Effects of hyperoxia on neutrophil adhesion.

S.R. THOM

Institute for Environmental Medicine and Department of Emergency Medicine University of Pennsylvania Medical Center, Philadelphia, PA 19104-6068

INTRODUCTION

This presentation will focus on the effects of oxygen and some reactive chemical species on interactions between circulating neutrophils and the vascular endothelium. It will also outline how we believe these actions may be related to some beneficial effects of hyperbaric oxygen.

HBO_2 and Neutrophil Adhesion

There are a number of animal models that have been used to look at the effects of hyperbaric oxygen on disease processes. Listed in Table 1 are investigations using animal models where neutrophils, in particular, have been linked to the progression of pathology and where a beneficial effect of hyperbaric oxygen has been related to inhibition of one or more neutrophil responses. Zamboni and others (1,2) have shown an antagonism of neutrophil adherence following ischemia-reperfusion injury in skeletal muscle. Others have demonstrated similar effects following ischemia-reperfusion injury in the brain (3), and neutrophil adhesion in the lung after an intestinal ischemia-reperfusion injury (4,5). Neutrophil adhesion is a component of the pathological responses to carbon monoxide poisoning (6), decompression sickness (7), and to lung injury as a consequence of smoke inhalation (8). Studies with each of these disorders have shown that beneficial effect of hyperbaric oxygen is linked to inhibition of neutrophil adhesion. Therefore, based on a rather wide sampling of disease processes, inhibition of neutrophil attachment to blood vessel appears to be a common theme to beneficial effects of hyperbaric oxygen.

Table 1. **Beneficial effects of hyperbaric oxygen associated with reduced neutrophil sequestration**.

Skeletal muscle ischemia-reperfusion injury (1,2)
Brain ischemia-reperfusion injury (3)
Lung after intestinal ischemia-reperfusion injury (4, 5)
Brain after carbon monoxide poisoning (6)
Brain after decompression sickness (7)
Lung after smoke inhalation (8)

Cell surface molecules called β_2 integrins mediate irreversible adherence by activated neutrophils (9). Some β_2 integrins are always present on the neutrophil surface, and additional molecules are mobilized from pre-packaged granules within the neutrophil during the activation process. These molecules interact with intercellular adhesion molecules (ICAM) on the endothelial surface to cause irreversible cell-to-cell adhesion. Diapedesis of neutrophils through the vessel wall commonly follows this interaction. The process is part of normal physiology and occurs when neutrophils perform their normal function of protecting the body from infectious agents. The worry arises, however, when this process occurs after other insults, such as ischemia-reperfusion injury. In this scenario, the neutrophil will precipitate further pathological events.

Shown in Figure 1 is a summary of some of our observations with the rat CO poisoning model in which neutrophil adhesion to brain vasculature can be documented (6). Neutrophil adhesion *in vivo* was quantified as myeloperoxidase activity. A marked elevation of sequestered neutrophils occurs after carbon monoxide poisoning, and this is not seen in animals treated with hyperbaric oxygen after carbon monoxide poisoning. The point with this model is that hyperbaric oxygen will inhibit neutrophil adhesion in the brain and this will reduce the magnitude of brain damage based on a variety of different parameters.

The second panel of Figure 1 shows increased neutrophil sequestration in lungs 24 hours after smoke inhalation (8), and significant reduction if rats are treated with hyperbaric oxygen shortly after the smoke insult. The effects of hyperbaric oxygen can also be shown *in vitro*.

The third panel of Figure 1 shows results of studies performed by taking blood from rats and passing it through columns packed with nylon fiber. Neutrophil adherence to the nylon is approximately 25 % in control rats, and it is unchanged if rats are exposed to 100 % O_2 at 1 atmosphere for 45 minutes before blood is taken. If rats are first exposed to 3 atmospheres absolute (ATA) O_2 for 45 minutes, neutrophil adherence to nylon is less than 5 % (6).

Figure 1. Neutrophil adhesion and its inhibition by hyperbaric oxygen.

