sure impair tissue perfusion and result in a further increase of blood lactate concentrations. Our results suggest that the basis for use of sodium bicarbonate in the therapy of hypoxic lactic acidosis should be reevaluated.

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Protection Against Lethal Hyperoxia by Tracheal Insufflation of Erythrocytes: Role of Red Cell Glutathione

Abstract. *Intact erythrocytes placed into the tracheobronchial tree of hyperoxic rats dramatically improved their chances for survival. Over 70 percent of the animals so treated survived more than 12 days during continuous exposure to 95 percent oxygen, whereas all of the control animals died within 96 hours. Lungs from erythrocyte-protected rats showed almost none of the morphologic damage suffered by untreated animals. Erythrocytes containing cyanomethemoglobin were as beneficial as normal erythrocytes, but cells in which glutathione was partially blocked were significantly less protective. Analogous results were obtained in vitro: ^{51}Cr-labeled target cells released 70 to 90 percent of their label when exposed briefly to hydrogen peroxide or to toxic oxygen species generated by phorbol ester–stimulated neutrophils. Addition of intact erythrocytes decreased release by approximately 75 percent, but significantly less than this if red blood cell glutathione was partially blocked. These results suggest that insufflated erythrocytes, through their recyclable glutathione, protect rats from toxic oxygen species engendered by hyperoxia.*

A Faustian compact is evident in the treatment of patients with severe respiratory distress who require oxygen at excessive pressures to preserve tissue oxygenation. That is, hyperoxia, itself, provokes pulmonary injury (*1*) by mechanisms not entirely understood. Hyperoxia is directly toxic to explanted lung parenchymal cells (*2*). However, our studies, and those of others (*3*), on the pathogenesis of the adult respiratory distress syndrome have suggested that stimulated inflammatory cells, particularly granulocytes, injure lungs through their production of toxic oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and the like. We have stressed the paradox that production of these oxidants might be amplified in the hyperoxic environment of lungs ventilated with oxygen at higher than normal pressures.

If toxic oxygen species are the cause of hyperoxic lung injury, scavenging them would seem worthwhile. In fact, levels of pulmonary superoxide dismutase (SOD), a dissipator of O_2^-, can be increased by exposing rats to 85 percent oxygen; such preadapted animals thereafter resist prolonged exposure to hyperoxia (*4*). In addition, prior administration of small doses of endotoxin also protects rats from oxygen toxicity; this again is associated with amplification of lung SOD levels, as well as with increases in other potentially protective moieties, including catalase and glutathione (GSH) (*5*). Glutathione is important in the resistance of diverse tissues to exhibited H_2O_2, organic peroxides, and the oxygen species released from inflammatory cells (*6*).

A more direct approach is intravenous injection of liposomes containing SOD plus catalase. This strategy increases the survival of rats exposed to 100 percent oxygen, whereas SOD and catalase in nonencapsulated form are ineffectual (*7*). Similar data from our laboratory indicate that liposome-encapsulated SOD or catalase when insufflated intratracheally also improves the survival of hyperoxic rats; 95 percent were alive after 72 hours as compared with 22 percent of control animals insufflated with "naked" liposomes (*8*).

We examined the possibility that intact erythrocytes placed in the tracheobronchial tree might act as more physiologic, encapsulated oxygen radical scavengers. Erythrocytes harbor large quantities of catalase, GSH, and SOD. Moreover, we reasoned that their enormous content of oxidizable heme groups might serve to detoxify labile oxygen species. We report that tracheal insufflation of small numbers of intact erythrocytes prevents oxidant lung injury and improves survival in hyperoxic rats, GSH being the beneficial erythrocyte constituent.

The tracheae of ether-anesthetized male Sprague-Dawley rats (275 to 325 g) were cannulated and insufflated with 1 ml of saline or with various red cell suspensions; thereafter, we placed the rats in a 100 by 30 by 30 cm exposure chamber suffused with 100 percent oxygen at 15 liters per minute. The oxygen tension in the chamber was greater than 0.95 atmosphere at all times. The rats were given free access to water and a standard rat diet and were killed after 3 days or 12 days for histologic examination of the lung.

