Free Rad. Res., Vol. 31, pp. 53–58
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Polynitroxyl $\alpha\alpha$-Hemoglobin (PNH) Inhibits Peroxide and Superoxide-Mediated Neutrophil Adherence to Human Endothelial Cells

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Accepted by Prof. B. Halliwell

(Received 20 December 1998; In revised form 20 January 1999)

Experimental hemoglobin-based O_2 carriers e.g. cross-linked $\alpha\alpha$-hemoglobin ($\alpha\alpha$-Hb), are under investigation as potential blood substitutes. However, some Hb-based products form strong oxidant species *in vivo* that may cause adverse clinical effects. We report the prototype of a new class of modified Hb-based O_2 carrier, polynitroxylated $\alpha\alpha$-Hb (PNH), which has antioxidant activities that may reduce inflammatory effects mediated by oxidant formation. We compared the effects of $\alpha\alpha$-Hb and PNH on xanthine oxidase and H_2O_2-induced neutrophil-endothelial adhesion *in vitro*. Both peroxide (> 0.1 mM), and superoxide/peroxide generated by xanthine oxidase (XO) (> 10 mU/ml) + 0.1 mM xanthine (X), increased endothelial-neutrophil adhesion. At 30 μM, $\alpha\alpha$-Hb significantly increased X/XO-mediated adhesion, while PNH inhibited peroxide or X/XO induced adhesion, with maximal inhibition at 10 μM PNH. These data indicate that PNH has antioxidant-anti-inflammatory properties that suggest its use as a potentially safer blood substitute in reperfusion injury, stroke, myocardial infarction and other forms of inflammation.

Keywords: Oxidant, superoxide, nitroxide, catalase

INTRODUCTION

An important aspect of ischemia/reperfusion (I/R) injury and inflammation in the microvasculature is the adhesion between neutrophils and the endothelium. In I/R, this process is triggered by oxidants like $O_2^{\bullet -}$, H_2O_2 and other reactive oxygen species (ROS) which are produced by leukocytes and endothelial cells.[1] Measuring neutrophil-endothelial adhesion *in vitro* is a useful model for screening the ability of therapeutic agents to attenuate I/R-mediated leukocyte entrapment within tissues associated with inflammatory injury in stroke and myocardial infarction.

Stable nitroxyl radicals (nitroxides) possess unusual superoxide dismutase (SOD) and catalase-like properties.[2,3] Several covalently nitroxylated proteins with high molar ratios of

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nitroxide/protein ("polynitroxylation") exhibit this high level of antioxidant activity.[2] We previously reported that polynitroxylated albumin (PNA) has striking protective effects in models of oxidant injury: (1) PNA inhibits leukocyte adhesion and emigration after ischemia-reperfusion;[3] (2) PNA inhibited superoxide/peroxide mediated neutrophil-adherence to endothelial cells;[3] and (3) reduced the severity of tissue injury in a stroke model.[4] Our experience with polynitroxylated albumin suggests that polynitroxylation of other proteins will yield novel compounds with antioxidant/anti-inflammatory properties.

Modified hemoglobin (Hb) compounds are being considered in clinical trials as O_2 carrying substitutes for blood. While currently available Hb products transport oxygen adequately, they lack antioxidant enzymes normally found in intact red cells (catalase, Cu/Zn SOD). Some of the reported problems with the use of such compounds may stem from the formation of potent oxidants in patients being treated with these compounds and lead to a wide range of adverse effects which may involve triggering of leukocyte-endothelial adhesion.

Here we show that polynitroxyl Hb (PNH), unlike native $\alpha\alpha$-Hb, does not promote leukocyte adhesion, and reduces oxidant stimulated leukocyte endothelial adhesion. Our data suggest that the use of poly-nitroxylated Hb O_2 carriers like PNH may present fewer problems in clinical settings than non-modified $\alpha\alpha$-Hb based on the antioxidant properties of PNH.

