PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **188**, 323–327 (1988)

Red Blood Cells Protect Endothelial Cells against H_2O_2-Mediated but Not Hyperoxia-Induced Damage (42741)

MIN-FU TSAN AND JULIE E. WHITE

Research Service, Veterans Administration Medical Center and Departments of Physiology and Medicine, Albany Medical College, Albany, New York 12208

Abstract. We studied the effect of intact red blood cells on the exogenous H_2O_2-mediated damage as well as on the hyperoxia-induced injury of cultured endothelial cells. Red blood cells protected endothelial cells against H_2O_2-mediated injury efficiently, but had no effect on the hyperoxia-induced damage. Failure of red blood cells to protect endothelial cells against hyperoxia-induced injury was not due to hemolysis. Furthermore, hyperoxia-exposed red blood cells were still capable of protecting endothelial cells against H_2O_2-mediated damage. © 1988 Society for Experimental Biology and Medicine.

Reactive oxygen species such as superoxide, H_2O_2, and hydroxyl radical play an important role in the pathogenesis of tissue injuries in a number of clinical situations including adult respiratory distress syndrome (1), oxygen toxicity (2), and reperfusion injury (3). Considerable efforts have been made attempting to scavenge these reactive oxygen species and thus prevent the development of tissue injury.

Red blood cells contain a high content of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. Since reactive oxygen species diffuse across the red cell membrane readily (4), red blood cells are powerful scavengers of reactive oxygen species. Thus, red blood cells have been shown to scavenge effectively extracellular H_2O_2 produced enzymatically (5, 6) or by activated neutrophils (7, 8) and protect endothelial cells against H_2O_2-mediated damage in tissue cultures (5, 9) as well as in an isolated lung perfusion system (5).

Recently, van Asbeck *et al.* (9) have shown that intratracheal insufflation of red blood cells protects rats against the lethal effect of hyperoxia. The reason for this protection is not clear. Reactive oxygen species are products of cellular oxidation–reduction processes. Hyperoxia is presumed to cause damage due to the enhanced production of reactive oxygen species which overwhelm the natural defense systems of the cells (2). Since reactive oxygen species are produced intracellularly under this circumstance, it is not clear whether red blood cells have any protective effects. In this report, we demonstrate that red blood cells protect endothelial cells against H_2O_2-mediated damage but not hyperoxia-induced injury.

Materials and Methods. *Culture of endothelial cells.* Bovine pulmonary artery endothelial cells (CCL-209) at the 16th passage were obtained from American Type Culture Collection (Rockville, MD) and cultured as described previously (10). Briefly, endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, Utah) and gentamycin (Shering Corp., Kenilworth, NJ) in Corning 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). Endothelial cell monolayers were then removed by gentle trypsinization (0.025% trypsin–EDTA–polyvinyl pyrrolidone, Sigma Chemical Co., St. Louis, MO) and suspended in the complete culture medium at a cell density of 2×10^5/ml. Confluent endothelial monolayers were then prepared by seeding 2×10^5 cells/well into Nunc 24-well plates (A/S Nunc Interme., Denmark) and cultured at 37°C in a 5% CO_2 incubator for 3 to 4 days to reach confluency before experiments. All experiments were done in duplicate and the results were averaged. Endothelial cells cultured as described above showed a homogenous population with cob-

0037-9727/88 $1.50
Copyright © 1988 by the Society for Experimental Biology and Medicine.
All rights reserved.

blestone appearance (11) and maintained a high degree of angiotensin-converting enzyme activity (12).

Preparation and treatment of red blood cells. Heparinized venous blood was obtained from normal volunteers. After removal of the buffy coat, packed red cells were washed once and resuspended in DMEM plus 1% FCS at a final hematocrit of 50% for further use. Labeling of red blood cells was done by incubating 1 ml of 25% red blood cells with 20 μCi of ^{51}Cr-sodium chromate at 37°C for 1 hr. At the end of incubation, red blood cells were washed three times with Hanks' balanced salt solution containing 5 m*M* glucose (HBSS). The labeled red cells were then resuspended in DMEM plus 1% FCS. Exposure of red blood cells to 95% O_2 and 5% CO_2 was carried out in an oxygen chamber as described below for the endothelial cells.

