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Functional Inhibition of Leukocyte B_2 Integrins by Hyperbaric Oxygen in Carbon Monoxide-Mediated Brain Injury in Rats

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Exposure to hyperbaric oxygen [3 atmospheres absolute (ATA) for 45 min] inhibited carbon monoxide (CO)-mediated lipid peroxidation in the brains of rats by preventing the conversion of xanthine dehydrogenase to oxidase, a conversion process known to be due to the action of leukocytes. The effect was the same whether treatment was given 24 hr before or up to 45 min after poisoning. Hyperbaric oxygen did not inhibit the initial interaction of leukocytes with brain microvasculature, based on measurements of myeloperoxidase (MPO) in microvessel segments, but persistent adherence, which is due to B_2 integrins, did not occur. Exposing rats to 3 ATA pressure (0.21 ATA O_2) after CO poisoning had no significant effects. A progressive reduction in brain microvessel MPO titers occurred with exposure to O_2 at 1, 2, or 3 ATA after CO poisoning, but 1 ATA O_2 treatment did not significantly inhibit xanthine oxidase formation or lipid peroxidation. *In vitro* studies with polymorphonuclear leukocytes (PMN) from rats exposed to hyperbaric oxygen corroborated the absence of PMN B_2 integrin function, but when these cells were stimulated they exhibited normal B_2 integrin expression on their surface and also normal elastase release and superoxide radical production. Adherence functions of PMN that do not require B_2 integrins appeared to remain intact after exposure to hyperbaric oxygen, as peritoneal neutrophilia in response to a glycogen challenge was not inhibited. B_2 integrin function could be restored by incubating cells with 8 bromo cGMP, and incubation with phorbol ester stimulated the adherence function of both control and hyperbaric oxygen-exposed PMN. These results provide a clear mechanism for the inhibition of CO-mediated brain lipid peroxidation by hyperbaric oxygen and indicate that hyperoxia causes a discrete disturbance of PMN adherence function.

Supplemental O_2 has long been regarded as a component of the treatment for carbon monoxide (CO) poisoning because it can hasten the rate of dissociation of CO from hemoglobin. Since 1960, hyperbaric O_2 between 2.0 and 3.0 atmospheres absolute (ATA) has been used increasingly, and a body of experience indicates that hyperbaric O_2 may reduce mortality and morbidity beyond that expected with sea level O_2 treatment (Goulon *et al.*, 1969; Myers *et al.*, 1985; Mathieu *et al.*, 1985). We have observed similar results in a small prospective clinical trial (Thom *et al.*, 1992). We have also reported that treatment of rats with hyperbaric O_2 at 3 ATA will prevent brain lipid peroxidation, whereas treatment with 1 or 2 ATA O_2, or 3 ATA normoxic gas (a pressure control) were of no benefit (Thom, 1990a). Tomaszewski *et al.* (1992) have shown that 3 ATA O_2 treatment can prevent functional neurological impairment in rats (ability to maneuver in a maze) and neuropathology after CO poisoning according to our model.

We have shown that CO precipitates physiological and biochemical changes in rat brains that are consistent with aspects of an ischemia-reperfusion injury. During the first 90 min after poisoning, xanthine dehydrogenase (XD) is converted to xanthine oxidase (XO) and O_2 radicals generated by XO are responsible for lipid peroxidation (Thom, 1992a). It is difficult to reconcile the apparent beneficial effects of exposure to elevated pressures of O_2 with this mechanism. Exposure to O_2 at partial pressures greater than ambient air, up to 1 ATA or 760 mm Hg, has been shown to exacerbate reperfusion injuries in animals and *in vitro* organ models (Mickel *et al.*, 1988; Fisher *et al.*, 1991). However, exposure to hyperbaric O_2 at 2 to 3 ATA for 45 min to 2 hr actually improves outcome from a number of ischemia-reperfusion injuries (Weinstein *et al.*, 1986, 1987; Nylander *et al.*, 1988; Thomas *et al.*, 1990; Zamboni *et al.*, 1992). The mechanism of this benefit is unknown.

The aim of this study was to investigate the mechanism of action of hyperbaric O_2 in antagonizing oxidative injury after CO poisoning. In a companion paper (Thom, 1993) we showed that leukocytes were responsible for XD conversion to XO following CO poisoning. Hence, the current investigation was focused largely on assays of leukocyte functions.

METHODS

Animals and reagents. Wistar male rats (Charles River Laboratories, Wilmington, MA) weighing 190–300 g were fed a standard diet and water *ad libitum*. Mouse monoclonal IgG_1 to rat CD-18 was purchased from

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Seikagaku America Inc. (Rockville, MD). This antibody has been shown to specifically bind CD-18, to block stimulated leukocyte aggregation, and to inhibit leukocyte binding to ICAM-1 (Tamatani *et al.*, 1991). Mouse monoclonal IgG_1 to rat CD-45, an antigen common to all rat leukocytes, was a product of SeraLab, Ltd., and was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Goat anti-mouse IgG (heavy and light chain) conjugated to fluorescein isothiocyanate (IgG–FITC) was purchased from Life Sciences Technologies–Gibco (Gaithersburg, MD). The following reagents were purchased from Sigma Chemical Co., (St. Louis, MO): cytochalasin B, bovine serum albumin, diaminobenzidine tetrahydrochloride (DAB), bovine erythrocyte superoxide dismutase (SOD), oyster shell glycogen, 8-bromo cGMP, phorbol 12-myristate 13-acetate (PMA), *N*-formyl-methionyl-leucine-phenylalanine (FMLP), and *N*-succinyl(ala)$_3$nitroanilide. Platelet activating factor (PAF) (1-*O*-octadecyl-2-*O*-acetyl-sn-glycero-3-phosphorylcholine) was purchased from Matreya Inc. (Pleasant Gap, PA). Percoll was purchased from Pharmacia Fine Chemicals inc. (Piscataway, NJ), and scrubbed nylon (Type 200L) was a product from DuPont Biotechnology Systems Division (Boston, MA). All other reagents were purchased from Fisher Scientific Corp. (Pittsburgh, PA). Compressed gases were purchased from Air Products and Chemicals Inc. (Allentown, PA).