MATERIALS AND METHODS

PNH Preparation

PNH was prepared from an $\alpha\alpha$-cross-linked Hb tetramer ($\alpha\alpha$-Hb) and 4-[2-bromoacetamido]-2,2,6,6-tetramethyl-piperidine-1-oxyl (BrAcTPO). $\alpha\alpha$-Hb was purchased from the US Army Research & Development Command. PNH was prepared by labeling $\alpha\alpha$-Hb with BrAcTPO at 40°C. The process was performed under carbon monoxide to stabilize $\alpha\alpha$-Hb to the heat used in the process. BrAcTPO labeling was self-terminating at molar ratio of 16 moles BrAcTPO per mole Hb tetramer. The formulation of PNH is 6 g/dl Hb in 0.9% sodium chloride solution; sterile filled under carbon monoxide (CO) to provide thermostability.

Human Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by 0.1% collagenase treatment as previously described.[5] The cells were cultured in Endothelial Growth Medium (EGM; Clonetics, San Diego, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT), thymidine (2.4 mg/l; Sigma, St. Louis, MO), glutamine (230 mg/l; Gibco, Gaithersburg, MD), heparin sodium (10 IU/ml; Sigma), antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.125 mg amphotericin B), and endothelial cell growth factor (80 ng/ml; Biomedical Technologies, Stoughton, MA). All other tissue culture reagents were obtained from Gibco. The cell cultures were incubated at 37°C in a 100% humidified atmosphere with 5% CO_2 and split with 0.25% trypsin/0.02% EDTA. Primary HUVEC were seeded into fibronectin-coated 48-well culture plates and used when confluent. Medium was replaced every second day. Only first-passage cultures were used. Cells were identified as endothelial by cobblestone appearance at confluence, and labeling with (1) Dil-Ac-LDL (Biomedical Technologies), and (2) mouse antihuman factor VIII (Calbiochem, San Diego, CA).

Human Neutrophil Isolation

Human polymorphonuclear leukocytes (PMNs) were isolated from venous blood from healthy adults, using dextran sedimentation and gradient separation on Histopaque 1077 (Sigma).[5] This

procedure yields cells which are 95–98% viable (by trypan blue) and 98% pure (by crystal violet). The Institutional Review Board approved the procedures used to obtain the endothelial cells and neutrophils. Each subject provided written consent and blood donors were paid for participating in the study.

Static Adhesion Assay and Treatment Protocols

Isolated PMNs were suspended in Hank's balanced salt solution (HBSS) and radiolabeled by incubating PMNs (at 2×10^7 cells/ml) with 30 μCi $Na^{51}CrO_4$ per ml PMN suspension at 37°C for 1 h. The cells were washed twice with HBSS at 4°C, centrifuged at 250*g* for 4 min to remove non-incorporated chromium, and resuspended in HBSS. HUVEC monolayers were exposed to test agents (oxidants and blockers) for 30 min at 37°C in HBSS. Labeled PMNs were then added and allowed to adhere for 30 min at 37°C in HBSS. Labeled PMNs were added to HUVEC monolayers at a PMN-to-HUVEC ratio of 10 : 1. After co-incubation, the percentage of added PMNs that adhered to the HUVEC monolayers was quantified by lysing the cells and determining the radioactivity in each of the wells.[5]

The chemical agents used in this study were: (1) 0–0.5 mM hydrogen peroxide; (2) 0 or 0.1 mM xanthine (X; Sigma) plus 0–20 mU/ml XO (Sigma); (3) 0–30 μM PNH (SynZyme Technologies Inc.) alone; (4) 0–30 μM αα-Hb (US Army Research & Development Command) alone; (5) 0.1 mM peroxide plus 0–30 μM PNH; (6) 0.1 mM peroxide plus 0–30 μM αα-Hb; (7) 0.1 mM X plus 10 mU/ml XO plus 0–30 μM PNH; (8) 0.1 mM X plus 10 mU/ml XO plus 0–30 μM αα-Hb.

Statistical Analysis

All values are expressed as mean ± SE. Data were analyzed using one-way ANOVA with Bonferroni's correction for multiple comparisons. Significance was accepted at $p < 0.05$.