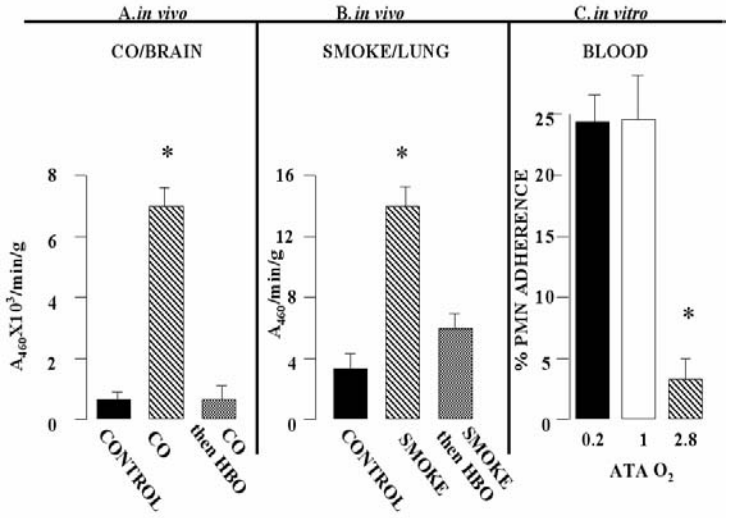

Fig. 1 Panel A: Myeloperoxidase activity in rat brain homogenates. Data are from (6) and reflect control values, values from rats killed 90 min after CO poisoning (CO), and those poisoned with CO, then exposed for 45 min 2.8 ATA O_2, so that they were killed at 90 min following the CO poisoning. **Panel B:** Myeloperoxidase activity in rat lungs. Data are from (8) and reflect control values, values from rats killed 24 h after smoke inhalation, and those exposed to smoke and to 2.8 ATA O_2 for 45 min, and then killed 24 h after the smoke inhalation. **Panel C**: Neutrophil adherence to nylon columns. Blood was removed from rats breathing air (0.2 ATA O_2), or after breathing pure oxygen at 1 or 2.8 ATA for 45 min. It was passed through columns following a technique to assess β_2 integrin-dependent neutrophil adherence (6). Columns reflect mean values $\pm$ SE (n=4 to 8 for all measurements),*$p<0.05$.

Once we demonstrated that inhibition of neutrophil adhesion could be monitored in blood drawn from animals exposed to hyperbaric oxygen, we extended our observations to humans. In

a study published in 1997 we showed that exposure to 2.8 or 3.0 ATA O_2 reduced β_2 integrin dependent adhesion to approximately 5 % of control and the effect persisted for approximately 12 hours (10). While our data indicate that there may be a benefit to hyperoxia with regard to temporary impairment of neutrophil adherence, we must be cognizant that more oxygen is not always good. Even with regard to neutrophil-endothelial interactions, there are instances when oxidative stress will actually increase cell adhesion. Superoxide and hydrogen peroxide have been shown to increase adhesion molecule expression and adherence between neutrophils and the endothelium in a cardiac ischemia reperfusion model (11-13). The point, therefore, is that oxidants can augment the inflammatory response. This issue must be reconciled with the other side, the antagonistic effects described above when determining fundamental mechanisms.

β_2 Integrin Adhesion Control Mechanisms

We have focused some effort on investigation of basic mechanisms for why hyperbaric oxygen inhibits β_2 integrin adhesion and how the beneficial versus harmful effects of reactive oxygen species may be reconciled. There are two pathways that have been demonstrated for how β_2 integrins are controlled in neutrophils. One pathway involves membrane receptors (14), and one was shown with membrane permeable agents that directly activate intracellular enzymes such as protein kinase C (PKC), to cause phosphorylation events leading to increased adhesiveness (15).