Animal procedures. Exposure to CO followed our model described in several previous publications (Thom, 1990a,b, 1992a). In brief, rats were placed in a 7-liter Plexiglas chamber into which a small volume of pure CO was injected to achieve a concentration of 1000 ppm. A mixture of 1000 ppm CO in air was then flushed through at a rate of 8 to 12 liters/min for 40 min, at which time the gas was switched to 3000 ppm CO in air and another CO bolus was added. The majority of rats lost consciousness in this environment within 20 min. In previous studies we have found that several seconds after loss of consciousness there occurs transient systemic hypotension (Thom, 1990b) and cerebral hypoperfusion (Mayevsky and Thom, 1992). Timing was found to be important for development of lipid peroxidation and for treatment. Therefore, to keep rats matched over months of study, any rats that did not fall unconscious by 20 min were rendered unconscious with a 60-cc pulse of pure CO. These rats showed the same cardiovascular and biochemical changes as rats that fell unconscious spontaneously (Thom, 1990b; 1992a). After losing consciousness, rats were removed from the chamber to breath air, and they regained consciousness in approximately 4 min.

Glycogen peritonitis was produced by injecting rats with 15 ml of sterile 0.33% (w/v) oyster shell glycogen. Four hours after the injection rats were killed with an overdose of pentobarbital and the abdomen was lavaged with 20 ml 0.9% (w/v) saline. Exactly the same technique was performed on all rats, and by using a large volume of fluid to lavage the cells from the abdomen, the volume of fluid recovered was within 0.2 ml for each rat. Results were expressed as number of PMN recovered per milliliter. Cell counts were performed using a phase-contrast microscope and counting chamber. In all instances the differential count performed with Wright's stained smears demonstrated >98% PMN in the fluid.

Biochemical assays. All manipulations and tissue biochemical assays have been described in detail in previous publications (Thom, 1992a, 1993). Rats were killed by decapitation at the times indicated in the text for assays of XD, XO, and conjugated dienes. The brains were immediately removed, bisected along the sagittal sinus, and frozen in liquid nitrogen within 45 sec of death. Frozen brain was homogenized in buffer designed to minimize artifactual conversion of XD to XO by either proteolytic or sulfhydryl oxidation mechanisms (50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and, with one half of each brain, 5 mM dithioerythritol (DTE) to convert sulfhydryl oxidized XO back to XD). Activity for XD and for total and DTE-irreversible XO was expressed as milliunits (mU)/mg soluble protein in the collected Sephadex G-25 eluates, with 1 unit being defined as the amount of enzyme required to convert 1 μmol of pterine, a substrate analog of xanthine, to the fluorescent product isoxanthopterine per minute. Conjugated dienes were assayed spectrophotometrically in extracted lipids and concentration was expressed as μmol/mg lipid as previously described (Thom, 1992a).

Microvessels from brain homogenates were prepared using a slight modification of the method originally described by Betz (1985). Our technique, described in detail in our companion paper (Thom, 1993), involved pouring a fraction of homogenized brain through a column of glass beads (25 to 33 μm in size). Microvessels adhere to the beads, and they were eluted off by washing with 10 ml 15 mM Hepes (hydroxymethylaminomethane), pH 7.4, containing 1 mM $MgCl_2$, 5 mM glucose, and 1% (w/v) bovine serum albumin. Based on assays of alkaline phosphatase, this technique achieved a 14-fold enrichment of blood vessels compared with whole brain homogenates.

Microvessels to be used for morphological studies (see below) were taken directly from the glass bead eluate. For assays of myeloperoxidase (MPO), the solution was centrifuged (5000*g* for 10 min), and the pellet was resuspended in 0.6 ml 50 mM potassium phosphate, pH 6.0, containing 0.5% (w/v) hexadecyl triethyl ammonium bromide. The solution was then sonicated in an ice-cold solution using a Heat Systems–Ultrasonics (Plainview, NJ) sonicator at a setting of 7 for three 30-sec cycles. The solution was centrifuged at 12,000*g* for 15 min, and the supernatant was used in the MPO assay.

The MPO assay was carried out in a dual-beam spectrophotometer (Hitachi Model 3210). The assay solution included 0.8 ml 50 mM potassium phosphate, pH 6.0, containing 0.5 mM *ortho*-dianisidine and 0.08 mM H_2O_2. The reaction was initiated by adding 0.2 ml of microvessel extract and absorbance at 460 nm (A_{460}) was measured for 5 min.

Morphologic studies. Microvessels were stained to detect peroxidase activity by incubating 20-μl samples with 60 μl of solution containing 50 mM Tris–HCl, pH 7.6, 1.4 mM DAB, and 380 mM hydrogen peroxide. The samples were then examined under a phase-contrast microscope after 1 hr. Samples were prepared for fluorescence studies as previously described (Thom, 1993), stained with a mouse anti-rat leukocyte specific antibody (CD-45) followed by counterstaining with goat anti-mouse IgG–FITC. Samples were visualized using a fluorescence microscope.