Measurement of H_2O_2 cytotoxicity. Hydrogen peroxide toxicity against endothelial cells was determined by two methods: ^{51}Cr release and the direct counting of viable cells using trypan blue stain. For the ^{51}Cr-release assay, confluent endothelial monolayers were labeled with 1 μCi of ^{51}Cr-sodium chromate in 1 ml of HBSS for 2 hr at 37°C. After three washes with HBSS, ^{51}Cr-endothelial monolayers were incubated for 3 hr at 37°C in a 5% CO_2 incubator in the presence or absence of red blood cells (0.01–0.5%) and/or H_2O_2 (5–30 m*M*) in 1 ml of DMEM plus 1% FCS. The supernate was then removed and centrifuged in a microcentrifuge at 13,000 rpm for 3 min at room temperature. The radioactivity in 0.5 ml of the clear supernate was determined in an autogamma counter (Packard Instrument Co., Downers, Grove, IL). The maximum releasable radioactivity was determined by incubating the endothelial monolayers with 1 ml of DMEM plus 1% FCS containing 1% Triton X-100 as described above and the amount of radioactivity in the supernate determined. Cytotoxicity was expressed as the percentage of ^{51}Cr release calculated by the formula as described previously (10):

$$\text{Percentage killing} = (A - B)/(C - B) \times 100,$$

where A was the radioactivity in the supernate of samples containing H_2O_2 and endothelial cells, B was the radioactivity in the supernate of samples containing endothelial cells alone, and C was the maximum releasable radioactivity of the endothelial cells.

For the assay using trypan blue stain, endothelial monolayers were incubated for 3 hr at 37°C in a 5% CO_2 incubator in the presence or absence of red cells and/or H_2O_2 in 1 ml DMEM plus 1% FCS. At the end of incubation, the monolayers were washed twice with HBSS and trypsinized, and the total number of viable endothelial cells determined by counting viable cells, e.g., trypan blue negative cells, in a hemocytometer.

Measurement of hyperoxia-induced injury. Due to the high rate of spontaneous release of ^{51}Cr by ^{51}Cr-labeled endothelial cells (8.5 $\pm$ 1.0% in 3 hr, n = 6), hyperoxia-induced injury against endothelial cells was determined only by counting viable cells using trypan blue stain as described previously (13). Briefly, endothelial monolayers in the presence or absence of red cells (0.01 to 1%) in 1 ml DMEM plus 1% FCS were exposed to normoxia or hyperoxia for up to 7 days. For the hyperoxic exposure, endothelial monolayers were placed in oxygen chambers (Bellco Glass, Inc., Vineland, NJ) which was flushed for at least 15 min with a mixture of 95% O_2 and 5% CO_2, or 80% 0_2, 5% CO_2, and15% N_2, and incubated in a warm room at 37°C. For the normoxic exposure, endothelial monolayers were incubated in a 5% CO_2 incubator at 37°C. The oxygen chamber was regassed daily. At 3 and 7 days after exposure, endothelial monolayers were washed twice with HBSS and trypsinized and the total number of viable cells determined as described above.

Statistical analysis. Statistical significance was calculated by Student's t test (14).

Results. *Effect of red blood cells on H_2O_2-mediated damage.* Figure 1 shows that exogenous H_2O_2 caused an enhanced release of ^{51}Cr from ^{51}Cr-labeled endothelial cells in a dose dependent manner. This cytotoxic effect of H_2O_2 was completely prevented by 0.1% red blood cells. Comparable results were obtained when H_2O_2 cytotoxicity was determined using the trypan blue stain (Fig. 2). Again, H_2O_2 toxicity was completely re-

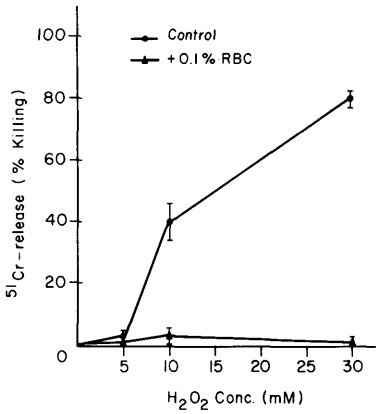

FIG. 1. Effect of red blood cells on the H_2O_2-mediated ^{51}Cr release by endothelial cells. Confluent ^{51}Cr-endothelial monolayers were incubated at 37°C for 3 hr in the presence or absence of H_2O_2 (5—30 m*M*) and/or 0.1% red blood cells in 1 ml DMEM plus 1% FCS. At the end of the incubation, ^{51}Cr release by endothelial cells was determined and percentage of killing calculated. The results are the mean ± SEM of three experiments.