Shown in Figure 2 are the effects of various agonists on neutrophil adhesion (16). Neutrophil suspensions were prepared from control rats, and also those that had first been exposed to hyperbaric oxygen. We found that cells incubated with N-formyl-methionyl-leucine-phenylalanine (FMLP), which will activate cells via membrane surface receptors, exhibit a slight elevation in adhesion over control, but there was no response in cells first exposed to hyperbaric oxygen. In contrast, if cells were incubated with phorbol ester (phorbol 12-myristate 13-acetate, PMA), to mediate cell activation through PKC, adherence was increased whether cells were obtained from control (air-exposed) rats, or those first exposed to hyperbaric oxygen. These findings imply that much of the cell's internal mechanisms for controlling adherence are intact after hyperbaric oxygen and that the effect may be mediated by one or more membrane-associated process. In additional studies we demonstrated that incubation with the sulfhydryl-reducing agent, dithioerythritol (DTE), reversed the effect of hyperbaric oxygen but had no effect on control cells. A similar effect was observed after incubation with a membrane-permeable analog of cyclic GMP, 8 bromo-cGMP. These findings led to the idea that the effect of hyperbaric oxygen involved cyclic GMP, and may be mediated through oxidative "stress" of one or more membrane-associated sulfhydryl group. Moreover, based on the lack of effect of FMLP, versus the stimulatory action of PMA, hyperoxia was presumed to inhibit receptor-dependent cell activation.

We next examined cyclic GMP synthesis by neutrophils and found that this was increased when control cells were incubated with either FMLP of PMA (Figure 3). When the same studies were done with cells taken from rats first exposed hyperbaric oxygen, the cells did respond to PMA but not to FMLP (16).

Given that cyclic GMP appears to be involved with the effect of hyperbaric oxygen on neutrophil adherence, we next examined how a neutrophil makes cyclic GMP. There are two different pathways (17). Nearly ninety-eight percent of the cyclic GMP produced is synthesized by the cytosolic guanylate cyclase, a process presumably stimulated by nitric oxide.

Figure 2. Neutrophil adherence to nylon columns.

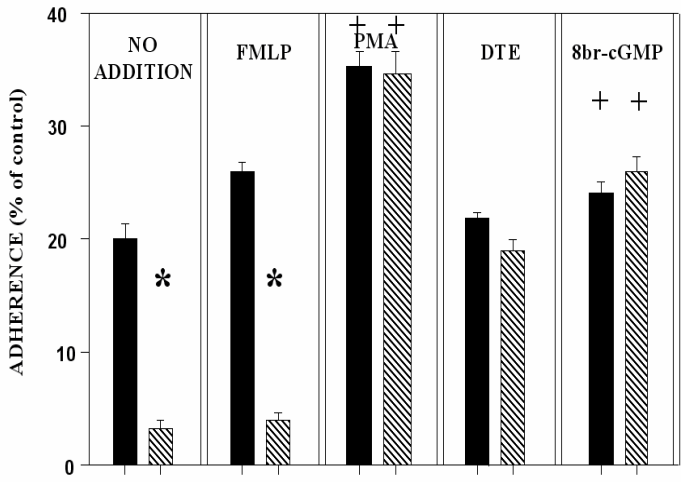

Fig. 2. Neutrophils obtained from peritoneal lavage of control rats or from rats first exposed to 2.8 ATA O_2 were incubated for 30 min with 0.1 μM FMLP or PMA, with 3 μM DTE, or 10 μM 8-bromo-cyclic GMP. A description of procedures and some data are from Chen et al. (16). Data represent percent of cells that adhered to nylon expressed as mean + SE (n=3 to 8 for each group), +P<0.05.

Figure 3. Neutrophil cyclic GMP (cGMP) content and effect of hyperbaric oxygen.