Oxygen exposures. Pure oxygen (medical grade, 98%) at 1 atmosphere absolute pressure or hyperbaric pressure (2 or 3 ATA), was delivered using a Bethlehem Steel Corp. Model G15-APSP hyperbaric chamber following established protocols (Thom, 1990a). When the hypoxic gas mixture was used, the air-filled chamber was first compressed to 3 ATA with nitrogen and then flushed with a 7% O_2/93% N_2 gas mixture.

The effect of 3 ATA O_2 on MPO activity was investigated by incubating microvessel extracts for 45 min at room temperature in a small, nonmagnetic metal chamber that was first flushed with pure O_2 and then compressed with O_2 to 3 ATA. This chamber has been described in detail in a previous publication (Thom, 1992b). After compression was complete, the chamber was placed on a magnetic stirrer so that the solution in the chamber could be stirred by a magnetic stirring bar present in the glass vessel holding the solution. After 45 min, the chamber was decompressed and the MPO activity was measured using our standard technique (described above).

In vitro PMN-associated assays. Heparinized (10 U/ml) blood was obtained from anesthetized rats (pentobarbital, 0.05 mg/g ip) by aortic puncture at the times indicated in the text. The PMN nylon adherence assay of MacGregor *et al.* (1974) was used, and our standard technique is described in the companion paper (Thom, 1993). In some instances heparinized blood was incubated with an additional chemical—PMA, FMLP, or PAF at a concentration of 1 μM, or 10 μM 8-bromo cGMP—at 37°C prior to filtration. Incubation times were based on preliminary studies and were the times required to achieve the maximum effects on PMN adherence. PMN incubation with PMA was carried out for 20 min, incubation with 8-bromo cGMP for 30 min, and PAF or FMLP for 10 min. In the case of the latter two chemicals, there were no significant changes in adherence whether the incubations were carried out for 2 to 30 min (data not shown).

Antibody effects on adherence were carried out using blood incubated for 5 min with mouse anti-rat CD-18 or CD-45 IgG (200 μg/ml blood). For assays involving flow cytometry, 200 μl of this blood was diluted with 3 ml ice-cold phosphate-buffered saline (PBS) (10 mM sodium phosphate, 27 mM KCl, 137 mM NaCl, pH 7.4) and centrifuged to pellet the leukocytes and erythrocytes (200*g* for 20 min). Binding of anti-CD-18 to unstimulated PMN was carried out using blood that was kept in an ice bath and that had not been passed through a nylon column. Following centrifugation, cells were washed once in PBS and then incubated with 2.8 μg goat anti-mouse IgG–FITC for 60 min at 4°C. As a control for each run, 200 μl blood was also incubated with mouse IgG that was not directed against a rat antigen and then stained with anti-mouse IgG–FITC to control for nonspecific binding. Following this cold incubation, the cells were suspended in 5 ml 0.87% (w/v) NH_4Cl, left to stand for 15 min at room temperature to lyse the erythrocytes, and then centrifuged at 1000*g* for 7 min. The pellet was washed with PBS and resuspended in 1 ml PBS for flow cytometry studies (Coulter Epics C clinical flow cytometer). Scans were gated to count only PMN and the results were expressed as mean channel linear fluorescence based on 5000 cells. Preliminary studies established saturating levels of the primary antibodies and experiments were performed using at least a twofold excess.

Elastase release from PMN was assayed using PMN separated from blood using standard techniques of sequential dextran sedimentation, separation through 43 and 53% plasma–Percoll gradients, and hypotonic lysis of the remaining erythrocytes. To obtain an adequate number of cells for each study, the blood from two rats was pooled. The resulting cell preparations were >95% PMN by Wright's stain, and >98% viable based on trypan blue dye exclusion. Elastase release was determined by suspending 2×10^6 cells in PBS with 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% albumin, followed by incubation for 10 min at 37°C with 5 μg/ml cytochalasin B. PMN were then incubated with 1 μM FMLP for 30 min at 37°C. Cells were pelleted by centrifugation at 400*g* for 20 min at 4°C, and the supernatant (I) was set aside on ice. Cells were resuspended in PBS containing $MgCl_2$, $CaCl_2$, and albumin, plus 0.05% (v/v) Triton X-100, and sonicated (Heat Systems–Ultrasonics sonicator Model W220-F at a setting of 7) on ice using three 2-minute cycles. Cellular debris was pelleted by centrifugation at 22,500*g* for 10 min at 4°C. The supernatant (II) was utilized to determine residual elastase left in cells after stimulation. Elastase was assayed using aliquots of supernatant suspended in solutions containing 0.5 M NaCl and 0.2 M Hepes, pH 7.5. The substrate, *N*-succinyl (ala)$_3$ paranitroanilide, was added from a stock solution in DMSO and the entire suspension was incubated at 37°C (total DMSO in the suspension was 5% v/v). The absorbance change at 405 nm was followed, and the reaction remained linear for at least 2 hr. Elastase activity was determined as milliunits (mU)/1×10^6 PMN (1 unit is defined as the amount of enzyme required to hydrolyze 1 μmol substrate/min). The responsiveness of different populations of PMN (control versus those exposed to 3 ATA O_2) was expressed as a percentage of total elastase activity released due to stimulation [(activity in supernatant I/activity in supernatants I + II) × 100].