A single insufflated bolus of 0.75 ml of erythrocytes (1 ml of a saline-washed erythrocyte suspension; hematocrit 75 percent) markedly improved the survival of hyperoxic rats: 75 percent of 89 erythrocyte-treated rats survived 96 hours in comparison with none surviving in the control group of 68 rats ($P < 0.001$) (Table 1). Of the animals that survived, some ($n = 24$) were still alive after 12 days of hyperoxia. The beneficial effect of erythrocytes was evidently dose dependent; halving the erythrocyte bolus

(0.375 ml of packed cells) decreased the 4-day survival to 25 percent in an experiment with 16 rats (not shown). In control rats (saline-insufflated or untreated), death occurred always within 96 hours. Intact erythrocytes were required; isologous rat erythrocytes and human erythrocytes were equally efficacious. In contrast, washed erythrocyte membrane "ghosts" were ineffective (Table 1), suggesting the toxic oxidant scavenger is a cytosolic constituent. Moreover, the scavenger evidently requires restoration by erythrocyte metabolism, as freeze-thawed hemolyzates provided insignificant protection in eight animals (data not shown).

Differences in lung histology were striking. Control animals treated with saline died after 3 to 4 days; these animals had heavy, virtually airless lungs, with marked hemorrhage, interstitial and intra-alveolar edema, inflammatory cell infiltration, and general disruption of architecture (Fig. 1A). In marked contrast, animals protected with insufflated red cells were healthy and, when killed after 12 days of hyperoxia, had virtually normal lungs (Fig. 1B); less than 15 percent of multiple sections from various lobes of these animals showed any damage, and this consisted of minimal edematous thickening of interstitial tissues. In some animals, tracheal catheters were purposely advanced as far as possible in an attempt to distribute red cells disproportionately to different lobes. In a representative animal, a lobe containing erythrocytes appeared histologically normal (Fig. 1C) whereas another lobe from the same animal, in which we could detect no alveolar red cells, showed marked damage (Fig. 1D).

We hypothesized that intact erythrocytes dissipate toxic oxygen species through oxidation of their abundant heme or GSH constituents—constituents which can then be readily reduced again in reactions catalyzed by the reduced forms of nicotinamide adenine dinucleotide (*9*). To investigate the putative role of heme, we treated erythrocytes with nitrite to generate methemoglobin and added excess cyanide to stabilize heme in the cyanomet form and to inhibit catalase. Insufflated erythrocytes containing cyanomethemoglobin protected animals just as well as untreated cells did; 93 percent of rats so treated survived 4 days or more of hyperoxia (Table 1). Similar results were obtained with insufflated erythrocytes containing carboxyhemoglobin (not shown) (*10*). Both cyanide and carbon monoxide form strong bonds with the sixth coordination position of iron in heme, preventing its interaction with oxygen species. Thus, a role for heme disposition of toxic oxygen by-products is not suggested by these studies.

In contrast, the GSH content of insufflated erythrocytes does seem relevant to their protective effects. We depleted 75 to 90 percent of GSH by incubating erythrocytes with predetermined amounts of chlorodinitrobenzene (*11*) or *N*-ethylmaleimide (*12*). As shown in Table 1, insufflation of depleted cells (the mean ± standard error of the mean for GSH was 0.82 ± 0.43 μmol per gram of hemoglobin) provided significantly less hyperoxia protection ($P < 0.01$) than untreated red cells (GSH, 4.82 ± 0.65 μmol/g). Results with rats that received erythrocytes treated with chlorodinitrobenzene were not significantly different