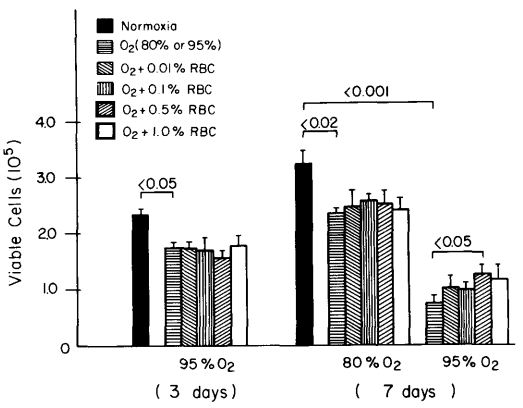

FIG. 3. Effect of red blood cells on the hyperoxia-induced injury against endothelial cells. Confluent endothelial monolayers were exposed to normoxia or hyperoxia in the presence or absence of red blood cells in 1 ml DMEM plus 1% FCS for up to 7 days. On days 3 and 7, the number of viable endothelial cells was determined. The results are the mean ± SEM of three to eight experiments.

versed by red blood cells at a concentration as low as 0.01%. At this concentration, the red cell to endothelial cell ratio under our experimental condition was about 5. Thus red cells were potent scavengers of exogenous H_2O_2.

Effect of red cells on the hyperoxia-induced injury. Figure 3 summarizes the effect of red cells on the hyperoxia-induced injury against endothelial cells. The number of endothelial cells in control monolayers was 2.35×10^5 on Day 3 and 3.24×10^5 on Day 7. Red blood cells at concentrations ranging from 0.01 to 1.0% had no effect on the number of endothelial cells under control conditions (data not shown). Exposure to hyperoxia caused a time and dose dependent reduction of the number of viable endothelial cells. Red blood cells (0.01 to 1.0%) did not protect endothelial cells against hyperoxia-induced injury by either 80 or 95% O_2. At 7 days after exposure to 95% O_2, there was a slight protection by the red cells, but only the group with 0.5% red cells reached statistical significance.

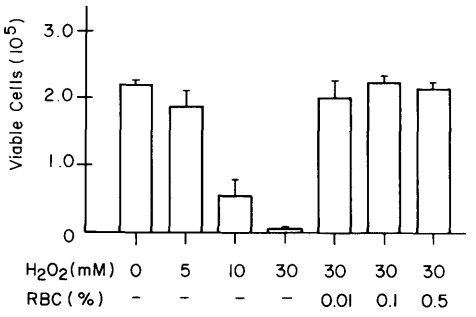

FIG. 2. Effect of red blood cells on the H_2O_2-mediated damage of endothelial cells. Confluent endothelial monolayers were incubated as described in Fig. 1. At the end of the incubation, the number of viable cells was determined using trypan blue stain. The results are the mean ± SEM of three experiments.

Effect of hyperoxia exposure on red blood cells. It is possible that failure of red blood cells to protect endothelial cells against hyperoxia-induced injury is due to inactivation of red cells by hyperoxia rendering them ineffective in scavenging reactive oxygen species and/or causing hemolysis. In the preceding experiments, gross hemolysis of red cells was not observed when red cells were exposed to as high as 30 m*M* H_2O_2 for 3 hr or exposed to 95% O_2 for 7 days. To further rule out the possibility of hemolysis as the cause of failure to protect against hyperoxia-in-

