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CELL-FREE HEMOGLOBIN REVERSES THE ENDOTOXIN-MEDIATED HYPORESPONSIVITY OF RAT AORTIC RINGS TO α-ADRENERGIC AGENTS

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SUMMARY: Hemoglobin (Hgb) and lysates of red blood cells act as vasoconstrictors in isolated vessels by a mechanism that may involve nitric oxide (NO·) scavenging. To determine if such a mechanism indeed occurs, we investigated the effects of cell-free Hgb, modified Hgb (met Hgb and cyan met Hgb), and red blood cells on the formation of NO· induced by endotoxin in rat aorta. Incubation of rat aortic rings with endotoxin induced a delayed and prolonged release of NO· that resulted in a decrease in the contractile response to phenylephrine. Hgb significantly potentiated contractions to phenylephrine in control rings and also reversed the hyporeactivity to this α_1-agonist in endotoxin-treated rings with and without endothelium. Lysed red blood cells but not whole red blood cells shifted the concentration-contraction response curves to phenylephrine significantly to the right in endotoxin-treated preparations. Neither picket-fence porphyrin-albumin (PFP-albumin) or metheme-albumin affected the contractile response to phenylephrine. Oxidation of Hgb to met Hgb did not alter the contractions to an α_1-agonist in endotoxin-treated rings. In contrast, the formation of cyan met Hgb abolished the action of Hgb on the vascular reactivity of endotoxin-treated preparations. Together, these results demonstrate that free Hgb can scavenge NO· produced in endotoxin-treated vascular tissue and that the ability to bind NO· requires a cell-free form of Hgb with an intact heme center capable of undergoing redox reactions.

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Nitric oxide, perhaps best known as an atmospheric pollutant, was first identified as an endothelium-derived relaxing factor by Furchgott et al.[1]. Recent discoveries have demonstrated that this gaseous ligand can also act as a powerful regulator of numerous biological functions. In addition to its vasodilatory actions, NO· can function as a cytostatic molecule [2]; a neuronal messenger [3], and a platelet adhesion inhibitor [4]. Nitric oxide formation associated with these diverse functions is regulated by a family of closely related isoenzymes that are either constitutively expressed and calcium dependent (neuronal and endothelial cell NO· synthases; [5-7] or induced by immunomodulators (macrophage and smooth muscle NO· synthases; [8-11]. The inducible isoform of NO· synthase can be demonstrated in a variety of cell types after exposure to endotoxin or cytokines. Previously we showed that endothelial cells can also express an inducible isoform of NOS that produces large amounts of NO· in response to cytokines [12]. This second enzyme isoform differs from the normally present constitutive endothelial cell enzyme by virtue of its calcium independence and lack of agonist regulation [13]. More important, NO· overproduction by the inducible enzyme appears to be related to cytokine-mediated hypotension in septic shock. Consistent with this hypothesis is the finding that inhibitors of NO· synthase, such as N^G-methyl-L-arginine (NMA), have potent antihypotensive effects in both animals and humans who develop hypotension as a result of endotoxin or cytokine exposure [14-18].

Hemoglobin (Hgb) can bind NO· and inhibit its biological effects. In the presence of oxygen, this reaction yields nitrates and met Hgb. We show here that the addition of Hgb, lysed red blood cells (RBC's), or met Hgb but not cyan met Hgb or whole red RBC's to aortic ring preparations that have been exposed *in vivo* to endotoxin can reverse the hyporeactivity to phenylephrine that is a consequence of NO· overproduction. This inhibition of the vasorelaxant properties of NO· appear to be due to the ability of cell-free Hgb to scavenge NO·.

METHODS

Bovine cell-free Hgb (5 g/dl) was obtained from Biopure (Boston, MA) Phenol-extracted endotoxin from Escherichia coli. serotype 0128:B12 was obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagles Medium was purchased from GIBCO (Grand Island, NJ); it contained less than 0.5 ng/ml of endotoxin as determined by the Limulus amebocyte assay (BioWhittaker, Walkersville, MD).