Production of superoxide (O_2^-) by PMN was determined using the method described by Johnston and Lehmeyer (1976). PMN (1×10^6) were suspended in PBS containing 0.08 mM ferricytochrome c with or without 70 U/ml bovine erythrocyte SOD. Cells were incubated at 37°C in a water bath with and without 1 μM FMLP or 1 μM PMA. Samples were taken, and the suspended cells were removed by centrifugation (1000*g* for 6 min at 4°C), or by rapid filtration through a Millipore filter (0.45-μm exclusion limit). Absorbance changes were measured at 550 nm ($E_{550} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$), and both separation techniques gave identical results. The rate of O_2^- production was taken as the difference between the cytochrome c reduction rates in the absence and presence of SOD. Incubations were carried out in polypropylene test tubes.

Results were expressed as mean ± SE. Statistical significance was determined by ANOVA followed by Scheffe's test (Snedecor and Cochran, 1980). The level of significance was taken as $p < 0.05$.

TABLE 1
Myeloperoxidase Activity in Brain Microvessels

Group	*n*	Activity ($A_{460} \times 10^3$/min/g brain)
Control	4	0.6 ± 0.3 (SE)
CO (immediate)	4	5.9 ± 0.9*
CO (90 min)	4	6.7 ± 1.0*
CO (3 ATA pressure)	3	4.5 ± 0.4*
CO (1 ATA O_2)	6	1.8 ± 0.3*,**
CO (2 ATA O_2)	3	1.3 ± 0.3**
CO (3 ATA O_2)	5	0.5 ± 0.2**
3 ATA O_2-CO (immediate)	4	6.7 ± 1.8*
3 ATA O_2-CO (90 min)	4	0.5 ± 0.1**

Note. Rats were poisoned with CO and killed immediately, 90 min later, or poisoned with CO and then exposed 45 min later to 3 ATA pressure (CO-3 ATA pressure), 1 ATA O_2 (CO-1 ATA O_2), 2 ATA O_2 (CO-2 ATA O_2), or 3 ATA O_2 (CO-3 ATA O_2), for 45 min and then killed. Two additional groups were exposed to 3 ATA O_2 for 45 min 24 hr before CO poisoning, and then killed either immediately after poisoning (3 ATA O_2-CO (immediate) or 90 min later (3 ATA O_2-CO (90 min)]. Values are mean ± SE. An MPO activity of 1.6×10^{-3} A_{460}/min was due to the presence of 1000 PMN based on a standard curve generated using techniques described in a companion paper (1993).

* $p < 0.05$, ANOVA versus control.

** $p < 0.05$ versus CO-90 min.

RESULTS

Myeloperoxidase activity. MPO titers in brain microvessel segments were measured as an index of leukocyte adherence using rats poisoned with CO and then treated for 45 min, beginning 45 min after CO exposure. Treatments were carried out with either 1, 2, or 3 ATA O_2, or, as a pressure control, 3 ATA of a gas mixture that resulted in normoxia (O_2 partial pressure 0.21 ATA) at 3 ATA total pressure (Table 1). All rats were killed 90 min after CO poisoning. This protocol was chosen because, in a previous study (Thom, 1990a), we found that 3 ATA O_2 inhibited lipid peroxidation, which is at a peak level 90 min after CO poisoning.

We have reported that leukocytes were identifiable in microvessels taken from CO-poisoned rats following staining for peroxidase activity or by immunofluorescence techniques (Thom, 1993). Using these techniques, we failed to observe leukocytes in microvessels taken from rats treated with 3 ATA O_2. The MPO results suggest, therefore, that O_2 treatments at 3 ATA were effective in preventing leukocyte sequestration in the microvasculature after CO poisoning. We also found that oxygen pressures less than 3 ATA were effective in reducing MPO activity after CO poisoning, although 1 ATA exposures were less effective than 3 ATA exposures.

Inhibition of MPO enzyme activity by hyperbaric oxygen is an alternative explanation for these data. To investigate

TABLE 2
Blood Cell Counts

Sample	n	WBC/μl ($\times 10^3$)	Differential (%)			HCT (%)
			PMN	Lymphocyte	Monocyte	
Control	8	6.3 ± 0.7	17 ± 3	76 ± 6	7 ± 4	43 ± 1
3 ATA O_2	18	6.9 ± 1.1	15 ± 2	75 ± 1	10 ± 2	45 ± 2

Note. Blood cell counts from control rats and from rats exposed to 3 ATA O_2 for 45 min. n = number of rats. (No significant differences between control and 3 ATA O_2.)

this possibility MPO assays were carried out on four microvessel extracts before and after exposure to 3 ATA O_2 for 45 min (see Methods). The values after oxygen exposure were exactly the same as the preexposure values in all four samples (data not shown), indicating that hyperbaric oxygen does not inhibit MPO activity.

