cGMP and cAMP cause pulmonary vasoconstriction in the presence of hemolysate

NORBERT F. VOELKEL,1 JENNY D. ALLARD,1 STEVEN M. ANDERSON,2 AND THOMAS J. BURKE3

1Pulmonary Hypertension Center, 2Department of Pathology, and 3Division of Renal Diseases and Hypertension, University of Colorado Medical School, Denver, Colorado 20262

Voelkel, Norbert F., Jenny D. Allard, Steven M. Anderson, and Thomas J. Burke. cGMP and cAMP cause pulmonary vasoconstriction in the presence of hemolysate. J. Appl. Physiol. 86(5): 1715–1720, 1999.—We recently reported that addition of a small amount of hemolysate to the salt solution that perfused isolated rat lungs hypersensitized the vasculature to subsequent additions of ANG II or exposure to hypoxia, and addition of NO gas (-NO) to the perfusate that contained hemolysate caused a strong vasoconstrictor rather than a vasodilator response. In the present study, we demonstrate that CO and the secondary messengers cGMP and cAMP (usually associated with vasodilation) exert similar effects in hemolysate-perfused lungs. Analogs of the cyclic nucleotides cGMP or cAMP (8-bromo-cGMP and dibutyryl-cAMP, respectively) caused profound vasoconstriction in the isolated rat lung perfused with a salt solution that contained hemolysate. The cGMP- or cAMP-analog-induced vasoconstriction was inhibited by chemically dissimilar Ca2+ antagonists, by the protein phosphatase inhibitor okadaic acid, and, to a lesser degree, by protein kinase inhibitor H-7. Antiphosphothreonine immunoblotting demonstrated that lungs perfused with hemolysate exhibit increased phosphorylation of several proteins. These data indicate that, in the presence of hemolysate, pulmonary vasculature responds to nominally vasodilatory stimuli, including analogs of cGMP and cAMP, with vasoconstriction rather than vasodilation. The importance of our finding is the paradoxical nature of the response to (analogs of) cyclic nucleotides because, to our knowledge, cyclic nucleotide-induced vasoconstriction has not been previously reported.

nitric oxide; carbon monoxide; calcium antagonists; hypersensitization

VARIOUS DISEASES in which hemolysis is a comorbid event are often life threatening. For example, in the hemolytic uremic syndrome, intense vasoconstriction is found in kidneys and in other organs as well. After subarachnoid hemorrhage occurs, vasoconstriction extends the cerebral area of the damage (13). Hemolysis and accompanying vasoconstriction were extremely severe with the first generation of cardiopulmonary bypass machines (27). A response to hemolysis itself may be crucial for development of vasoconstriction because, in rats, injection of distilled water into a carotid artery results in acute renal dysfunction (11), and hemolytic crises in humans are often accompanied by hypertension and/or increased vascular resistance, usually in one or more organ systems (7). Small amounts of hemolyzed blood induce vasoconstriction in the isolated perfused kidney (11) and isolated perfused lung (26).

Several compounds, including but not limited to vanadate, endothelin (13), oxyhemoglobin (1), adenine nucleotides (principally ATP), and vasoactive lipids (8) contained within red blood cells (RBC), individually induce vasoconstriction. It is likely that each of these chemically dissimilar components of RBC causes vasoconstriction through unique mechanisms. However, there may be additional as-yet-unrecognized factors within RBC that affect vascular reactivity.

Under normal physiological conditions, net vascular reactivity is manifested by a balance between vasoconstrictive and vasodilatory agonists. One of the potent vasodilatory agents to which resistance vessel smooth muscle cells (VSMC) respond is nitric oxide (NO); however, NO production and bioavailability in hemolytic crises may lead to vasospasm (21). We recently reported that, in the presence of human and rat blood hemolysate, the lung vascular response to hypoxia, angiotensin II (ANG II), KCl, and 4-aminopyridine is augmented (26). In that study, we also observed that in the hemolysate-perfused lung, NO provoked a paradoxical vasoconstriction rather than vasodilation. Because of our interest in determining the mechanism of pulmonary vasoconstriction in patients with sickle cell disease, we used human blood (24). Because both hemolysed human and hemolysed rat blood elicit the same hypersensitization response in the isolated perfused rat lung preparation (26) and because human blood is often used in such cross-species studies (16, 18, 19), we have continued to use human blood as the source of hemolysate.