Met Hgb was prepared by adding an equimolar amount of sodium nitrite to cell-free Hgb (5 gm/dl). After incubation at 4°C for 30 min, the Hgb was dialyzed in 2 changes of 100 volumes each of phosphate-buffered saline, pH 7.4.

Cyan met Hgb was prepared from human Hgb A isolated by chromatography on Whatman DE-52 anion exchange medium. Hgb A was eluted in 0.01 M Tris buffer, pH 8.3. The isolated Hgb was oxidized in elution buffer with a 3-fold molar excess (over heme) of sodium nitrite. The cyan met Hgb derivative was then prepared by adding a slightly more than 1 M excess (over heme) of sodium cyanide. Unreacted cyanide was removed by batch treatment of the mixture with Amberlite MB-3 mixed-bed ion-exchange resin. Prior to use of the cyan met Hgb, we tested for the potential presence of cyanide by taking an aliquot of the Hgb derivative and concentrating it in a membrane concentrator (Amicon Centricon). To insure complete removal of free cyanide, an aliquot of the cyan met Hgb solution was concentrated in a membrane concentrator (Amicon Centricon). The Hgb-free filtrate was added to a met Hgb solution, and the resulting preparation was analyzed by visible-UV spectroscopy. No cyan met Hgb optical absorption bands were observed in the test solution.

Met heme-albumin was prepared by adding hemin chloride to a solution of human serum albumin (HSA). Hemin chloride was first dissolved in a minimal volume of a solution of dimethyl sulfoxide/ 0.15 M phosphate-buffered saline, pH 7.4 (3:5, v/v) containing 1/60th volume of 1 N NaOH. A 25% solution of HSA (Albumin-USP 25%; Cutter Pharmaceuticals, Cutter, USA) was then added by vigorous vortex mixing. The resultant deep amber (but crystal clear) solution of met heme-albumin was used without further purification. Iron picket fence porphyrin (Porphyrin Products, Provo, UT) was dissolved in the same solvent used for hemin chloride. Then, HSA was again added as described above and the preparation was used without further purification. Canine (RBC's) were obtained from blood collected from the saphenous veins into heparin-containing tubes. RBCs from 5 ml of whole blood (6 X 10^9 RBCs/ml) were collected by centrifugation, washed twice in phosphate buffered saline, pH 7.4 (PBS), and resuspended in 200 µl. PBS by vortex mixing. The intact RBC suspension was further diluted with either 2.5 ml PBS (unlysed cells) or 2.5 ml of distilled water (lysed RBCs).

Measurement of vascular reactivity

Male Wistar rats (300-400 g) were euthanized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The thoracic aortas were excised and stored in cold modified Krebs-Ringer solution containing NaCl 118.3 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25.0 mM, Ca EDTA 16 µM, and glucose 11.1 mM (control solution). Arteries were cleaned of fat and connective tissue and cut into rings. For some experiments the endothelium was removed mechanically by placing rings on filter paper wetted with the control solution, inserting the tip of a forceps into the lumen, and rolling the ring back and forth on the filter paper. The presence of the endothelium was confirmed by determining the relaxation to acetylcholine (10^{-6} M) in arteries contracted with phenylephrine (10^{-6} M). The rings with or without endothelium were placed in 24-well multiwell plates with Dulbecco's Modified Eagle's Medium and Ham's F-12 Medium (DMEM/F12) (1 ml), in the presence or absence of endotoxin (LPS) 200 ng/ml, for 4 h (rings with endothelium) or 6 h (rings without endothelium). After incubation, the rings were suspended in organ chambers containing 10 ml of control solution (37° C, pH 7.4) and aerated with 95% O_2 and 5% CO_2. Rings were stretched progressively to 2.5-3 g of tension. Changes in isometric tension were recorded with a force transducer connected to an analog-to-digital input board (Scientific Solutions, Inc., Solon, OH) in an IBM 386/30 mHz personal computer. The aortic rings were rinsed three times with warm control solution, rested for 30 min, and then incubated with bovine cell-free Hgb, heme-HSA cyan met Hgb, PFP- HSA,

met Hgb, venous RBC's, or lysed RBC's for 5 min before a concentration-contraction curve to phenylephrine (10^{-9} - 10^{-5} M) was obtained.

