Rapid Publications

Intact Human Erythrocytes Prevent Hydrogen Peroxide-mediated Damage to Isolated Perfused Rat Lungs and Cultured Bovine Pulmonary Artery Endothelial Cells

Karen M. Toth, Dennis P. Clifford, Elaine M. Berger, Carl W. White, and John E. Repine
Departments of Medicine and Pediatrics, and the Webb-Waring Lung Institute, University of Colorado Medical Center, Denver, Colorado 80262

Abstract. Addition of untreated or glutaraldehyde-fixed human erythrocytes decreased hydrogen peroxide (H_2O_2)-mediated acute edematous injury in isolated rat lungs, H_2O_2-induced damage to cultured bovine pulmonary artery endothelial cells, and H_2O_2-dependent oxidation of reduced cytochrome C in vitro. The results suggest that intact erythrocytes can scavenge H_2O_2, and as a result, protect the lung and possibly other tissues from damage.

Introduction

Acute edematous lung injury, such as that seen in the adult respiratory distress syndrome (ARDS),[1] is an important clinical problem whose pathophysiology is poorly defined (1). However, recent evidence suggests that toxic oxygen metabolites may contribute to endothelial cell injury and acute edematous lung injury (1). The source of these O_2 metabolites is unclear, but they may be made by lung cells and/or by neutrophils that have been recruited to the lung and activated (1). Erythrocytes (RBC) are also commonly seen in intravascular, interstitial, and alveolar spaces during the development of ARDS but their significance is unknown (1–3). However, since RBC contain large amounts of superoxide dismutase (SOD), catalase, glutathione peroxidase, and reduced hemoglobin, which could scavenge O_2 metabolites (4–10), we hypothesized that RBC could decrease acute edematous lung injury that was caused by O_2 metabolites. We tested this premise in three systems. First, we perfused isolated rat lungs with varying concentrations of human RBC and then exposed these lungs to superoxide anion and hydrogen peroxide (H_2O_2), which were generated by hypoxanthine (HX) and xanthine oxidase (XO). Second, we measured the effect of adding RBC on H_2O_2-mediated damage to cultured bovine pulmonary artery lung endothelial cells. Third, we assessed the influence of RBC on H_2O_2-dependent oxidation of reduced cytochrome C in vitro. Our results show that RBC decrease acute edematous injury in isolated lungs that were perfused with HX and XO, decrease damage to cultured endothelial cells treated with H_2O_2, and decrease oxidation of cytochrome C by H_2O_2 in vitro.

Methods

Preparation of human RBC. Venous blood was collected by a two-syringe technique from healthy, human volunteers who were not taking any medications. RBC were separated by hetastarch sedimentation (Hespan, American Hospital Supply Corp., McGaw Park, IL), triply-washed, and resuspended in Hank's balanced salt solution. For some experiments, RBC were pretreated with glutaraldehyde (GLUT-RBC, 0.01%, Sigma Chemical Co., St. Louis, MO) for 60 min (11) or with diethyldithiocarbamic acid (DDC-RBC, 50 mM, sodium salt, Sigma Chemical Co.), for 45 min (7, 10, 12). Untreated or glutaraldehyde-

Portions of the work were presented by Ms. Toth at the Annual Meeting of the Western Society for Clinical Investigation, where she was awarded the Western Section-American Federation of Clinical Research Medical Student Pulmonary Subspecialty Prize. The work has also been selected for presentation at meetings of the American Society for Clinical Investigation and at the National Medical Student Research Forum.

Address correspondence to Dr. Repine.

Received for publication 24 February 1984.

1. *Abbreviations used in this paper:* ARDS, adult respiratory distress syndrome; AMT-catalase, 3-amino-1:2:4-triazole; DDC-RBC, diethyldithiocarbamic acid pretreated RBC; DMTU, dimethylthiourea; RBC, erythrocyte; GLUT-RBC, glutaraldehyde pretreated RBC; H_2O_2, hydrogen peroxide; HX, hypoxanthine; LDH, lactate dehydrogenase; M199, serum-free medium 199; SOD, superoxide dismutase; XO, xanthine oxidase.

J. Clin. Invest.

0021-9738/84/07/0292/04 $1.00
Volume 74, July 1984, 292–295

treated RBC did not release LDH after treatment with HX and XO or with H_2O_2 (10). DDC-treated RBC had undetectable SOD activities (≪3 U/g Hgb) when compared with untreated RBC (>1,100 U, [13]).