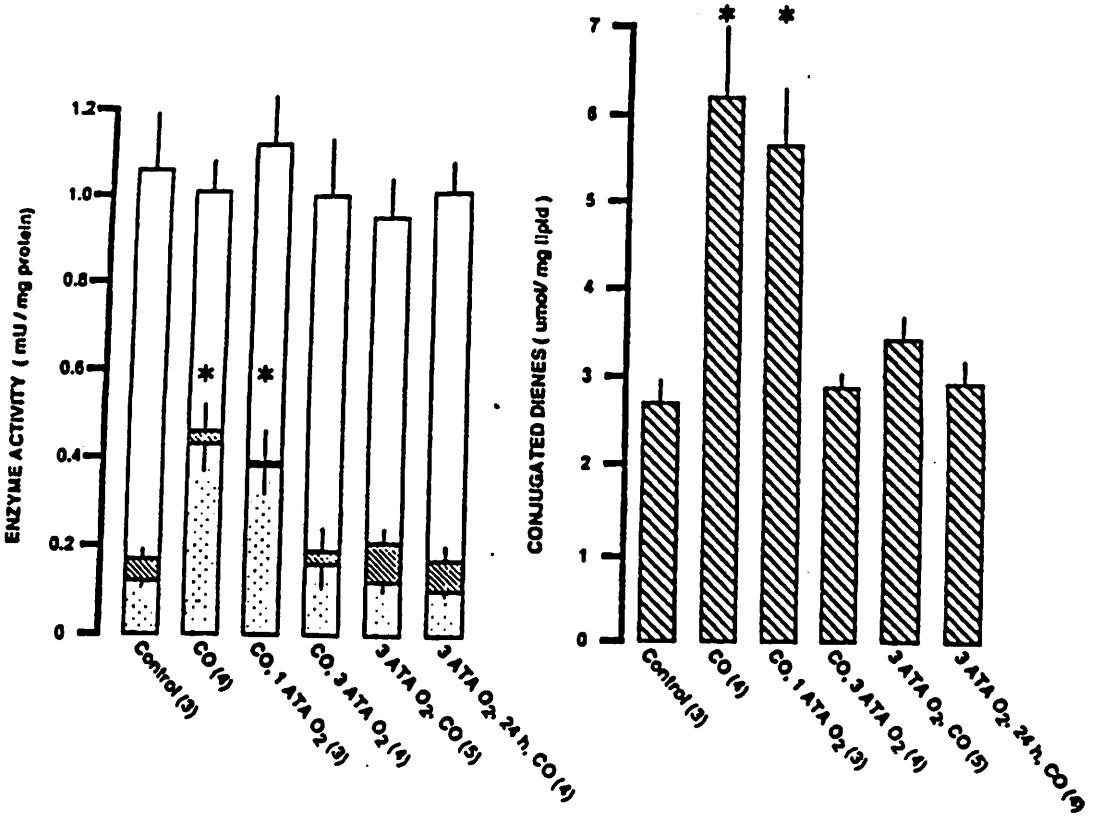
Blood cell counts were performed on animals exposed to 3 ATA O_2 to ascertain whether the effect of hyperbaric oxygen might be attributable to a marked change in the numbers of circulating leukocytes. As shown in Table 2 there were no significant differences between blood cell counts for control rats and for those exposed to 3 ATA O_2.

CO binding to heme-proteins is involved with CO pathophysiology, and hyperbaric O_2 is known to hasten the dissociation of CO from heme-proteins (Shephard, 1983). If this action of hyperbaric O_2 were involved in our observations, we reasoned that pretreatment would not have an effect on the MPO titer. In fact, the microvessel MPO titers from the brains of rats treated with 3 ATA O_2 24 hr before CO poisoning and killed 90 min after poisoning were not significantly elevated above the control level (Table 1). We conclude therefore, that hastened CO dissociation from heme-proteins is not involved. To investigate the effect of hyperbaric O_2 on the initial interaction between leukocytes and the microvascular endothelium, rats were treated with 3 ATA O_2 24 hr before CO poisoning and killed immediately after poisoning. As shown in Table 1, hyperbaric O_2 did not inhibit the early increase in the MPO titer of microvessels.

XD to XO conversion and lipid peroxidation. · The effects of O_2 treatment on CO-mediated biochemical changes, XD conversion to XO, and lipid peroxidation assessed as conjugated diene formation are outlined in Fig. 1. A group of control rats and a group of rats poisoned with CO and then killed either immediately or 90 min later were included for this series of studies. A group of five rats was also studied after being exposed to only 3 ATA O_2 (no CO poisoning) to assess whether hyperoxygenation altered the baseline biochemical profile. XD, total and sulfhydryl-irreversible XO, and conjugated diene levels in these rats were the same as in control rats (data not shown). As shown in Fig. 1, rats poisoned with CO and then treated with 3 ATA O_2 exhibited virtually identical values as control rats exposed only to air. In contrast, 1 ATA O_2 treatment did not prevent enzyme conversion or lipid peroxidation. We also found that treatment with 3 ATA O_2 was equally as effective whether administered 24 hr before poisoning, immediately before poisoning, or 45 min after poisoning.

Effects of exposure to CO or 3 ATA O_2 on in vitro PMN adherence. When blood is filtered through nylon fibers, a fraction of the PMN adhere to the nylon, and we have found that this adherence was nearly completely inhibited by monoclonal IgG to the CD-18 epitope of B_2 integrins (Thom, 1993). Therefore, we have used this procedure to assay for B_2 integrin function. To ease comparisons of the adherence characteristics of PMN, data from our previous study (Thom, 1993) on blood from control rats and the effect of incubating blood from control rats with anti-CD-18 IgG, and as a control, blood incubated with an antibody specific to another leukocyte antigen, CD-45, are included in Fig. 2.

There was no detectable PMN adherence to nylon by cells from rats that were exposed to 45 min of 3 ATA O_2 administered either 24 hr or immediately before blood was obtained. PMN binding function could be restored by pretreating blood with 8-bromo cGMP, and PMA exposure also markedly increased adherence of cells from rats exposed to 3 ATA O_2 as well as cells from control rats. Nylon binding by PMN in blood from rats exposed to 3 ATA O_2 and treated with 8-bromo cGMP or PMA was also dependent on CD-18. If anti-CD-18 IgG was added to this blood, PMN adherence to nylon was negligible. In three trials carried out with blood incubated with 8-bromo cGMP, PMN adherence was 0 ± 0 (SE) %, and in three trials with PMA, 10 ± 5% ($p < 0.05$ versus control, ANOVA). Preincubation of blood with PAF or FMLP modestly increased adherence of PMN from control rats and had no effect on adherence of PMN from rats exposed to 3 ATA O_2. Figure 2 also shows the binding characteristics of the PMN from rats poisoned with CO and killed either immediately, 45 min, or 90 minutes later. These data were reported in our previous paper (Thom, 1993). Among the experiments run