RESULTS

Effects of Hydrogen Peroxide and X Plus XO on PMN Adherence to Endothelial Cells

Hydrogen peroxide at 0.1 and 0.5 mM significantly increased endothelial-neutrophil adherence (Figure 1). XO at 10 and 20 mU/ml also significantly enhanced PMN adherence in the presence of 0.1 mM xanthine (Figure 2). Neither 0.5 nor 0.1 mM peroxide nor XO at concentrations up to 20 mU/ml increased lactate dehydrogenase (LDH) release from endothelial cells within 60 min (data not shown).

Effects of PNH and αα-Hb on PMN Adherence to Endothelial Cells

PNH (0–10 μM) did not increase endothelial-neutrophil adherence, however 30 μM PNH did slightly increase PMN adhesion (Figure 3). In contrast, αα-Hb did not enhance PMN adherence at the concentrations tested in this study (Figure 3).

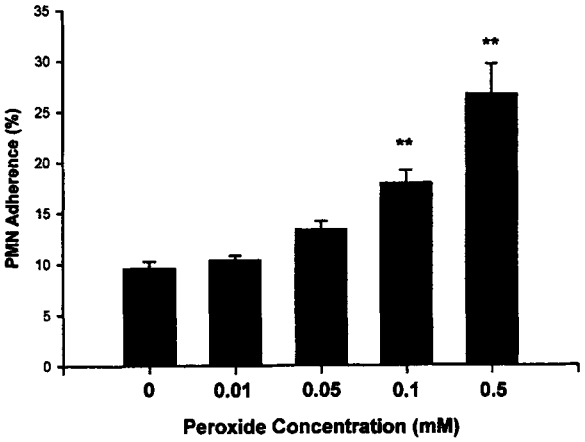

FIGURE 1 Effects of hydrogen peroxide on endothelial-neutrophil adherence. HUVEC monolayers were pre-incubated with 0–0.5 mM peroxide for 30 min. ^{51}Cr-labeled PMNs were then added to monolayers, and PMN adherence was determined 30 min later. Values are means ± SE, $n = 4$. $^{**}p < 0.01$ compared with treated with 0 mM peroxide.

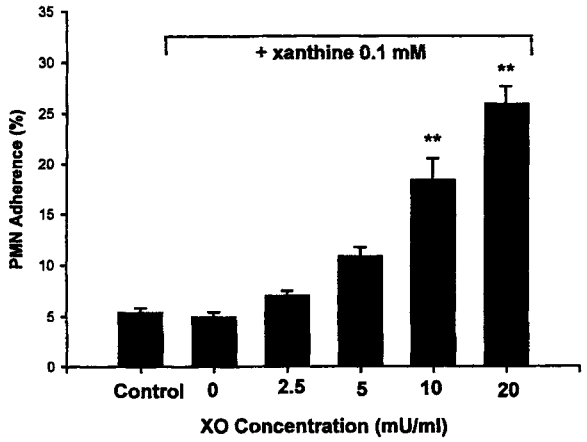

FIGURE 2 Effects of X/XO on endothelial-neutrophil adherence. HUVEC monolayers were pre-incubated with 0–20 mU/ml XO in the presence or absence (control) of 0.1 mM X for 30 min. ^{51}Cr-labeled PMNs were then added to monolayers, and PMN adherence was determined 30 min later. Values are means ± SE, $n = 4$. $^{**}p < 0.01$ compared with control.