Isolated perfused rat lung preparation and measurement of acute edematous lung injury. Healthy, male Sprague-Dawley rats (350–450 g, Charles River Breeding Laboratories, Inc., Wilmington, MA) were allowed to acclimate in Denver for 7 d. Rats were then anesthetized with pentobarbital (30 mg) and a tracheotomy was performed, which permitted ventilation with an animal respirator (model 6700, Harvard Apparatus Co., Inc., S. Natick, MA) at a rate of 75 breaths/min with 95% air, 5% CO_2 mixture, under 9 cm H_2O pressure, and positive end expiratory pressure of 1.5 cm H_2O (14–15). Subsequently, a median sternotomy was performed and heparin (200 U, Panheparin, Abbott Laboratories, N. Chicago, IL) was injected into the right ventricle and allowed to circulate for 3 min. Cannulas were then placed in the pulmonary artery and left atrium. The latter was left open to maintain a left atrial pressure of zero. Subsequently, lungs were flushed with buffered (pH 7.4) Kreb's-Henselite solution (50 cm^3) which contained albumin (4.5%). Next, the heart and lungs were excised en bloc and placed in a humidified chamber at 37°C. A perfusion circuit was instituted with Kreb's-Henselite albumin solution (70 cm^3). Lungs were then perfused at a rate of 0.04 ml/g of rat body weight per minute. Lung weights were measured using a Grass force displacement transducer (FD-10, Grass Instruments Co., Quincy, MA). Pulmonary artery perfusion pressures were measured by a pressure transducer (Bell & Howell Co., Pasadena, CA, type 4-327-0010) (14–15). Each measurement was recorded continuously on a multichannel recorder (VR-6, Electronics for Medicine, Pleasantville, NY). After a 15-min equilibration period, the perfusate was changed to a buffered Kreb's-Henselite albumin (4.5%) solution which contained hypoxanthine (Sigma grade, 3 mM). In some experiments, RBC, SOD (type I, 2,650 U/mg protein, Sigma Chemical Co.), catalase (bovine liver 2,000 U/mg protein, Sigma Chemical Co.), or catalase, which had been inactivated by 3-amino-1:2:4-triazole (AMT-catalase, [16]) were also added. After another 10-min period, xanthine oxidase (0.03–0.10 U/ml, grade I buttermilk, Sigma Chemical Co., or Calbiochem-Behring Corp., San Diego, CA) was added to the perfusates. Preparations were then monitored for an additional 35 min. At the end of the 60-min perfusion period, lungs were removed from the apparatus and lavaged with warm saline (5 ml). Afterwards, lavages were recovered and centrifuged at 500 *g* for 8 min. Supernatants were then collected and analyzed for albumin concentrations by the bromcresol green method (14–15).

Source and culture of bovine pulmonary artery endothelial cells and measurement of lactate dehydrogenase (LDH) release. Heart and lungs with attached great vessels were obtained from cows that were slaughtered <45 min previously (17). The pulmonary artery was then dissected free from pericardium and aorta, stripped of fat and connective tissue, and transected near the pulmonic valve and just distal to the bifurcation of the left and right pulmonary arteries. The artery segment was then immersed for 10 min in Puck's saline G, which contained antibiotics and antimycotics, slit open longitudinally, and placed cell side down in a dish containing serum-free medium 199 (M199, Gibco Laboratories, Grand Island, NY) with collagenase (0.1%, 131 μg/ml, Gibco Laboratories). After incubation for 10 min (37°C, 5% CO_2), the luminal surface was lightly scraped with a sterile Q-tip. Cells that accumulated on the Q-tip were placed in a sterile conical tube that contained M199 plus fetal calf serum (20%, Biocell Laboratories, Carson, CA), thymidine (10^{-5} M, Sigma Chemical Co.), and antibiotics. Endothelial cells that were suspended in M199 were then distributed among 25-cm^2 culture flasks and placed in a humidified incubator (37°C, 5% CO_2). Endothelial cells were identified by their characteristic cobblestone monolayer structure, typical ultrastructure, and the presence of Factor VIII antigen, which used indirect immunofluorescence (17). All experiments were done with first passage endothelial cells. At the beginning of each experiment, endothelial cells were washed and suspended by treatment with trypsin (0.02–0.05%, Gibco Laboratories). Identical numbers of endothelial cells were then added to each tube with or without RBC and/or H_2O_2 (J. T. Baker Chemical Co., Phillipsburg, NJ) in a final volume of 1 ml of Hank's balanced salt solution. After each ingredient was added, the tubes were then capped and mixed by gentle rotation end over end at 37°C for 60 min. After incubation, each tube was spun at 800 *g* for 10 min, and then supernatants were recovered. Samples that contained comparable numbers of endothelial cells were disrupted by sonication. LDH activity was then determined in paired supernatants and sonicates using a spectrophotometric method to measure NADH-dependent conversion of pyruvate to lactate (17). LDH release was then calculated from the formula: LDH release (% total) = (LDH in supernatant)/(LDH in sonicate) × 100. LDH was not measurable in samples containing catalase.