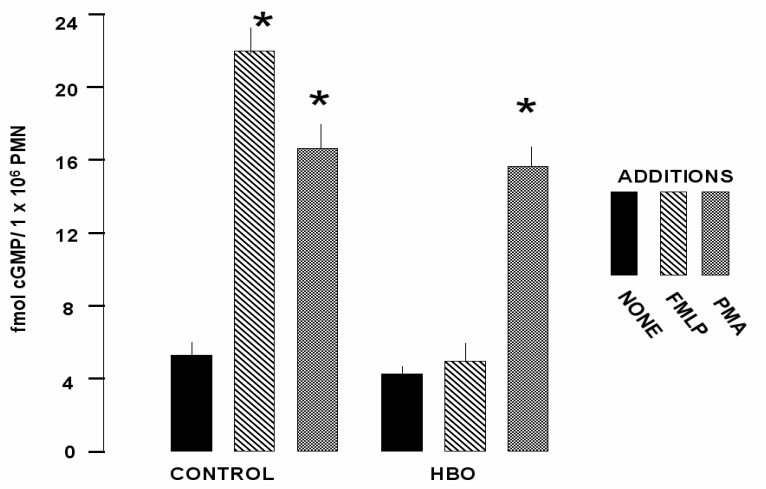

Fig. 3. Neutrophils were obtained by peritoneal lavage of rats exposed to air (control) or to 2.8 ATA O_2 for 45 min (HBO_2). They were plated onto plastic and exposed for 1 min to 0.1 μM FMLP or PMA. Data and procedures are from (16). Values are mean ± SE, n=7 to 34 for each group, *p<0.05.

Only about 2 percent of cellular cyclic GMP is produced by a membrane-bound guanylate cyclase. This is a very different protein from the cytosolic enzyme. It has no heme moiety and is not activated by nitric oxide. The enzyme can be activated by atrial natriuretic peptide, although whether this is physiologically relevant is unclear. If cells were incubated for 2 minutes in buffer containing 50 μM nitric oxide, cyclic GMP was increased by 18 ± 5% (SE, n=3), whether neutrophils were obtained from control animals or those first exposed to hyperbaric oxygen. This suggested that cytosolic guanylate cyclase was not inhibited by hyperoxia. In contrast, if neutrophil membranes were isolated to assay membrane guanylate cyclase a very different picture emerged.

Membrane fragments from control neutrophils exhibited significant production of cyclic GMP when incubated with FMLP, but this was not observed with membranes obtained after exposure to hyperbaric oxygen (Figure 4). Similarly, incubation with atrial natruretic peptide (plus ATP) stimulated cyclic GMP synthesis by membrane fragments from control cells but not those from animals exposed to hyperbaric oxygen. These data lead to the conclusion that membrane guanylate cyclase was inhibited by hyperbaric oxygen, and that impairment of membrane-associated cyclic GMP synthesis plays a role with inhibiting β_2 integrin adherence.

Figure 4. Guanylate cyclase activity of isolated membrane fragment from neutrophils obtained by peritoneal lavage of rats exposed to air (control) or to 2.8 ATA O_2 for 45 minutes (HBO_2).

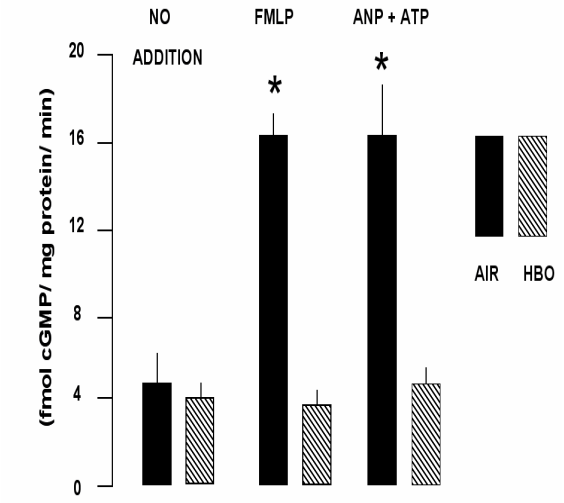

Fig. 4. Procedures are exactly as described in (25). Values reflect cyclic GMP (cGMP) after incubation for 10 minutes with 50 μM GTP (no addition of agonist), GTP and 0.1 μM FMLP, or GTP and 0.1 μM atrial natriuretic peptide (ANP) plus 330 (M ATP. Values are mean + SE, n= 4 to 17 for each sample, *p<0.05.

The studies outlined above were performed with rats, but similar findings have also been made using human neutrophils (10). Neutrophils were obtained from human volunteers before and after exposure to 2.8 ATA O_2 for 45 minutes. Control cells exhibited membrane guanylate cyclase activation by FMLP or ANP + ATP, but neither agonist had an effect on membranes from cells exposed to hyperbaric oxygen. Hyperbaric oxygen also inhibited ANP binding that was analyzed by Scatchard plot. There are approximately 7,300 ANP binding sites/cell, they form a single class, and they have a dissociation constant (Kd) of 450 pM (10). Following exposure to hyperbaric oxygen, however, no Scatchard analysis was possible since only a random pattern of points in the region of the plot origin was found.