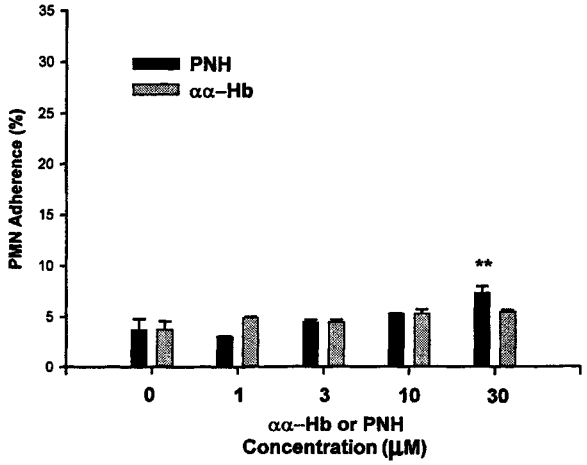

FIGURE 3 Effects of native hemoglobin ($\alpha\alpha$-Hb) and PNH on endothelial-neutrophil adherence. HUVEC monolayers were pre-incubated with either $\alpha\alpha$-Hb or PNH at 0–30 μM for 30 min. ^{51}Cr-labeled PMNs were added to monolayers, and PMN adherence was determined at 30 min. Values are means ± SE, $n = 4$. $^{**}p < 0.01$ compared with untreated controls.

Effects of PNH and $\alpha\alpha$-Hb on Endothelial-Neutrophil Adherence Induced by X/XO

The endothelial-neutrophil adherence induced by XO (10 mU/ml with 0.1 mM xanthine) was significantly inhibited by 1–30 μM PNH. Half-maximal inhibition of adhesion (IC_{50}) was observed at a PNH concentration of 10 μM (Figure 4). 1–10 μM $\alpha\alpha$-Hb did not prevent this adhesion, and 30 μM $\alpha\alpha$-Hb, actually increased XO induced adhesion (Figure 4).

Effects of PNH and $\alpha\alpha$-Hb on Endothelial-Neutrophil Adherence Induced by Peroxide

The endothelial-neutrophil adherence induced by 0.1 mM peroxide was significantly inhibited only by 10 μM PNH (Figure 5). $\alpha\alpha$-Hb (0–30 μM) did not block this adhesion (Figure 5).

DISCUSSION

Importance of Co-Incubation of Both Endothelial Cells and Neutrophils with Oxidants

In this model, maximum PMN adhesion is achieved when both endothelial cells and neutrophils were exposed to oxidants. We have previously reported that exposure of only endothelial cells only (but not neutrophils), to 0.1 mM hydrogen peroxide did not increase endothelial-neutrophil adherence in 30 min.[6] Exposure of endothelial cells only to 0.1 mM peroxide or 10 mU/ml XO for 60 min caused only a 1.6× and 1.9× increase respectively in endothelial-neutrophil adherence (data not shown). Exposure of neutrophils alone to the same concentrations of peroxide or XO for 30 min caused only a 1.6-fold increase in adherence (data not shown).

Here, exposure of both endothelial cells and neutrophils to these same concentrations of either peroxide or XO for 60 min gave maximum PMN adhesion, with increases of 2–5-fold with peroxide, (Figure 1) and a 4–10-fold increase with XO, (Figure 2) compared to baseline adhesion. These results suggest a synergistic effect on PMN adherence when both cell types are exposed to

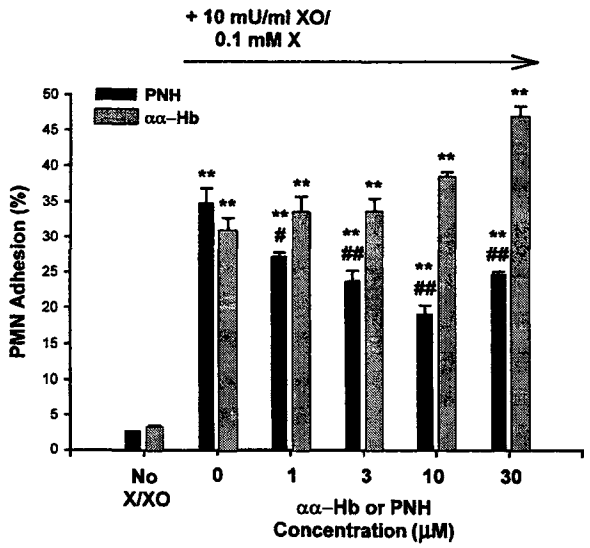

FIGURE 4 Effects of $\alpha\alpha$-Hb or PNH on endothelial-neutrophil adherence induced by X/XO. HUVEC monolayers were pre-incubated with either $\alpha\alpha$-Hb or PNH at 0–30 μM in the absence ('No X/XO') or presence of 10 mU/ml XO/0.1 mM X for 30 min. ^{51}Cr-labeled PMNs were added to monolayers, and adherence determined at 30 min. Values are means ± SE, $n = 4$. $^{**}p < 0.01$ compared with control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with monolayers treated with X/XO alone.

oxidants. Because both cell types are exposed to oxidants *in vivo*, this model may be the most relevant for investigating the pathophysiology of microvascular I/R effects in stroke and myocardial infarction, for example.