Measurement of cytochrome C oxidation in vitro. Assay of the oxidation of reduced cytochrome C was used to measure H_2O_2 in vitro. Briefly, sodium hydrosulfite (Sigma Chemical Co.) was added to cytochrome C (5 mg, Sigma Chemical Co.) and dissolved in 0.05 M KPO_4 buffer (1 ml) with 10^{-4} M EDTA (pH 7.8). Reduced cytochrome C was then quickly passed over a Sephadex G25 column that was equilibrated with phosphate buffer and the eluent was adjusted to yield an absorbance of 0.800±0.020 at 550 nm. Mixtures were then prepared that contained 0.05 M cytochrome C, 10^{-4} M KPO_4 with EDTA, and H_2O_2 and/or RBC. Samples were then mixed on ice and incubated at 37°C for 20 min in a shaking waterbath. After 20 min, samples were centrifuged at 300 *g* for 5 min and the absorbance at 550 nm was determined spectrophotometrically. Cytochrome C oxidation was calculated by dividing the difference between samples that contained H_2O_2 and non-H_2O_2-containing controls by 0.021.

Results

Effect of RBC on edema formation in isolated lungs perfused with HX and XO. In the absence of any additions, isolated perfused lungs did not gain weight or develop increases in lung lavage albumin concentrations (Table I). In contrast, after perfusion with HX and XO, isolated lungs developed acute edematous injury which were manifested by significant lung weight gains and lung lavage albumin increases. In parallel, addition to perfusates of increasing concentrations of human RBC progressively decreased lung weight gains and lung lavage albumin increases in isolated lungs that were perfused with HX and XO. Administration of lysed samples of RBC also decreased acute edematous injury in isolated lungs that were perfused with HX and XO (data not shown), but RBC lysis was not essential, since glutaraldehyde-fixed RBC also decreased HX- and XO-mediated acute edematous injury in isolated perfused lungs (Table I). The mechanism that was responsible for acute edematous injury in isolated lungs perfused with HX and XO appeared to involve H_2O_2, rather than superoxide anion, since addition of DDC-treated, SOD-inactivated RBC or catalase (but not AMT-inactivated catalase or SOD) prevented injury (Table I). Moreover, pulmonary artery perfusion pressure increases in lungs

Table I. Effect of Human RBC on Edema Formation in Isolated Rat Lungs Perfused with HX and XO

Test conditions (substances added to perfusates)	Lung weight gains (*g*)	Lung lavage albumin concentrations (*mg/dl*)
None	0.3±0.1 (12)*‡	220±15 (12)‡
HX + XO	17±1.0 (10)	2,090±260 (10)
HX + XO + RBC (0.25%)	16±0.2 (5)§	2,110±250 (5)§
HX + XO + RBC (0.5%)	6.2±0.7 (5)‡	485±60 (5)‡
HX + XO + RBC (1%)	0.8±0.5 (10)‡	205±50 (10)‡
HX + XO + RBC (5%)	0.4±0.1 (5)‡	250±60 (5)‡
HX + XO + GLUT-RBC (1%)	0.5±0.4 (10)‡	290±45 (10)‡
HX + XO + DDC-RBC (1%)	0.8±0.3 (5)‡	275±95 (5)‡
HX + XO + catalase (100 μg/ml)	0.8±0.2 (5)‡	250±45 (5)‡
HX + XO + AMT-catalase (100 μg/ml)	16 (2)§	2,050 (2)§
HX + XO + SOD (100 μg/ml)	16±0.9 (5)§	1,985±260 (5)§
HX	0.3 (2)‡	220 (2)‡
XO	0.3 (2)‡	220 (2)‡

* Mean±SE (number of determinations).
‡ Value significantly different ($P < 0.05$) from value obtained with HX + XO alone.
§ Value not significantly different ($P > 0.05$) from value obtained with HX + XO alone.

treated with HX, XO and RBC (7.0±2.3 mm Hg) were the same as increases seen in lungs that were perfused with HX and XO (7.1±2.7 mm Hg).

Effect of RBC on LDH release from endothelial cells treated with H_2O_2. Addition of increasing amounts of H_2O_2 progressively increased the amounts of LDH in supernatants from cultured, passage-one, bovine pulmonary artery endothelial cells (Table II). In parallel, addition of increasing numbers of RBC progressively decreased the amounts of LDH present in supernatants from endothelial cells exposed to H_2O_2 (30 mM). Addition of glutaraldehyde-treated RBC also decreased LDH concentrations in supernatants from endothelial cells treated with H_2O_2.