In the present study, we wished to determine whether the paradoxical vasoconstrictive effect of NO in hemolysate-perfused lungs is unique and/or whether the gas itself or its second messenger cGMP is responsible. Our results demonstrated that another guanylyl cyclase activator, namely carbon monoxide (CO), as well as the cell-permeable compound 8-bromo-cGMP (8-BrcGMP) also induce vasoconstriction. We also found, in the isolated lung, that addition of dibutyryl-cAMP (DBcAMP) to the perfusate that contained hemolysate caused vasoconstriction. Thus conventional second messenger function/activity in pulmonary vascular tissue is unexpectedly altered by some factor released during the lysis of RBC.
MATERIALS AND METHODS

Isolated rat lung preparation. Sprague-Dawley rats (300 g body weight) raised on a regular diet were used for these experiments. The rats were anesthetized with an injection of pentobarbital sodium (80 mg/kg i.p.), and the lungs and heart were isolated as described previously (9, 26). The lungs were perfused with Earle's balanced salt solution (EBSS) at a constant rate of 0.03 ml·g body weight\(^{-1}\)·min\(^{-1}\) at 37°C. After a standard 30-min equilibration period, pulmonarypressor responses were elicited with either a 0.1-µg bolus injection (in 100 µl of EBSS) of ANG II (Sigma Chemical, St. Louis, MO) or exposure to alveolar hypoxia, which was achieved by switching from one gas bag that contained 21% oxygen to another that contained 0% oxygen (26). Mean pulmonary artery pressure (Ppa) measurements were obtained from a Statham pressure transducer attached to a strip recorder. In every experiment, after the reaction of a lung to these two vasoconstrictive stimuli was tested, either hemolysate (in EBSS) or an equal volume of EBSS was added to the perfusion reservoir. Thirty minutes later, responses to ANG II and CO were determined, and then another challenge was performed.

Preparation of hemolysate. Hemolysate was generated from the blood, obtained by routine venipuncture, of a human volunteer. Ten milliliters of whole blood were centrifuged at 1,000 rpm for 10 min, and then the buffy coat and plasma were decanted. The remaining RBCs were washed twice with sterile 0.9% saline. The washed RBCs were lysed as follows: 175 µl of packed RBCs were diluted to 2.0 ml with cold, sterile, distilled water. The hemolysed cells were centrifuged for 1 h at 100,000 g, and the entire volume of membrane-free supernate was added to the lung-perfusate reservoir, which contained 50 ml of EBSS. Dose-dependency studies carried out previously showed that effects of hemolysate begin to be apparent at >17.5 µl, with maximal effects noted at 175 µl (26). As a point of reference, total blood volume would be ~24 ml in a 300-g rat with a blood volume of 8.0% of body weight. With a hematocrit of 50%, there would be ~12 ml of packed RBCs. We have used the equivalent of 85–90 µl of lysed, packed RBCs/25 ml of EBSS perfusate. This amount of hemolysate is considerably smaller than would be observed in clinical situations, such as hemolytic anemia.