effect of oxygen treatment on CO-mediated biochemical changes in brain. Rats were poisoned with CO for 1 hr where indicated and er. Others were treated with 1 ATA O_2 (CO-1 ATA O_2) or 3 ATA O_2 (CO-3 ATA O_2) for 45 min beginning 45 min after CO poisoning, exposed to 3 ATA O_2 for 45 min immediately prior to CO poisoning (3 ATA O_2-CO), or 24 hr prior to CO poisoning (3 ATA O_2-24 : activities of both enzymes (XD + XO) are shown as open blocks. Sulfhydryl-irreversible XO activities are shown as stippled blocks, and :O activity reversed back to XD by DTE treatments are shown as diagonally hatched blocks. Enzyme activities are expressed as mU/g Conjugated diene levels (diagonally hatched blocks) are expressed as μmol/mg lipid. All values represent mean ± SE; number in e number of rats. *Significantly greater sulfhydryl-irreversible XO activity and conjugated diene level than in control rats (ANOVA).

ly with trials involving studies of rats killed 90 poisoning were three trials where rats were 1 CO, but then breathed air for 45 min fol- nin with 3 ATA O_2 before being killed at 90 soning. No binding was measurable in these

anion production and elastase release. The ıulated production of superoxide anion (O_2^-), 1 μM FMLP or 1 μM PMA, were measured btained from the blood of rats that had been TA O_2 for 45 min. We also measured degran- sed as elastase release in response to 1 μM own in Table 3, the PMN responses were of nitude as in control cells. It is notable that, FMLP did not increase nylon adherence of s exposed to 3 ATA O_2, it did stimulate elas- d O_2^- production.

etry studies. Expression of CD-18 on the was investigated by flow cytometry to evalu- is correlated with the inhibitory effect of hy- n on adherence (Table 4). The response of ntrol rats to passage through nylon was as- sessed by adding anti-CD-18 IgG to the blood prior to filtration to prevent cells from binding to the column. Because cells from rats exposed to hyperbaric oxygen did not bind to nylon, antibody could be added before or after filtration and the results were the same. Data in the figure were obtained from experiments where anti-CD-18 was added prior to filtration. Mean channel fluorescence was doubled by nylon filtration, indicating that cells were stimulated to increase B_2 integrin expression on the membrane surface. The same values were measured using cells from either control or hyperbaric oxygen-exposed rats. We then examined the response of cells to stimulation with 1 μM FMLP or PMA, and again found no differences between the control group and those exposed to hyperbaric oxygen. From these data we conclude that hyperbaric oxygen does not inhibit increased expression of B_2 integrins when PMN are stimulated.

Glycogen peritonitis. The ability of PMN to undergo chemotaxis and transendothelial migration in response to peritoneal glycogen instillation was investigated. Lavage samples were taken 4 hr after injection and the number of PMN in lavage from control rats was compared with that