Effect of RBC on cytochrome C oxidation by H_2O_2 in vitro. Addition of increasing concentrations of H_2O_2 progressively oxidized reduced cytochrome C in vitro (Table III). In parallel, H_2O_2-mediated cytochrome C oxidation was progressively decreased by addition of increasing concentrations of RBC. Glutaraldehyde-treated RBC also decreased H_2O_2-mediated oxidation of cytochrome C in vitro. In addition, addition of catalase (but not aminotriazole-inactivated catalase or SOD) decreased oxidation of cytochrome C by H_2O_2 in vitro.

Discussion

Previous studies have focused on the susceptibility of RBC to oxidant injury rather than on their ability to scavenge O_2 metabolites and protect other tissues (4–10). Our results show that

Table II. Effect of Human RBC on LDH Concentrations in Supernatants from Cultured Bovine Pulmonary Artery Endothelial Cells Treated with H_2O_2

Test conditions (substances added to cultures)	LDH concentrations (% total)
None	13±0.1 (22)*‡
H_2O_2 (3 mM)	19±1.5 (4)‡
H_2O_2 (10 mM)	23±1.2 (4)‡
H_2O_2 (30 mM)	41±4.0 (14)
H_2O_2 (30 mM) + RBC (0.1%)	38±2.5 (4)§
H_2O_2 (30 mM) + RBC (0.5%)	20±1.8 (4)‡
H_2O_2 (30 mM) + RBC (1%)	17±0.1 (8)‡
H_2O_2 (30 mM) + RBC (5%)	7±4.0 (4)‡
H_2O_2 (30 mM) + GLUT-RBC (1%)	17±1.0 (6)‡
H_2O_2 (30 mM) + SOD (100 μg/ml)	40±4.4 (7)§

* Mean±SE (number of determinations).
‡ Value significantly different ($P < 0.05$) from value obtained with H_2O_2 (30 mM) alone.
§ Value not significantly different ($P > 0.05$) from value obtained with H_2O_2 (30 mM) alone.

human RBC can decrease H_2O_2-induced injury to both isolated perfused lungs and cultured lung endothelial cells.

The mechanism responsible for protection by RBC appears to involve inactivation of H_2O_2 by intracellular RBC scavengers (4–10). First, complete protection occurred after addition of glutaraldehyde-fixed RBC, which do not release their intracellular contents under these conditions. Second, DDC-treated, SOD-

Table III. Effect of Human RBC on Oxidation of Reduced Cytochrome C by H_2O_2 In Vitro

Test conditions (substances added in vitro)	Oxidation of reduced cytochrome C (nm oxidation per 20 min)
None	0 (20)*‡
H_2O_2 (1 μM)	3±0.2 (5)‡
H_2O_2 (10 μM)	7±0.3 (6)‡
H_2O_2 (20 μM)	15±0.3 (30)
H_2O_2 (20 μM) + RBC (0.001%)	15±0.2 (5)§
H_2O_2 (20 μM) + RBC (0.01%)	8±0.3 (6)‡
H_2O_2 (20 μM) + RBC (0.05%)	0.5±0.1 (15)‡
H_2O_2 (20 μM) + GLUT-RBC (0.05%)	0.5±0.1 (15)‡
H_2O_2 (20 μM) + catalase (100 μg/ml)	0 (8)‡
H_2O_2 (20 μM) + AMT-catalase (100 μg/ml)	12±0.6 (4)§
H_2O_2 (20 μM) + SOD (100 μg/ml)	15±0.5 (7)§

* Mean±SE (number of determinations).
‡ Value significantly different ($P < 0.05$) from value obtained with H_2O_2 (20 μM) alone.
§ Value not significantly different ($P > 0.05$) from value obtained with H_2O_2 (20 μM) alone.

inactivated RBC decreased acute edematous injury as well as untreated RBC. Third, addition of catalase (but not SOD) decreased acute edematous injury in isolated lungs that were perfused with HX and XO. The most likely RBC scavengers of H_2O_2 are catalase, glutathione peroxidase, and/or hemoglobin. However, the exact contribution of each of these RBC scavengers to protection is very difficult to determine because of their complex interactions (4–10).