Data analysis

Significance was determined by comparing the data using the Student's T-test; p values less than 0.05 were considered statistically significant.

RESULTS

Phenylephrine induced a dose-dependent increase in tension in control aortic rings with endothelium. Endotoxin treatment for 4 h *in vitro* induced a significant right-ward shift of the phenylephrine concentration-response curve and decreased the maximal response (Figure 1). In control and endotoxin-treated rings (with endothelium), Hgb significantly shifted the phenylephrine concentration-contraction curves to the left without modifying the maximal contraction. Hgb also potentiated the phenylephrine-induced contraction in endotoxin-treated rings without endothelium (Figure 2).

The effect of fresh lysed RBC's on vessel contraction was similar to that of bovine cell-free Hgb (Figure 2). In brief, the addition of lysed red blood cells but not whole RBC's significantly reversed the hyporeactivity to phenylephrine induced by endotoxin in rings without (Figure 2) and with (Figure 3) endothelium.

To investigate the role of other porphyrin and heme structures, endotoxin treated rings with endothelium were studied in the presence of PFP-albumin or met heme-albumin. Neither PFP-albumin or metheme-albumin affected the

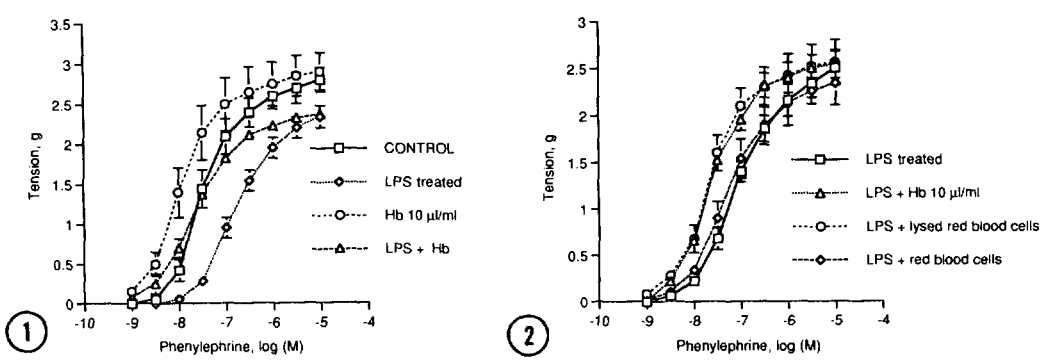

Figure 1. Effects of hemoglobin on the concentration-contraction curves evoked by phenylephrine in rat aortic rings with endothelium incubated in the absence or presence of endotoxin (200 ng/ml; LPS) for 4 hrs. Results are presented as the mean ± SEM of 6 different experiments.

Figure 2. Effects of red blood cells and lysed red blood cells on the concentration-contraction curves evoked by phenylephrine in rat aortic rings without endothelium incubated for 4 hrs in culture medium containing endotoxin (LPS 200 ng/ml). Results are presented as the mean ± standard error of the mean of 7 different experiments.