Potential for ROS Formation in the Presence of Hb-Based Compounds

Experimental HBOC compounds, including $\alpha\alpha$-Hb, are currently under investigation as blood substitutes and therapeutic agents. However, Hb-based compounds may promote neutrophil adhesion by triggering the formation of reactive oxygen species. An important example of this is the $OH^{\bullet}$ formed when heme iron reacts with peroxide catalyzing Fenton chemistry.[4] In this study, some concentrations of $\alpha\alpha$-Hb exacerbated XO-mediated PMN adhesion and had no effect on peroxide-mediated adhesion. It is not clear why $\alpha\alpha$-Hb alone did not exacerbate peroxide-mediated adhesion. In other previous pilot studies, we observed that some lots of $\alpha\alpha$-Hb did in fact promote adhesion. The differences observed here may reflect differences in $\alpha\alpha$-Hb mode of preparation, age or lot to lot variation. However, our data do indicate that while $\alpha\alpha$-Hb does not promote injury in the absence of an oxidant stress, it does enhance oxidant mediated inflammation in certain circumstances.

Antioxidant Activity of PNH

Suzuki *et al.*,[7] Kurose *et al.*,[8] Yoshida *et al.*[5] and Ichikawa *et al.*[9] have reported that SOD and catalase have protective effects against leukocyte adhesion to venules or endothelial cells induced by I/R or oxidants. We reported that polynitroxylated albumin has ability to inhibit leukocyte adhesion in models of inflammation.[3] Here, we examined a polynitroxylated form of Hb, PNH. PNH inhibited XO-mediated PMN adhesion to endothelial cells *in vitro*, and may attenuate peroxide mediated adhesion at some concentrations. In this respect, PNH exhibited SOD and perhaps, catalase like activities.

Mechanistic Considerations on the Anti-Inflammatory Effect of PNH

The PNH in this study was $\alpha\alpha$-Hb covalently labeled with a 16:1 molar ratio of nitroxide to Hb. We have shown with albumin that polynitroxylation of proteins 'fixes' the antioxidant activity on the target protein and prolongs its biological half-life. It is reasonable to expect a similar effect when nitroxide is linked to Hb. In addition to the bioavailability benefit of linking nitroxide to polypeptides, there may be another unique benefit in using Hb as the platform for polynitroxylation. Krishna *et al.*[10] described mechanisms by which nitroxide (i.e. free, not protein-bound), in the presence of heme iron, has antioxidant catalytic activities not observed with nitroxide alone. These include catalase and

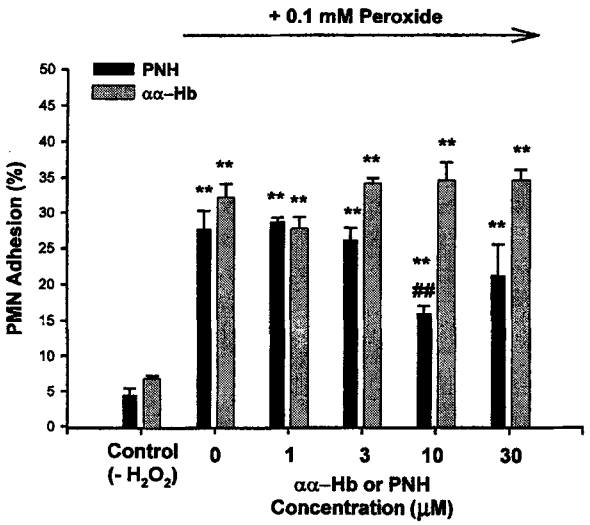

FIGURE 5 Effects of αα-Hb on endothelial-neutrophil adherence induced by 0.1 mM peroxide. HUVEC monolayers were pre-incubated with either αα-Hb or PNH at 0–30 μM in the absence ('control – H_2O_2') or presence of 0.1 mM peroxide for 30 min. ^{51}Cr-labeled PMNs were added to monolayers, and adherence determined at 30 min. Values are means ± SE, $n = 4$. $^{**}p < 0.01$ compared with control.