Our findings raise the possibility that RBC can decrease O_2 metabolite-mediated damage to the lung. Many studies now implicate O_2 metabolites as mediators of some forms of acute edematous lung injury, such as that seen in ARDS (1). For example, when phorbol myristate acetate is injected into rabbits, an acute edematous lung injury occurs that is characterized by neutrophil sequestration in the lung, which is prevented by coaddition of dimethylthiourea (DMTU), an H_2O_2 and hydroxyl radical scavenger (14, 18). In addition, when stimulated by phorbol myristate acetate, normal neutrophils, but not O_2 metabolite-deficient chronic granulomatous disease neutrophils, cause acute edematous injury in isolated perfused lungs, which is also prevented by coaddition of DMTU (14). Furthermore, when a chemical system is used (such as HX and XO) to generate O_2 metabolites, DMTU inhibitable acute edematous injury also occurs in isolated perfused lungs (15).

The present studies show that RBC can decrease H_2O_2-mediated acute edematous injury in isolated perfused lungs, H_2O_2-mediated damage to cultured lung endothelial cells, and H_2O_2-mediated oxidation of cytochrome C in vitro. The ability of RBC to scavenge H_2O_2 may be important in the pathophysiology of lung and other diseases that are mediated by toxic O_2 metabolites.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health, American Heart Association, American Lung Association, Council for Tobacco Research, Swan, Hill, and Kleberg, and R. J. Reynolds Foundations.

References

1. Tate, R. M., and J. E. Repine. 1983. Neutrophils and the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 128:552–559.

2. Bachofen, M., and E. R. Weibel. 1977. Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. *Am. Rev. Respir. Dis.* 116:589–615.

3. Stewart, G. J., W. G. M. Ritchie, and P. R. Lynch. 1974. Venous endothelial damage produced by massive sticking and emigration of leukocytes. *Am. J. Pathol.* 74:507–521.

4. Cohen, G., and P. Hochstein. 1964. Generation of hydrogen peroxide in erythrocytes by hemolytic agents. *Biochem.* 3:895–899.

5. Goldberg, B., A. Stern, and J. Peisach. 1976. The mechanism of superoxide anion generation by the interaction of phenylhydrazine with hemoglobin. *J. Biol. Chem.* 251:3045–3051.

6. Goldberg, B., and A. Stern. 1976. Superoxide anion as a mediator of drug-induced oxidative hemolysis. *J. Biol. Chem.* 251:6468–6470.

7. Lynch, R. E., and I. Fridovich. 1978. Effects of superoxide on the erythrocyte membrane. *J. Biol. Chem.* 253:1838–1845.

8. Weiss, S. J. 1982. Neutrophil-mediated methemoglobin formation in the erythrocyte. *J. Biol. Chem.* 257:2947–2953.

9. Paglia, D. E., and W. N. Valentine. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* 70:158–168.

10. Eaton, J. W., M. Boraas, and N. L. Etkin. 1972. Catalase activity and red cell metabolism. *In* Hemoglobin and Red Cell Structure and Function. G. J. Brewer, editor. Plenum Publishing Co., New York. 121–131.

11. White, J. G., and J. E. Repine. 1978. Fine structural alterations in erythrocytes by phorbol myristate acetate. *Am. J. Pathol.* 91:571–578.

12. Heikkila, R. E., F. S. Cabbat, and G. Cohen. 1976. In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J. Biol. Chem.* 251:2182–2185.

13. McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244:6049–6053.

14. Shasby, D. M., K. M. VanBenthuysen, R. M. Tate, S. S. Shasby, I. F. McMurtry, and J. E. Repine. 1982. Granulocytes mediate acute edematous lung injury in rabbits and isolated rabbit lungs perfused with phorbol myristate acetate. Role of oxygen radicals. *Am. Rev. Respir. Dis.* 125:443–447.

15. Tate, R. M., K. M. VanBenthuysen, D. M. Shasby, I. F. McMurtry, and J. E. Repine. 1982. Oxygen radical mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. *Am. Rev. Respir. Dis.* 126:802–806.

16. Feinstein, R. N., S. Berliner, and F. O. Green. 1958. Mechanism of inhibition of catalase by 3-amino-1:2:4-triazole. *Arch. Biochem. Biophys.* 76:32–44.

17. Bowman, C. M., E. N. Butler, and J. E. Repine. 1983. Hyperoxia damages cultured endothelial cells causing increased neutrophil adherence. *Am. Rev. Respir. Dis.* 128:469–472.

18. Jackson, J. H., C. W. White, D. P. Clifford, E. M. Berger, and J. E. Repine. 1983. Dimethylthiourea, a scavenger of toxic oxygen metabolites, prevents acute edematous injury in rabbits following injection of phorbol myristate acetate. *Am. Rev. Respir. Dis.* 127:286. (Abstr.)