In summary, these results lead to the conclusion that hyperbaric oxygen inhibits (2 integrin-dependent neutrophil adhesion by inhibiting membrane guanylate cyclase. How cyclic GMP synthesis regulates (2 integrin function is not yet clear. Neutrophil membrane receptors, such as the one binding FMLP, appear to be linked to membrane-bound guanylate cyclase via one or more G-proteins (16). Others have reported a cyclic GMP-dependent protein kinase that will phosphorylate cytoskeletal elements that may coordinate function of β_2 integrins (18).

NEW DIRECTIONS

These results were all published some years ago. So what is new? I'll share with you some of our more recent findings in the last few minutes. Starting from a slightly different angle, there are precedents in the literature that the free radical, nitric oxide ($^{\cdot}NO$) will inhibit neutrophil adhesion (19-21). In fact, there are several different adhesion molecules affected by $^{\cdot}NO$, including P-selectin as well as β_2 integrins (22-25). Our interest was looking at how $^{\cdot}NO$

inhibits β_2 integrins function. In a series of studies conducted in a manner similar to the hyperbaric oxygen experiments shown in Figure 3, neutrophils were exposed to a flux of ·NO to inhibit β_2 integrins -dependent neutrophil adhesion (26). If the ·NO-incubated cells were then exposed to FMLP, a membrane receptor-dependent activator, there was no effect. In contrast, if the cells were incubated with PMA to activate the intracellular kinase pathway, enhanced adhesion was seen. Similarly, when cells were incubated with a sulfhydryl reducing agent, this reversed the effect of ·NO. Quite surprisingly, cells could also be incubated with membrane-permeable 8-bromo cyclic GMP to reverse the effect of ·NO. The paradox here, of course, is that one might have argued that exposure to ·NO should elevate cyclic GMP, so how can supplying supplemental cyclic GMP reverse the anti-adhesion effect?

Figure 5 shows the cyclic GMP content of neutrophils exposed to a flux ·NO, allowed to adhere to plastic plates, and then stimulated with either FMLP or PMA (25). The point is that the pattern of effects with ·NO looks much the same as we saw using hyperbaric oxygen.

Figure 5. Neutrophil cyclic GMP (cGMP) content and effect of nitric oxide (·NO).

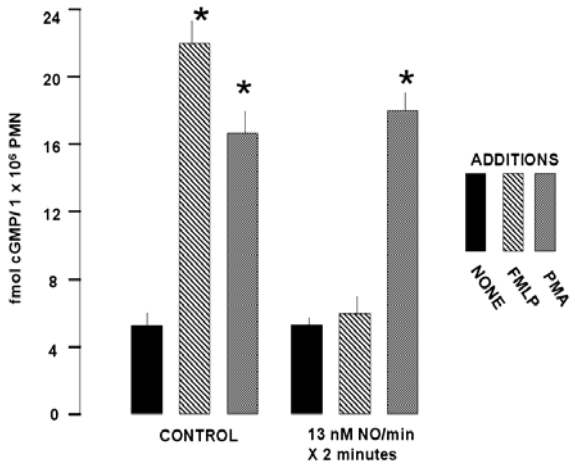

Fig. 5. Rat neutrophils were obtained by peritoneal lavage, plated onto plastic and, where indicated, exposed to 50 nM diethylamine NONOate to cause a 13 nM flux of nitric oxide/minute for 2 minutes. All cell samples were then rinsed and, where indicated, exposed to 0.1 μM FMLP or PMA for 1 minute. Data and procedures are from (25). Values are mean + SE, n=5 to 2 for each group, *p<0.05.

Looking directly at the membrane guanylate cyclase function of neutrophils before and after exposure to ·NO, we see that this inhibits enzyme activity whether membrane fragments are exposed to no agonist, as well as to FMLP or ANP/ATP (Figure 6).