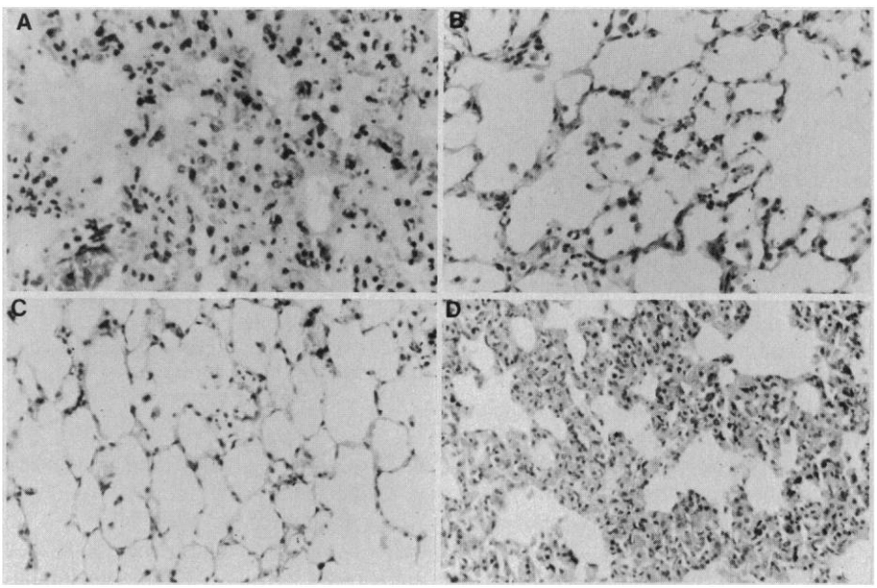

Fig. 1. Effects of various treatments on histology of rat lungs after hyperoxia. Light photomicrographs from representative sections of lungs 3 days (A) or 12 days (B to D) after continuous exposure of rats to more than 95 percent oxygen. In rats treated with tracheal insufflation of saline, lungs were markedly damaged (A), whereas in rats treated with tracheal insufflation of 0.75 ml of red blood cells, lungs were normal (B). In separate experiments the tracheal catheter was advanced as far as possible to achieve disproportionately distributed red cells. The left lower lobe of one such animal contained alveolar erythrocytes and showed minimal damage (C). The right upper lobe of the same animal (D), in which no insufflated red cells were detectable, showed damage similar to that seen in the saline-treated rat. Magnification, ×200.

Table 1. Effect of tracheal insufflation of erythrocytes on survival of rats exposed to more than 95 percent oxygen. Rats were treated with tracheal insufflation of saline or 0.75 ml of intact or altered erythrocytes. Erythrocyte ghosts were prepared by osmotic lysis (*19*). Erythrocytes containing cyanomethemoglobin were prepared by incubating a 37.5 percent erythrocyte suspension with sodium nitrite (1 mg/ml) for 30 minutes, then with 20 m*M* potassium cyanide for 5 minutes. Glutathione-depleted erythrocytes were prepared by incubating a 25 percent erythrocyte suspension with either 2 m*M* 1-chloro-2,4-dinitrobenzene (*11*), or 2 m*M* *N*-ethylmaleimide (*12*) for 60 minutes. Cells treated with the anion channel blocker 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) (*14*) were prepared by incubation of a 37.5 percent erythrocyte suspension with 1 m*M* DIDS for 30 minutes. All incubations were done at 37°C. After each of the incubations, treated and control erythrocytes were washed five times with large volumes of saline and made up into 75 percent suspensions. Results are presented as percent survival after 72 and 96 hours of hyperoxia; the number of rats is in parentheses. *P* values are given for the data after 96 hours (Student's *t*-test). N.S., not significant.

