react with critical molecules such as the lipids in cell membranes and the proteins which regulate all cellular processes. Endogenous defenses against toxic oxygen radicals include a variety of scavenging enzyme systems. Glutathione is a ubiquitous, largely intracellular antioxidant that is believed to play a key role in the defense against oxygen-derived free radicals [17, 18]. In the presence of oxygen radicals, reduced glutathione is oxidized to GSSG. In normal plasma, levels of GSSG are very low

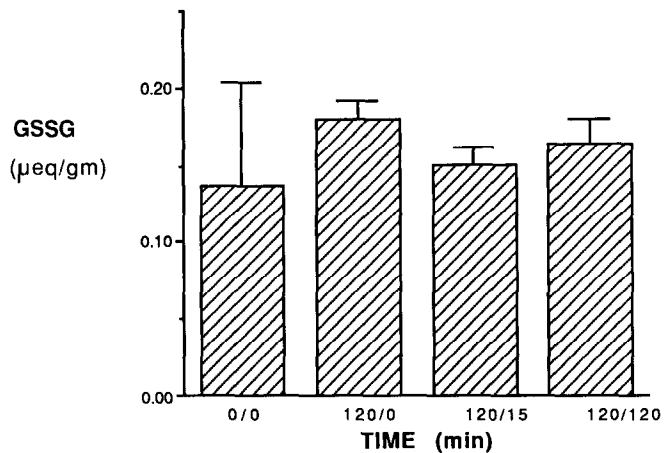


FIG. 3. Lung tissue GSSG remained at low levels and did not change during the experimental periods, suggesting rapid transfer of GSSG from the intracellular compartment to plasma after oxidation. Time points on the x-axis represent minutes of intestinal ischemia/minutes of intestinal reperfusion (e.g., 120/15 indicates 120 min of ischemia, 15 min of reperfusion). ($P > 0.05$ for all time points.)

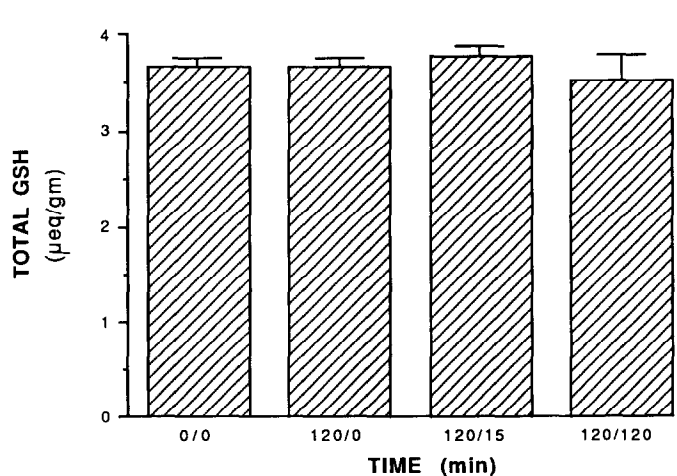


FIG. 5. Total GSH in lung tissue did not change during the experimental periods. Time points on the x-axis represent minutes of intestinal ischemia/minutes of intestinal reperfusion (e.g., 120/15 indicates 120 min of ischemia, 15 min of reperfusion). ($P > 0.05$ for all time points.)

or undetectable by enzymatic assay [21]. It has been suggested that cells transport GSSG into the extracellular compartment to keep the intracellular concentrations of GSH and GSSG roughly constant [10, 22, 23]. Thus, plasma GSSG levels have theoretical appeal as a potential indicator of intracellular oxidant stress. Indeed, the liver has been shown to transport GSSG into blood following free radical generation induced by drug overdose [21]. A variety of drugs which are oxidatively metabolized are known to increase levels of GSSG in plasma [10, 11, 12, 13]. Finally, arteriovenous differences in GSSG after paraquat administration have been reported [9]. Collectively, these observations provide reason to expect that tissue injured oxidatively as a result of inflammation may be a source of plasma GSSG.

The lung injury generated by this animal model may be mediated in part by neutrophils. The evidence for this includes morphometric evaluation, myeloperoxidase accumulation in lung parenchyma, and increased pulmonary microvascular leak as previously reported [6]. Others have provided evidence that this type of pulmonary endothelial cell injury is neutrophil dependent and oxygen radical generated *in vivo* [24–27] and *in vitro* [28].

The data provided in Fig. 2 for GSSG in plasma represent a relatively direct approach for evaluation of oxidant stress *in vivo*. Significant increases in plasma GSSG occur with intestinal reperfusion. Given the body of literature which suggests that oxygen radicals participate in reperfusion injury to the intestine, this is not a surprising observation [29, 30]. The significant step up in aortic GSSG suggests that an additional oxidant-injured “target” is interposed between venous and arterial compartments. The pulmonary microvasculature is a possible target capillary bed. This is consistent with previous data which suggest a role for sequestered neutrophils in alveolar capillaries [6] and with the work of others suggesting this common mechanism in other similar injuries [1, 24, 25, 26, 31]. The time-dependent rise in GSSG through 120 min of reperfusion suggests ongoing reperfusion injury. The loss of compartmental differences may reflect the fact that the circulation has had sufficient time to equilibrate and that the compartmentalization is, of course, incomplete. This suggests that discrete, organ-specific, oxidant-mediated injuries may be localized by early compartmental plasma sampling and that whole-body or multiple-organ oxidant stress may be assessed at later times. The data in Fig. 3 show that lung tissue GSSG is unaffected during oxidant stress and remains at low levels. This is consistent with the hypothesis that the generation of intracellular GSSG is rapidly followed by translocation into the plasma to preserve the intracellular environment.

Total plasma GSH, unlike GSSG, does not change significantly following reperfusion. Lung tissue total GSH and GSSG remain constant throughout the experimental period. The intracellular environment (GSH/GSSG ratio) is preserved. These data are in contrast to earlier work demonstrating a drop in total tissue GSH in a model of

acute inflammatory injury to the lung [32]. Two factors may account for the different results. The earlier work was in rabbits, as opposed to rats in this study, and pulmonary injury was induced directly in the rabbit model whereas this model involved an indirect means of initiating the pulmonary injury. Thus, the plasma determinations of GSSG may represent a sensitive means of measuring what may be a more subtle oxidant stress. Whereas GSSG determinations are a measure of only oxidized glutathione, total GSH is a measure of the sum of oxidized plus reduced glutathione. Total glutathione levels may then reflect increases in GSSG, increased excretion of GSH out of cells into plasma, and perhaps altered capacity of the kidney to excrete GSH normally [10]. In light of these findings, plasma GSSG may be a more sensitive indicator of oxidant injury than total plasma glutathione or even tissue glutathione.

In summary, plasma GSSG appears to be a sensitive early indicator of oxidant injury. In contrast to total GSH levels, normal plasma GSSG levels are very low or undetectable, and any elevation of GSSG in plasma suggests pathology. These data suggest that GSSG determinations in plasma may reflect oxidant stress *in vivo*. Early sampling from specific vascular compartments may allow assessment of specific target organs; in particular, arteriovenous differences may reflect pulmonary microvascular injury. Later sampling particularly from systemic venous sites may reflect whole-body oxidant stress. The potential clinical use of this approach has great appeal. Further experimental and clinical studies are needed to confirm this preliminary investigation.

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