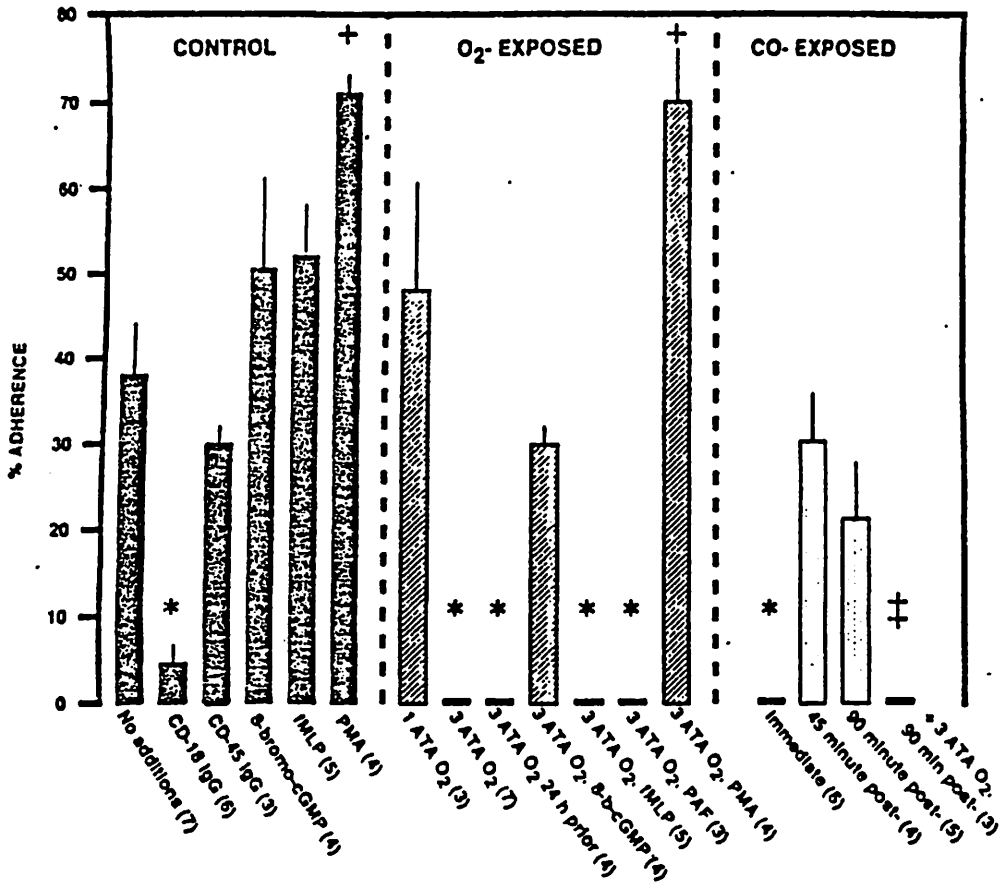


FIG. 2. The adherence of PMN to nylon columns. Labels at the top of the figure indicate gas exposure of animals prior to obtaining blood. Rats were exposed to only air (control), to 45 min of O_2 at 1 ATA or 3 ATA, or to CO per the standard protocol. Rats were anesthetized, and blood was removed within 10 min following gas exposure, except in cases noted as being delayed at the bottom of the figure. Prior to passage through the nylon column, blood from some rats was incubated, as described under Methods, with anti-CD-18 or anti-CD-45 IgG (200 μg/ml), with 10 μM 8-bromo cGMP, or with 1 μM FMLP, PAF, or PMA. Data represent % PMN that adhered to nylon expressed as mean ± SE. *Significantly less than control (ANOVA). †Significantly greater than control. ‡Significantly less than 90 min post-CO without 3 ATA O_2.

from rats that had been exposed to 3 ATA O_2 24 hr before glycogen injection. Hyperbaric O_2 did not inhibit the PMN response to the glycogen challenge. The number of PMN/ml of lavage fluid in control rats was 3.6 ± 0.9 (SE) × 10^6/ml (n = 4), and in rats exposed to hyperbaric O_2 it was 4.7 ± 1.0 × 10^6/ml (n = 4).

DISCUSSION

Hyperbaric O_2 was effective in blocking CO-mediated biochemical changes including XD conversion to XO. We have shown that O_2 radicals from XO cause brain lipid peroxidation (Thom, 1992a), hence, the absence of XO formation explains how hyperbaric O_2 can protect against CO-mediated lipid peroxidation. In our companion paper we established that leukocytes were required for XD conversion to XO, and so we focused the current work on the effects of hyperbaric O_2 on leukocyte function. The MPO activities in microvessel segments obtained from brains immediately after rats were poisoned with CO were the same between untreated rats and those that were exposed to 3 ATA O_2 before CO poisoning. This indicates that the initial interaction of leukocytes with the endothelium was not effected by hyperbaric treatment. However, the MPO level among rats pretreated with 3 ATA O_2 and killed 90 min after CO poisoning was nearly the same as the control (unpoisoned) level. Moreover, treatment even 45 min after poisoning was effective. These results indicate that the later adhesion event, which we have shown to occur approximately 45 min after CO poisoning and to be due to B_2 integrin function (Thom, 1993), is inhibited by hyperbaric O_2. Inhibition of CD-18 dependent adherence by hyperbaric O_2 is corroborated in the *in vitro* PMN adherence assays.

The MPO titers in brain microvessels from rats killed 90 min after CO poisoning were lower in those rats that had been exposed to 1 ATA O_2 after CO poisoning. The level was still significantly greater than in control (unpoisoned) rats, however, and 1 ATA O_2 treatment had no significant effect on XD-to-XO conversion and lipid peroxidation

TABLE 3
Elastase Release and Superoxide Production by Peripheral Blood Neutrophils

Group	Elastase release (%) due to 1 μM FMLP	Total elastase (mU/1 × 10^6 PMN)
Control	21 ± 5 (7)	33 ± 4 (10)
3 ATA O_2	28 ± 8 (5)	32 ± 3 (8)

	O_2^- production (nmol/min/1 × 10^6 PMN)		
	No stimulus	1 μM FMLP	1 μM PMA
Control	0.5 ± 0.3 (4)	1.2 ± 0.5 (5)	38 ± 11 (4)
3 ATA O_2	0.5 ± 0.6 (4)	2.1 ± 0.8 (3)	54 ± 12 (4)

Note. PMN were isolated from peripheral blood and incubated with either 1 μM FMLP or 1 μM PMA as described under Methods. n = number of neutrophil preparations pooled from two rats. Results listed are mean ± SE; no values were significantly different from control (t test).

which follow CO poisoning. Impaired PMN adherence following 1 ATA O_2 exposure was not observed in the *in vitro* experiments, which suggests that 1 ATA O_2 may have been acting on endothelium rather than on PMN to change the adherence response. We have not investigated the mechanism for this effect in this manuscript, but an attractive possibility relates to the enzyme nitric oxide synthase, which is present in the microvascular endothelium. Nitric oxide (NO) will inhibit PMN binding to endothelium (Kubes *et al.*, 1991), and hyperoxia is known to enhance NO production by NO synthase from bovine cerebellum (Rengasany and Johns, 1991).

Exposure to 3 ATA O_2 before CO poisoning was equally as effective as treatment following CO poisoning in preventing CO-mediated leukocyte adherence and biochemical changes. From this finding we conclude that the mechanism of action of hyperbaric O_2 is not based on heme-CO dissociation, but rather is due to an alteration in CD-18-dependent PMN adherence. The flow cytometry assays demonstrate that PMN from rats exposed to hyperbaric O_2 express the CD-18 epitope (B_2 integrin) when stimulated, but the nylon binding studies indicate that the receptors do not function. PMN adherence phenomena require both receptor expression, as well as clustering in the plane of the membrane and possibly other qualitative changes. Several investigations have shown that quantitative changes in surface expression are insufficient to account for PMN aggregation or adherence (Detmers *et al.*, 1987; Buyon *et al.*, 1988; Lo *et al.*, 1989).