NO or CO addition to perfusate. Only trace amounts of NO (~70 nM) were added to the perfusate (26). One hundred microliters of NO from a tank with a concentration of 800 parts per million, balance nitrogen, was aspirated into a 1.0-ml syringe which had been coated with EBSS and then injected into the perfusate reservoir. In the CO experiments, a mylar balloon was filled with CO from a tank that contained 100% CO; 100 µl of the gas were aspirated into a 1.0-ml EBSS-coated syringe and were injected into the perfusate reservoir. In three lungs perfused with hemolysate, the effects of addition of CO and NO were tested, and then ANG II (0.1-µg bolus) was again injected via the pulmonary arterial cannula to compare the magnitude of the ANG II pressor response to hemolysate alone and to hemolysate followed by trace amount of the gas(es). Either CO or NO each caused a large (50 and 53 mmHg, respectively) sustained pressor response. An ANG II bolus now produced a potentiated vasoconstriction. In lungs perfused under identical conditions with EBSS without hemolysate, neither CO nor NO caused vasoconstriction (results not shown). A representative experiment is shown in Fig. 1.

Cyclic nucleotide studies. For the remainder of the isolated lung experiments, the exact protocol described above was followed but, rather than NO or CO, analogs of cyclic nucleotides were added to hemolysate-perfused lungs. Either 8-BrcGMP or DBcAMP, at final concentrations between 10\(^{-4}\) and 10\(^{-5}\) M (8, 14, 23) was added, in a volume of 100 µl, to the perfusate reservoir, and the change in mean Ppa was recorded. The cyclic nucleotide analogs were dissolved in EBSS. In several experiments, we have documented that sequential addition of either 8-BrcGMP or DBcAMP at 20 min intervals each elicited a large increase in Ppa. Because we found pressor responses to NO, CO, or their secondary mediator cGMP in lungs perfused with hemolysate, we next attempted to block the hemolysate-associated pressor responses. For these studies, we first determined the magnitude of the pressor response to 8-BrcGMP or DBcAMP and then added H-7 (10\(^{-7}\) M) (17), or the Ca\(^{2+}\)-entry blocker diltiazem (10\(^{-5}\) M), or verapamil (10\(^{-5}\) M) to the perfusate. Five minutes later, the lungs were rechallenged with 8-BrcGMP or DBcAMP.

Additional experiments involving the phosphatase inhibitor okadaic acid were conducted in an effort to link the functional pressor responses to NO, CO, or cGMP to an enhanced phosphorylation evoked when hemolysate was present in the perfusate. Okadaic acid (10\(^{-7}\) M; Sigma Chemical) (15) was added 5 min before the second of two sequential 8-BrcGMP additions in hemolysate-perfused lungs, and the pressor response was recorded before and after okadaic acid was added.

Rat lung protein phosphorylation. The impressive pressor effects of analogs of cGMP and cAMP suggested that kinase-mediated phosphorylation of one or more cell proteins may have been altered in the presence of hemolysate. Therefore, rat lungs were perfused without or with hemolysate for 1 h and were then snap frozen in liquid nitrogen. The frozen tissue was pulverized with a mortar and pestle, lysed in lung lysis buffer [in mM: 50 KPO\(_4\), 50 NaF, 1 EDTA, 1 EGTA, 1 sodium orthovanadate, 150 NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, supplemented with 0.1 PeFabloc (Boehringer Mannheim, Indianapolis, IN), 1% kallikrein inhibitor (Calbiochem, San Diego, CA), 50 mg/ml pepstatin-A (Boehringer Mannheim), and 50 mg/ml leupeptin (Boehringer Mannheim)]. The lysate was homogenized for 2 min at full
speed by using a Beckman Polytrofe. Lysates were clarified by centrifugation at 13,000 rpm for 30 min in a refrigerated Savant microfuge. Protein concentrations were determined with the biocinchonic acid protein assay (Pierce Chemical, Rockford, IL). Identical amounts of protein (250 μg/ lane) were loaded onto a 7.5% SDS polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to Immobilon (Millipore, Bedford, MA). The filter was blocked in Tris-buffered saline-Tween 20 (TBS-T; 150 mM NaCl, 50 mM Tris, pH 7.4; and 0.5% Tween-20) with 5% nonfat dried milk (Carnation, Glendale, CA). The filter was washed three times with TBS-T and then incubated overnight with a 1:100 dilution of antiphosphothreonine antibody (no. 3555, Sigma Chemical). The filter was washed three times with TBS-T and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG. The antibody was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**Results.** Results are given as means ± SE. Comparisons are made with paired t-test for pressor responses to a single maneuver in control or hemolysate-treated lungs. The number of experiments are indicated in the figure legends.