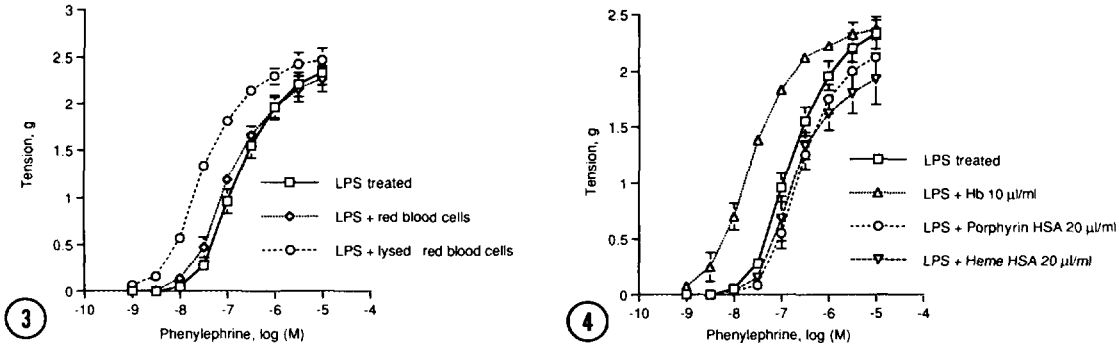

Figure 3. Effects of red blood cells and lysed red blood cells on the concentration-contraction curves evoked by phenylephrine in rat aorta rings with endothelium, incubated for 4 hrs in culture medium containing endotoxin (LPS) 200 ng/ml. Results are presented as mean ± SEM of 7 different experiments.

Figure 4. Effects of bovine cell-free hemoglobin (Hgb), picket-fence porphyrin human serum albumin (PFP HSA) and heme human serum albumin (heme HSA) on the concentration-contraction curves evoked by phenylephrine in rat aorta rings with endothelium, incubated for 4 hrs in culture medium containing endotoxin (LPS) 200 ng/ml. Results are presented as mean ± SEM of 4 different experiments.

hyporeactivity to phenylephrine when compared with cell-free Hgb, which significantly potentiated α_1-agonist-mediated vascular contraction (Figure 4).

To further investigate the structure-activity relationship for Hgb, both met Hgb and cyan met Hgb were added to organ chambers containing endotoxin-treated rings with endothelium. Oxidation of Hgb to met Hgb did not alter the ability of the Hgb molecule to reverse the endotoxin-induced hyporeactivity to an α_1- adrenergic agonist (Figure 5). In contrast, the formation of cyan met Hgb abolished the activity of Hgb on the vascular reactivity of endotoxin-treated rings (Figure 5).

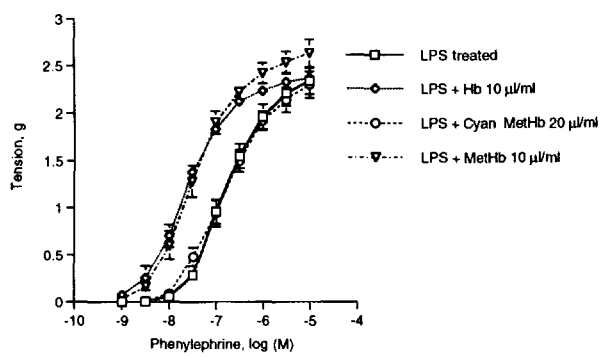

Figure 5. Effects of bovine cell-free hemoglobin (Hgb), cyan met hemoglobin (cyan met Hgb) and methemoglobin (met Hgb) on the concentration-contraction curves evoked by phenylephrine in rat aorta rings with endothelium, incubated for 4 hrs in culture medium containing endotoxin (LPS) 200 ng/ml. Results are presented as mean ± SEM of 4 different experiments.

DISCUSSION

Administering endotoxin to rats or incubating intact vessels with endotoxin results in a decreased vascular reactivity to α-adrenergic agents *in vitro*. That this vascular hyporeactivity is due to vascular production of NO· was first shown by Fleming, et al. [19] who demonstrated that analogues of arginine such as N^G-methyl-L-arginine (NMA) were able to restore the contractile responses to norepinephrine in aorta from endotoxemic rats. Consistent with this report, we also found that nitro-L-arginine reverses the effects of endotoxin on the reactivity of vascular rings (data not shown).