ferryl heme reductase activities. It appears that the same synergy occurs when the nitroxide is covalently bound to heme proteins, like Hb. Thus, PNH may be a new compound that combines both the properties of an Hb-based red cell substitute and a potent antioxidant.

In conclusion, this study shows that PNH significantly inhibits some forms of oxidant-mediated neutrophil adhesion to cultured endothelial cells. The polynitroxylation of Hb to create PNH converts the Hb from a compound with pro-oxidant potential, to a compound with anti-oxidant and potential therapeutic properties. Potential clinical indications include ischemic and inflammatory diseases such as stroke and myocardial infarction.

Acknowledgments

This study was supported in part by National Institutes of Health grants HL-47615 P01-DK-43785, HL-17655, HL-38324, HL-53860 and a contract from the O.N.R. of the U.S. Navy contract number N00014-98-C0140. The opinions and assertions contained herein are those of the authors and are not to be construed as the official, or reflecting the views of the U.S. Navy.

References

[1] T. Matsubara, T. Ziff (1986). Superoxide anion release by human endothelial cells: synergism between a phorbol ester and a calcium ionophore. *Journal of Cell Physiology* **127**: 207–210.

[2] P. Kuppusamy, P. Wang, J.L. Zweier, M.C. Krishna, J.B. Mitchell, L. Ma, C.E. Trimble and C.J.C. Hsia (1996). Electron paramagnetic resonance imaging of rat heart with nitroxide and polynitroxyl-albumin. *Biochemistry* **35**: 7051–7057.

[3] J. Russell, N. Okayama, J.S. Alexander, D.N. Granger and C.J.C. Hsia (1998). Pretreatment with polynitroxyl albumin (PNA) inhibits schemia-reperfusion induced leukocyte-endothelial cell adhesion. *Free Radical Biology and Medicine* **25**: 153–159.

[4] C. Beaulieu, E. Busch, J. Rother, A. de Crespigny, C.J. Hsia and M.E. Moseley (1998). Polynitroxyl albumin reduces infarct size in transient focal cerebral ischemia in the rat: potential mechanisms studied by magnetic resonance imaging. *J. Cereb. Blood Flow Metab.* **18**(9): 1022–1031.

[5] N. Yoshida, D.N. Granger, D.C. Anderson, R. Rothlein, C. Lane and P.R. Kvietys (1992). Anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *American Journal of Physiology* **262** (*Heart Circulatory Physiology* **31**): H1891–H1898.

[6] N. Okayama, H. Ichikawa, L. Coe, M. Itoh and J.S. Alexander (1998). Exogenous NO enhances hydrogen peroxide-mediated neutrophil adherence to cultured endothelial cells. *American Journal of Physiology* **274** (*Lung Cellular Molecular Physiology* **18**): L820–L826.

[7] M. Suzuki, M.B. Grisham and D.N. Granger (1994). Leukocyte-endothelial cell adhesive interactions: role of xanthine oxidase-derived oxidants. *Journal of Leukocyte Biology* **50**: 488–494.

[8] I. Kurose, R. Wolf, M.B. Grisham and D.N. Granger (1994). Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. *Circulation Research* **74**: 376–382.

[9] H. Ichikawa, S. Flores, P.R. Kvietys, R.E. Wolf, T. Yoshikawa, D.N. Granger and T.Y. Aw (1997). Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Circulation Research* **81**: 922–931.

[10] M.C. Krishna, A. Russo, J.B. Mitchell, S. Goldstein, H. Dafni and A. Samuni (1996). Do nitroxide antioxidants act as scavengers of superoxide or as SOD mimics. *Journal of Biological Chemistry* **271**: 26026–26031.