Tracheal insufflate	Percent survival after		*P*
	72 hours	96 hours	
Saline	19	0 (68)	
Erythrocyte ghosts	50	0 (6)	N.S.*
Normal erythrocytes	90	75 (89)	<0.001*
Cyanomethemoglobin erythrocytes	93	93 (15)	N.S.†
GSH-depleted erythrocytes	54	37 (24)	<0.01†
DIDS-treated erythrocytes	87	80 (15)	N.S.†

*Significance of differences in survival compared to rats insufflated with saline. †Significance of differences in survival compared to rats insufflated with normal erythrocytes.

from results with rats that received erythrocytes treated with *N*-ethylmaleimide; assayable levels of catalase and SOD were unaffected by the partial thiol blockade of either inhibitor. Thus, it is unlikely that diminished protection reflected catalase or SOD blockade or resulted from hemolysis of GSH-depleted erythrocytes; less than 10 percent of GSH-depleted cells hemolyzed after 72 hours of incubation in vitro in physiologic levels of glucose at 37°C, nor were GSH levels restored during this period.

Since an earlier study of genetically acatalasemic erythrocytes showed that GSH is the primary defender of erythrocytes from damage by H_2O_2 (*13*), our results suggest that H_2O_2 may be particularly involved in hyperoxic lung injury. That superoxide anion is probably not involved was shown as follows: we treated insufflated erythrocytes with the anion channel blocker, diisothiocyanostilbene disulfonate (DIDS), which binds irreversibly to band 3, the erythrocyte membrane protein, thereby blocking transport of superoxide and other anions across erythrocyte membranes (*14*). Erythrocytes treated with DIDS were as effective as untreated erythrocytes in protecting the hyperoxic animals (Table 1). Hence superoxide is not a toxin in hyperoxic lung damage.

To strengthen our studies with intact animals, we performed two different in vitro studies. In the first study, cultured, radiolabeled endothelial cells harvested from human umbilical veins were incubated with increasing concentrations of H_2O_2 (*15*). Cytotoxicity (release of label) steeply increased with exhibited H_2O_2 greater than 0.09 m*M* but not if intact red cells were also added (Fig. 2). In the second study, using techniques of Weiss (*16*), we exposed ^{51}Cr-labeled target cells to phorbol ester–stimulated granulocytes, as perhaps more physiologically relevant toxic oxygen generators (*17*). Cytotoxicity was inhibited 75 ± 2 percent (n = 10) with the addition of untreated red cells. As in our intact animals, significantly less inhibition of target cell lysis (52 ± 6 percent, n = 6) was observed when GSH-depleted red cells were added ($P < 0.05$).

The importance of erythrocyte GSH suggests that H_2O_2 oxidation of glutathione in a glutathione peroxidase–catalyzed reaction and subsequent reduction by nicotinamide adenine dinucleotide–dependent glutathione reductase may be involved. Earlier studies in genetically acatalasemic erythrocytes showed that this antiperoxide sequence is more important in red blood cells than is catalase

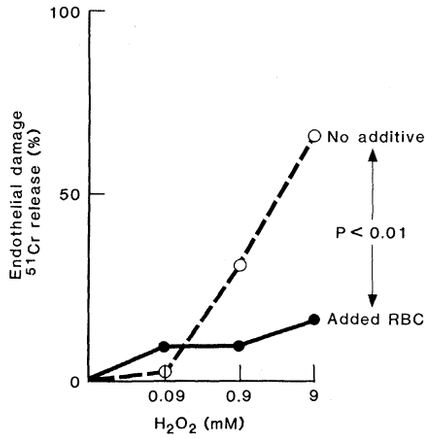

Fig. 2. Protection against H_2O_2-mediated damage of cultured endothelial cells by added erythrocytes. Endothelial cells were obtained from human umbilical cord veins, cultured to confluence and labeled with $Na_2{}^{51}CrO_4$ (*15*). They were then incubated in 0.3 ml of Hanks buffered salt solution containing 0.5 percent albumin with various concentrations of H_2O_2 at 37°C (5 percent CO_2 in air), for 90 minutes in the presence (●) or absence (○) of 10^6 added erythrocytes. Results are presented as the percentage of ^{51}Cr released.

dissipation of H_2O_2 (*13*). This conclusion is supported by the present studies in which cyanide was used in excess in studies of cyanomethemoglobin red cells. In these cells, catalase was inhibited and hemoglobin was altered; yet these cells were as effective as untreated erythrocytes in protecting against hyperoxia. Ample support for the concept that H_2O_2 dissipation is critical to tissue protection from various oxidant assaults comes from the studies of Weiss and colleagues [see, for example (*17*)]. Using a continuously recording electrode specific for H_2O_2 (and no other O_2 species), they demonstrated that added red cells scavenge approximately 75 percent of the H_2O_2 emitted from activated granulocytes (*18*).