Figure 6. Guanylate cyclase activity of isolated membrane fragment from rat neutrophils obtained by peritoneal lavage.

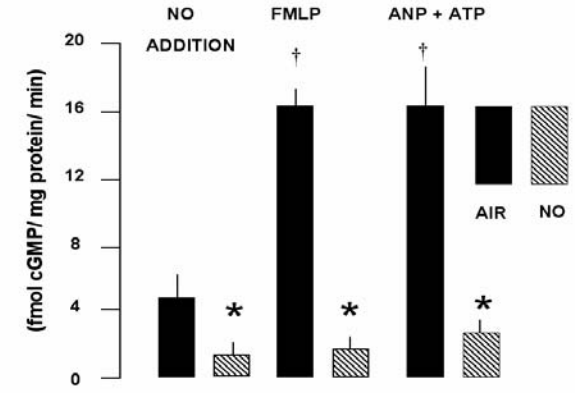

Fig. 6. Samples were obtained from control neutrophils (black bars) or from cells first exposed to 50 nM diethylamine NONOate to cause a 13 nM flux of nitric oxide/minute for 2 minutes (hatched bars). Procedures are exactly as described in (25). Values reflect cyclic GMP (cGMP) after membrane fragments were incubated for 10 minutes with 50 μM GTP (no addition of agonist), GTP and 0.1 μM FMLP (column marked FMLP), or GTP and 0.1 μM atrial natriuretic peptide (ANP) plus 330 μM ATP (column marked ANP + ATP). Values are mean ± SE, n= 5 to 17 for each sample, *p<0.05 versus air sample for each group, † p<0.05 versus Air, no addition.

The effect of ·NO on neutrophil adhesion is very much dose-dependent. Figure 7 shows neutrophil adhesion in control cells, and in cells exposed to differing fluxes of ·NO ranging from 2 to 133 μM/min. We think, based on work by others, that a level of approximately 20 to 30 μM may be close to that found in the perivascular zone of most vascular beds in the body. We think it notable that if cells are exposed to extreme concentrations of ·NO, such as those found in intense inflammatory zones like abscess cavities, ·NO does not inhibit neutrophil adhesion.

Figure 7. Dose effect of nitric oxide on neutrophil adherence.

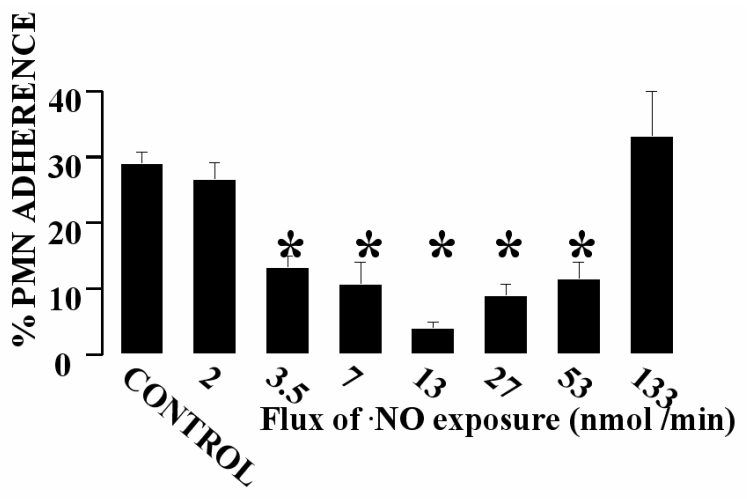

Fig. 7. Rat neutrophils were obtained by peritoneal lavage and exposed for 2 min to diethylamine NONOate at concentrations sufficient to cause a flux of nitric oxide ranging from 2 to 133 μM/min. After this exposure, cells were passed through nylon columns. Data represent percentage of cells that adhered to nylon expressed as mean ± SE (n=4 to 33 for each group), *$p<0.05$. Data and procedures are from (25).