Since Hgb scavenges NO·, we investigated its effects in isolated, endotoxin--treated vascular rings. NO· reacts with Hgb to form nitrosyl-Hgb adducts. The nitrosyl-Hgb thus formed reacts with oxygen to yield nitrates and met Hgb which in turn is reduced by met Hgb reductase [20]. Formation of nitrosyl-Hgb has been observed in animals and humans given endotoxin and nitroglycerin respectively, as determined by electron paramagnetic resonance spectroscopy [21, 22]. NO· - Hgb interactions have also been thought to explain the inhibition of acetylcholine-induced relaxation in isolated vessels from animals and humans. Our results demonstrate that Hgb is able to potentiate the contraction of untreated rings by an α_1-agonist, probably by inhibiting the action of NO· formed by basal release [23, 24]. Thus, the ability of cell-free Hgb to reverse endotoxin-mediated vascular hyporeactivity to α_1-agonists observed in our study further supports a role for NO· in vasorelaxation.

Interestingly, cell-free Hgb reverses endotoxin-induced vascular hyporesponsivity, however, an equivalent amount of Hgb sequestered in RBC's has no effect. This data supports previous studies showing that Hgb, but not RBC's inhibits vascular relaxation caused by acetylcholine in dog femoral arteries [25]. Furthermore, others have shown that only 0.8% of the Hgb contained in RBC's obtained from endotoxic mice is nitrosylated [21]. One explanation for the difference between RBC's and cell-free Hgb may be that vasoactive NO· production is inaccessible to Hgb contained in RBCs. Since smooth muscle cells can produce NO· in autocoid fashion, cell-free Hgb may be a more efficient scavenger of NO· due to its penetration into the interstitial cell space. However, a difference in intracellular diffusion of Hgb does not appear to fully explain the difference between RBC-sequestered Hgb and cell-free Hgb since removing the endothelial cell layer, which allows more direct access to the smooth muscle cell surface, does not result

in an RBC-mediated reversal of vascular hyporeactivity. Alternately, the intact RBC membrane may interfere with the Hgb-NO· interaction. NO· may form nitroxyl ion [26] which would not readily diffuse across the RBC membrane. Cell-free met Hgb; however will react with nitroxyl ion [27].

Another possible explanation for these observations may be the loss of 2,3 diphosphoglycerate (2,3 DPG) after lysis of the RBCs. This would result in an increase in both the oxygen and NO· affinity. Addition of inositol hexaphosphate, which binds even more strongly to the 2,3 -DPG site, did not alter the Met Hgb-mediated reversal of endotoxin-induced vascular hyporeactivity (data not shown).

Studies with PFP-HSA and heme-albumin failed to demonstrate a reversal of vascular hyporesponsivity suggesting that neither the stable ferrous or ferric iron group can function as effective NO· scavengers; an effect which appears to require a Fe^{2+} to Fe^{3+} oxidation to provide reducing power for the reaction with NO·. Alternately, the binding sites of heme and iron picket-fence porphyrin in albumin may restrict access of NO· to the iron atoms of their active centers.

Whereas, both Hgb and met Hgb appeared to be effective in reversing vascular reactivity, cyan met Hgb, which is modified to prevent heme-NO· adduct formation, was ineffective, also suggesting that access of NO· to the heme iron is required for action as an NO· scavenger in the vascular ring preparation. Confirming this hypothesis, previous investigators have shown that infusion of diaspirin-cross-linked Hgb (DCLHB) increased the blood pressure in rats while cyan met-DCLHB had no effect [28]. The ability of met Hgb to scavenge NO· may be dependent on the reduction of Met Hgb to Hgb by NO·.

Recent clinical trials have demonstrated that agents which inhibit NO· formation have significant antihypotensive effects [17, 18]. Additional strategies to bind NO· with heme-based scavengers have the added advantage that other toxic radicals will be removed, such as peroxynitrite which may accentuate endotoxin-induced damage to the endothelium. Reversal of the pathological vasodilation that occurs as a result of excess NO· production may be a useful therapeutic strategy in the treatment of cytokine-mediated septic shock. Experiments presented here suggest that the contribution of the vascular smooth muscle to production of vasoactive NO· may not significantly limit the effectiveness of an agent which sequesters NO·. Furthermore, these results suggest that blood substitutes using modified Hgbs may have clinical efficacy in the treatment of septic shock.

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