The first few hours of hyperoxic exposure seem to be critically damaging to lungs. Animals surviving 96 hours with insufflated erythrocyte protection were at no further risk of death for as long as 12 days of continual exposure to more than 95 percent oxygen. Moreover, histologic examination of lungs from such animals showed neither the inflammatory cell infiltration nor the hemorrhage characteristic of untreated animals that died spontaneously in shorter periods of time. We cannot exclude a continuing benefit of alveolar erythrocytes through undiminished cycling of GSH oxidation-reduction for 12 days or more. In fact, intact erythrocytes were seen in the alveoli of animals surviving this period. Nevertheless, we wonder whether adaptive increases in endogenous pulmonary antioxidant defenses (for instance, SOD, catalase, or unsaturated fatty acid release) may come into play in extended survivors.

An ironic, and previously unsuspected, connotation of the presented results is that small amounts of spontaneous alveolar hemorrhage, a common feature in respiratory distress situations, may actually be beneficial, particularly in patients ventilated with oxygen at high inspired tensions. Clinical trials of purposeful tracheobronchial insufflation of erythrocytes in such patients might be considered in the future.

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Distinct Hepatic Receptors for Low Density Lipoprotein and Apolipoprotein E in Humans

Abstract. *Since the liver is a central organ for lipid and lipoprotein synthesis and catabolism, hepatic receptors for specific apolipoproteins on plasma lipoproteins would be expected to modulate lipid and lipoprotein metabolism. The role of hepatic receptors for low density lipoproteins and apolipoprotein E–containing lipoproteins was evaluated in patients with complementary disorders in lipoprotein metabolism: abetalipoproteinemia and homozygous familial hypercholesterolemia. In addition, hepatic membranes from a patient with familial hypercholesterolemia were studied and compared before and after portacaval shunt surgery. The results establish that the human liver has receptors for apolipoproteins B and E. Furthermore, in the human, hepatic receptors for low density lipoproteins and apolipoprotein E are genetically distinct and can undergo independent control.*

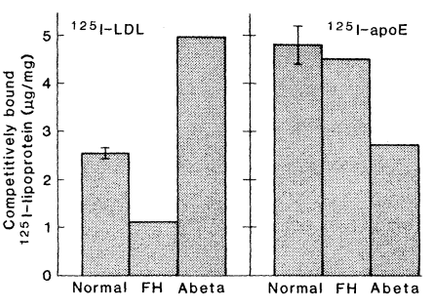
In addition to solubilizing lipid for transport in the blood, the plasma apolipoproteins affect various specific physiologic functions (*1*). Apolipoprotein B (apoB), the primary protein constituent of the cholesterol-carrying low density lipoproteins (LDL), mediates cholesterol delivery to cells by interacting with a specific protein receptor (*2*). Although apolipoprotein E (apoE) is important in the metabolism of triglyceride-rich lipoproteins (*3, 4*), the exact mechanism by which it modulates triglyceride-rich lipoprotein catabolism has not been clearly defined. Lipoproteins containing apoE bind to cultured fibroblasts (*5*) as well as to canine liver (*6*). The binding of apoE-containing lipoproteins can be competed for by apoB-containing lipoproteins; this receptor has therefore been designated the hepatic B, E, or LDL receptor. The LDL receptor is affected by drug treatment, biliary diversion, and diet (*6, 7*). A separate hepatic apoE receptor has been reported, but the physiologic importance of the hepatic apoE receptor in mammals has not been definitely established.