Our previous work suggested that leukocyte-derived proteases were responsible for conversion of XD to XO after CO poisoning (Thom, 1993). In this study we have found that PMN from rats exposed to 3 ATA O_2 functioned normally with regard to elastase release and O_2^- production, as well as surface expression of CD-18 when stimulated. These findings are in agreement with Gadd *et al.* (1990), who found that exposure to 2.4 ATA O_2 did not impair murine PMN functions including phagocytosis and oxidative burst. We conclude that prior exposure to hyperbaric O_2 does not disturb the stimulus–response coupling for primary granules (elastase), tertiary granules (CD-18), or NADPH-oxidase activation. Our findings are consistent with the *in vitro* work of Phan *et al.*, (1992), who found that the close contact between PMN and endothelial cells facilitated by B_2 integrins was necessary for PMN proteases (principally elastase) to cause XD conversion to XO.

PMA stimulated equally the PMN from control or 3 ATA O_2-exposed rats. The ability of PMA to elicit PMN responses has been attributed to the activation of a Ca^{2+}-sensitive, phospholipid-dependent protein kinase. Phosphorylation states of a large number of proteins change upon stimulation with PMA (White *et al.*, 1984). We utilized 8-bromo cGMP in our study because of the observation by MacGregor *et al.* (1978) that it increased the adherence of PMN to nylon. In contrast to their work, we did not detect an increase in binding to nylon by cells from control rats, which may be due to species differences. However, this agent did restore CD-18-dependent binding of PMN from rats exposed to hyperbaric O_2. Analogues of cGMP, including 8-bromo cGMP, have been used to demonstrate the presence of a cGMP-dependent protein kinase in vascular smooth muscle. Activation leads to the phosphorylation of 9 to 11 proteins, as well as the dephosphorylation of myosin (Rapaport *et al.*, 1983). We speculate that activation of a cGMP-dependent protein kinase by addition of 8-bromo cGMP may be the basis for the restoration of CD-18 func-

TABLE 4
B_2 Integrin Expression on PMN

Group	Fluorescence
Unstimulated	51 ± 3 (5)
Post-nylon column	
Control	97 ± 27 (4)
3 ATA O_2	87 ± 6 (4)
FMLP	
Control	99 ± 12 (3)
3 ATA O_2	97 (2)
PMA	
Control	154 ± 46 (3)
3 ATA O_2	223 ± 45 (3)

Note. Flow cytometry analysis of blood from control rats or from rats exposed to 3 ATA O_2 for 45 min. Expression of the CD-18 epitope was recorded as mean channel linear fluorescence using unstimulated cells and cells stimulated by passage through a nylon column, or incubation with either 1 μM FMLP or 1 μM PMA. Values indicate mean ± SE; number in parentheses indicate number of rats. All stimulated values were greater than the unstimulated value (ANOVA). There are no significant differences between the control and 3 ATA O_2 groups.

tion in PMN from rats exposed to hyperbaric O_2. It is possible that hyperbaric oxygen may cause partial denaturation of the protein kinase, leading to a decrease in its affinity for cGMP. Several cGMP analogues, including 8-bromo-cGMP, have a higher affinity for this enzyme than native cGMP (Corbin *et al.*, 1986). Alternatively, the normal enzymatic source of cGMP, guanylate cyclase, may be inhibited by hyperbaric oxygen. This enzyme is known to be O_2-labile due to damage to the nonregulatory (nonheme containing) subunit (Brune *et al.*, 1990).

Glycogen peritonitis was investigated to assess whether the antagonistic effect of hyperbaric O_2 on PMN–endothelial cell interactions could be shown with another stimulus. PMN recruitment following glycogen injection is inhibited by anti-CD-18 antibodies (Mulligan *et al.*, 1992a); however, there are additional mechanisms for PMN–endothelial cell interactions following a glycogen challenge that are independent of B_2 integrins. Interactions mediated through activated complement and E selectin also participate (Mulligan *et al.*, 1991, 1992b). Apparently, these redundant mechanisms allow for PMN recruitment despite the inhibitory influence of hyperbaric O_2.

We have not measured MPO activity in microvessels taken from rats treated with hyperbaric oxygen and killed more than 90 min after CO poisoning. Hence, we cannot say whether leukocyte adherence to the endothelium might occur at some later time. We think that this is unlikely, however, because lipid peroxidation was not detected in rats poisoned with CO, treated with 3 ATA O_2, and then killed 2.25, 6, 24, or 48 hr later (Thom, 1990a). Moreover, in the current study we found that the effect of hyperbaric oxygen on B_2 integrin function persisted for at least 24 hr, and Tomaszewski *et al.* (1992) failed to find pathologic changes in rat brains 4 days following treatment with hyperbaric oxygen.

This investigation provides evidence which explains the beneficial action of hyperbaric O_2 in CO-mediated oxidative brain injury. Hyperbaric O_2 causes a rather discrete disturbance in PMN adherence function, and this mechanism may be involved in a number of other studies where hyperbaric O_2 has been shown to be of benefit in ischemia-reperfusion injuries (Weinstein *et al.*, 1986, 1987; Nylander *et al.*, 1988; Thomas *et al.*, 1990; Zamboni *et al.*, 1992). Leukocytes are not necessarily involved in all such injuries, and this may explain why supplemental O_2 can exacerbate the injury in some models (Mickel *et al.*, 1988). Enhanced injury by O_2 in *in vitro* studies may be explained by an absence of PMN in organ perfusion model systems (Fisher *et al.*, 1991). Further studies are necessary to identify the intracellular target(s) for O_2 in PMN. The effects of even relatively low O_2 partial pressures on CD-18-dependent adherence are an additional finding in this study that may have broad physiological significance; and it also requires additional investigation.

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