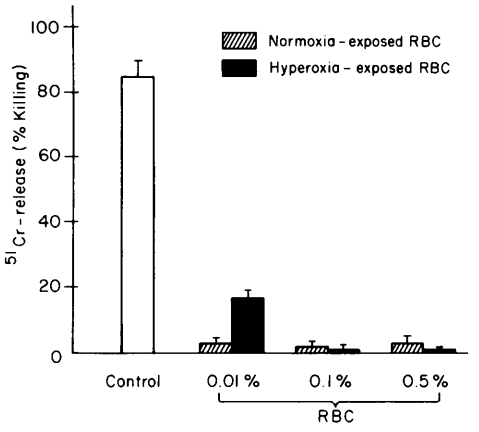

FIG. 4. Effect of hyperoxia-exposed red blood cells on the H_2O_2-mediated ^{51}Cr release by endothelial cells. Confluent ^{51}Cr-endothelial monolayers were incubated as described in Fig. 1 except that red blood cells were previously exposed to normoxia or hyperoxia (95% O_2 and 5% CO_2) for 7 days. The results are the mean ± SEM of three experiments.

duced injury, red cells were labeled with ^{51}Cr and then exposed to normoxia or 95% O_2 for 7 days. This experiment was possible because of the slow rate of spontaneous release of ^{51}Cr from ^{51}Cr-labeled red cells. There was no difference in the ^{51}Cr release by ^{51}Cr red cells in the two groups (15.8 ± 0.6% vs 16.3 ± 0.7%, n = 3).

Figure 4 is a comparison of control and hyperoxia-exposed red cells on the protection against H_2O_2 toxicity. After exposure to 95% O_2 for 7 days, red cells were less effective in protecting endothelial cells against 30 mM H_2O_2. However, this effect was seen only at a red cell concentration of 0.01%. Upon the addition of H_2O_2 (30 mM), hemolysis was noted on hyperoxia-exposed but not control red cells. At 0.1 and 0.5%, but not at 0.01%, sufficient red cells were left to completely protect the endothelial cells against H_2O_2 toxicity.

Discussion. The results presented demonstrate that human red blood cells, though protecting endothelial cells against exogenous H_2O_2 toxicity efficiently, do not protect endothelial cells against hyperoxia-induced injury. Failure of hyperoxia-exposed red cells to protect endothelial cells against hyperoxia-induced injury was not due to hemolysis. In addition, hyperoxia-exposed red cells were still capable of protecting endothelial cells against H_2O_2-mediated damage, though they were less efficient than the control red cells. These observations asre consistent with the current concept that under hyperoxic conditions, reactive oxygen species are produced intracellularly as products of cellular oxidation–reduction processes (2). Thus exogenous reactive oxygen species scavengers would have no protective effect unless they are incorporated into the cells. Freeman *et al.* (15, 16) have shown that liposome-encapsulated superoxide dismutase and catalase which are taken up by the cells, but not free enzymes, are able to protect endothelial cells against hyperoxia-induced injury *in vitro* and protect mice against the lethal effect of hyperoxia *in vivo*.

Our results also suggest that protection of rats against the lethal effect of hyperoxia by intratracheal insufflation of red cells as reported by van Asbeck *et al.* (9) is not due to the direct scavenging of reactive oxygen species produced by the cells under elevated partial pressures of oxygen. In rats exposed to 95% O_2, an influx of neutrophils into the lung parenchyma precedes the death of animals (17). Furthermore, when rabbits are made neutropenic, the mortality from hyperoxia decreases (18), suggesting the important role of neutrophils in the hyperoxia-induced injury under *in vivo* conditions. Thus, the insufflated red cells may protect the animals by scavenging reactive oxygen species produced by activated neutrophils as suggested by van Asbeck *et al.* (9). However, ultrastructural studies have revealed that endothelial cells are the prime target of hyperoxia-induced injury *in vivo* (19, 20). Since the insufflated red cells stay in the alveoli, it is not clear how they protect pulmonary endothelial cells by scavenging reactive oxygen species in the alveolar space. In animals surviving the lethal dose of hyperoxia, there was no neutrophil infiltration in the lungs (9). It is possible that the insufflated red cells prevent the production of chemotactic factors in the alveolar space, thus preventing the influx of neutrophils and subsequent injuries. Further studies are necessary to clarify these points.