Lipoprotein transport in normal subjects and patients with dyslipoproteinemia can be elucidated by evaluating individuals with specific inborn errors of lipoprotein metabolism. Patients with abetalipoproteinemia do not secrete lipoproteins containing apoB (*8*). The normal ligand in the LDL-receptor cholesterol transport system is missing in these patients, and therefore compensatory changes in the lipid and lipoprotein transport system are required for lipid homeostasis (*8*). Individuals with homozygous familial hypercholesterolemia (FH) lack the LDL receptor (*9*). Therefore, from the standpoint of the interaction of LDL with its receptor, abetalipoproteinemia and FH are complementary genetic defects. We evaluated the hepatic LDL and apoE receptors in humans by analyzing the binding of apoB- and apoE-containing lipoproteins to hepatic membranes from normal subjects and patients with abetalipoproteinemia and homozygous FH.

Patients with abetalipoproteinemia and homozygous FH showed the clinical, biochemical, and genetic criteria for their respective diagnoses (*8, 9*). A therapeutic portacaval anastomosis was performed on the subject with FH in order to decrease the concentration of plasma LDL cholesterol. Hepatic tissues were obtained from normal subjects, from the patient with abetalipoproteinemia, and from the patient with FH after informed consent had been given. Biopsy specimens were immediately placed in a chilled beaker, and hepatic membranes were prepared at 4°C as described (*10*). The membrane pellets were frozen in dry ice and stored in liquid nitrogen until they were used for binding assays.

For lipoprotein quantitation, blood obtained from the subjects after a 12- to 14-hour overnight fast was collected in 0.1 percent EDTA, and the plasma was separated at 4°C in a refrigerated centrifuge. Plasma cholesterol, triglycerides, and lipoproteins were quantitated on an analytic spectrophotometer (Gilford 3500) as described (*10, 11*). Apolipoproteins B and E were determined by radial immunodiffusion and radioimmunoassay, respectively (*11, 12*).

Low density lipoproteins (density, 1.030 to 1.050 g/ml) were used for the binding studies of apoB-containing lipoproteins. These lipoproteins were isolated (*13*), dialyzed against phosphate-buffered saline (*p*H 7.0), and sterilized by filtration. Radioimmunoassay showed that LDL was devoid of apoE. The LDL



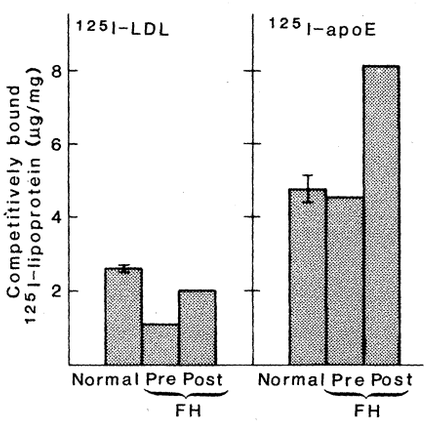


Fig. 1 (left). The binding of apoB (LDL) and apoE particles to hepatic membranes from three normal, one FH, and one abetalipoproteinemia (Abeta) subject. Hepatic membrane protein (0.1 to 0.2 mg) from normal and dyslipidemic subjects was incubated for 30 minutes at 37°C with 10 μg of ^{125}I-labeled lipoprotein. Bound and free ^{125}I-lipoproteins were then separated by a 3-minute, 100,000*g* ultracentrifugation. Competitively bound lipoprotein is the difference in binding observed in the absence and presence of tenfold excess unlabeled homologous lipoprotein. Values for normal subjects represent the mean ± standard error of the mean of the three samples. Fig. 2 (right). The binding of apoB (LDL) and apoE lipoproteins to three normal and one homozygous FH hepatic membrane before (pre) and after (post) portacaval shunt surgery. The competitive binding of ^{125}I-lipoproteins was determined as the difference in binding in the absence and presence of tenfold excess unlabeled lipoprotein to 100 to 200 μg of membrane proteins. Normal values represent the mean ± standard error of the mean of the three separate samples.