Based on the previous work, the inhibition that we see is linked presumably to inhibition of the membrane bound guanylate cyclase. Even at a markedly elevated flux of ·NO the membrane guanylate cyclase will be inhibited, but we believe β_2 integrins function because there is adequate ·NO to diffuse into the cell and elevate intracellular cyclic GMP level by activating the cytosolic guanylate cyclase. This would negate the membrane-surface effects.

Because of the similarity in molecular mechanisms we have found between hyperbaric oxygen and ·NO, we have questioned whether the effect of hyperbaric oxygen may be mediated through ·NO. The way we tested this hypothesis was to look at neutrophil adhesion after rats were treated with an inhibitor of nitric oxide synthase, L-nitroarginine methyl ester (L-NAME). Figure 8 shows that whereas treatment with L-NAME had little impact on neutrophil adhesion by itself, pre-treated rats exposed to hyperbaric oxygen did not exhibit impaired neutrophil adhesion. Also shown are the effects of first incubating blood with antibodies that block β_2 integrin function. The inhibitory effect on control blood demonstrates the specificity of the assay for β_2 integrin-specific neutrophil adherence. The inhibitory effect on blood taken from rats first treated with L-NAME and then hyperbaric oxygen shows that the adherence seen in neutrophils from these rats was also due to β_2 integrins, and not to some alternative adhesion process.

Figure 8. Neutrophil adherence to nylon columns.

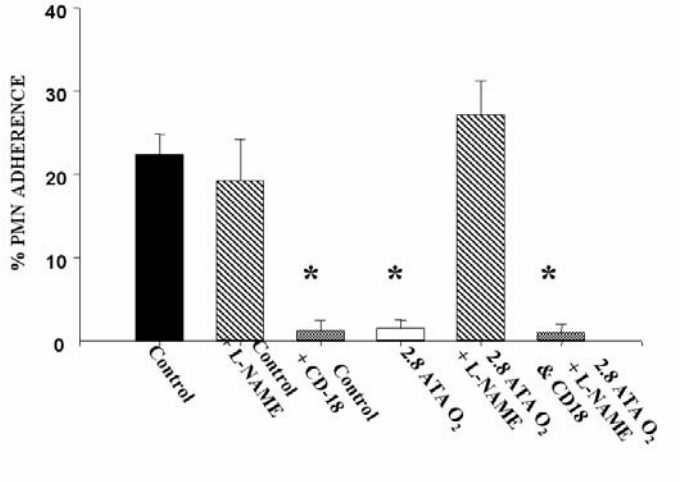

Fig. 8. Blood was obtained from control rats, or rats first exposed to 2.8 ATA O_2 and passed through nylon columns as described in (6). Where indicated, rats were injected with 40 mg/kg L-nitroarginine methyl ester (L-NAME) 2 hours and 45 minutes before sacrifice, or 2 hours before exposure to hyperoxia. Where indicated, anti-CD-18 antibodies (200 mg/ml) were added to blood prior to passage through columns to block adhesion dependent on β_2 integrins. Data represent percent of cells that adhered to nylon expressed as mean $\pm$ SE (n=4 to 11 for each group), *P<0.05.

CONCLUSIONS

We conclude from these findings that hyperbaric oxygen appears to act via $^{\cdot}NO$ to inhibit neutrophil β_2 integrin function. Additional studies are underway to elucidate the mechanism for augmented $^{\cdot}NO$ synthesis by hyperoxia.

We, and others, have demonstrated the ability of hyperbaric oxygen to inhibit neutrophil adhesion in humans (10, 26). The effect appears remarkably discrete, but there may be additional perturbations to cell function under some circumstances (26,27). We believe that the reason for the relatively discrete perturbations may relate to the localized effect of hyperoxia on membrane guanylate cyclase.

In closing, it should be acknowledged that there are still other effects of hyperbaric oxygen, and still more work to be done. Clearly, there are effects of hyperoxia on the endothelium that may have some effects with regard to inhibiting ischemia-reperfusion or other injuries (28). There is also a need for a tighter evaluation of the dose-response relationship with hyperbaric oxygen. It is well known that with extreme oxidative stress, neutrophil adhesion is increased (29).

ACKNOWLEDGMENTS

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