This work was supported in part by the Veterans Administration Medical Research Service and a grant from the U. S. Public Health Service HL-32418. The authors thank Denise Fournier for secretarial assistance.

1. Rinaldo JE, Rogers RM. Adult respiratory-distress syndrome. Changing concepts of lung injury and repair. N Engl J Med **306**:900–909, 1982.
2. Frank L, Massaro D. Oxygen toxicity. Amer J Med **69**:117–126, 1980.
3. McCord JM. Oxygen-derived radicals in postischemic tissue injury. N Engl J Med **312**:159–163, 1985.
4. Lynch RE, Fridovich I. Permeation of the erythrocyte stroma by superoxide radical. J Biol Chem **253**:4697–4699, 1978.
5. Toth KM, Clifford DP, Berger EM, White CW, Repine JE. Intact human erythrocytes prevent hydrogen peroxide mediated damage to isolated perfused rat lungs and cultured bovine pulmonary artery endothelial cells. J Clin Invest **74**:292–295, 1984.
6. Agar NS, Sadrzadeh SMH, Hallaway PE, Eaton JW. Erythrocyte catalase. A somatic oxidant defense. J Clin Invest **77**:319–321, 1986.
7. Test ST, Weiss SJ. Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. J Biol Chem **259**:399–405, 1984.
8. Winterbourn CC, Stern A. Human red cells scavenge extracellular hydrogen peroxide and inhibit formation of hypochlorous acid and hydroxyl radical. J Clin Invest **80**:1486–1491, 1987.
9. van Asbeck B, Hoidal S, Vercellotti GM, Schwartz BA, Moldow CF, Jacob HS. Protection against lethal hyperoxia by tracheal insufflation of erythrocytes: Role of cell glutathione. Science **277**:756–759, 1985.
10. Tsan MF, Danis EH, Del Vecchio PJ, Rosano CL. Enhancement of intracellular glutathione protects endothelial cells against oxidant damage. Biochem Biophys Res Commun **127**:270–276, 1985.
11. Phillips PG, Higgins PJ, Malik AB, Tsan MF. Effect of hyperoxia on the cytoarchitecture of cultured endotheliual cells. Amer J Pathol, in press.
12. Del Vecchio PJ, Lincoln DW II. Phenotypes of cultured endothelium, expression of angiotensin-converting enzyme activity. In: Thilo-Korner DGS, Freshney, RI, Eds. The Endothelial Cell—A Pluripotent Control Cell of the Vessel Wall. Basel, Karger, pp67–83, 1983.
13. Tsan MF, Phillips PG. L-2-Oxothiazolidine-4-carboxylate protects cultured endothelial cells against hyperoxia-induced injury. Inflammation **12**:113–121, 1988.
14. Croxton FE. Elementary Statistics with Applications in Medicine and Biological Sciences. New York, Dover, pp 235–244, 1953.
15. Freeman BA, Young SL, Crapo JD. Liposome-mediated augmentation of superoxide dismutase in endothelial cells prevent oxygen injury. J Biol Chem **258**:12534–12542, 1983.
16. Turrens JF, Crapo JD, Freeman BA. Protection against oxygen toxicity by intravenous injection of liposome-entraped catalase and superoxide dismutase. J Clin Invest **73**:87–95, 1984.
17. Fox RB, Hoidal JR, Brown DM, Repine JE. Pulmonary inflammation due to oxygen toxicity: Involvement of chemotactic factors and polymorphonuclear leukocytes. Amer Rev Respir Dis **123**:521–523, 1981.
18. Shasby DM, Fox RB, Harada RN, Repine JE. Reduction of the edema of acute hyperoxic lung injury by granulocyte depletion. J Appl Physiol **52**:1237–1244, 1982.
19. Bowden DH, Adamson IYR. Endothelial regeneration as a marker of the differential vascular responses in oxygen-induced pulmonary edema. Lab Invest **30**:350–357, 1974.
20. Crapo JD, Barry HA, Foscue HA, Shelburne J. Structural and biochemical changes in rat lungs occuring during exposures to lethal and adaptive doses of oxygen. Amer Rev Respir Dis **122**:123–143, 1980.

Received January 20, 1988. P.S.E.B.M. 1988, Vol. 188.
Accepted March 23, 1988.