**RESULTS**

Pressor responses to NO, CO, or cyclic nucleotide analogs. Addition of ANG II or exposure to hypoxia in the absence of hemolysate increases Ppa by 1–5 mmHg (9, 14, 23, 26). This increase was confirmed in the present studies before the addition of hemolysate to the perfusate. Injection of 100 μl of CO gas into the perfusate that contained hemolysate (n = 3) caused a prompt, large pulmonary pressor response comparable to that observed after injection of NO. A representative experiment and the typical pressor response are shown in Fig. 1; the response to NO is similar to previously published data from our laboratory (26). The pressor responses (Ppa, shown in Fig. 1 after CO or NO addition) were estimated to be >50 mmHg. Both the magnitude and the duration of the pressor response were greater for NO and CO than for ANG II.

Having established that two gaseous guanylyl cyclase activators cause vasoconstriction in the presence of EBSS that contained hemolysate, we added a cell permeable cGMP analog, 8-BrcGMP (10−4 M), to the perfusate, and vasoconstriction was again observed (Fig. 2A). Similar pressor responses were found after addition of a lower dose of 8-BrcGMP (10−5 M; Fig. 2B) or addition of 8-BrcAMP (10−4 M; Fig. 2C). Figure 2, A-C, shows representative experiments. The pressor responses after addition of either cyclic nucleotide analog to lungs exposed to hemolysate were of substantial magnitude, were sustained for 8–10 min before recovering toward baseline, and occurred repeatedly in the same preparation (Fig. 2D). Three or four separate additions of DBcAMP, 15 min apart, each evoked a large pressor response.

The pressor response induced by 8-BrcGMP or DBcAMP was inhibited by the Ca2+-entry blocker diltiazem (Fig. 3, A and B). H-7, the nonspecific protein kinase (PK) inhibitor (Fig. 3C) blocked the pressor response to 8-BrcGMP. Figure 3 shows typical tracings of these experiments. Figure 4 indicates the mean responses for all experiments. The results with 8-BrcGMP were similar to those with DBcGMP (Fig. 4).

In two experiments, verapamil also blocked the pressor effect of 8-BrcAMP (Fig. 4).

Furthermore, in addition to causing vasoconstriction rather than vasodilation in the hemolysate-treated
lungs, cyclic nucleotide analogs enhanced the subsequent pressor response to a bolus injection of ANG II (Fig. 2B). Exposure to hemolysate also appears to hypersensitize the vasculature to doses of ANG II that would otherwise only cause a modest pressor response (Figs. 1 and 2B).

Effect of hemolysate on lung protein phosphorylation. PKs may act as a link between the increase in vasoactive cyclic nucleotides and altered vascular tone. Treatment with hemolysate resulted in an increase in the phosphorylation of several lung proteins that contain threonine. Two proteins, with apparent molecular masses of 140 and 155 kDa, appeared to be especially affected by hemolysate treatment (Fig. 5; compare left and right lanes). The increase in protein phosphorylation might have been due to a hemolysate-mediated increase in threonine-specific kinase activity, or to a decrease in threonine-specific phosphatase activity, or to some combination thereof. We were unable to detect a change in tyrosine phosphorylation when the lung tissue was immunoblotted with the antiphosphotyrosine antibody 4G10 (data not shown), and it was not possible to analyze changes in phosphoserine-containing proteins because of a high background staining encountered when we probed with a monoclonal antibody directed against phosphoserine (data not shown).

DISCUSSION

We showed previously that the effects of hemolysate on lung vascular tone regulation cannot be reproduced by purified hemoglobin, ATP, ADP, or other small molecules, such as glutathione, and we suggested that the mechanisms may include the alteration of the lung cell mitochondrial energy metabolism and/or ion-channel activities (26). In the present study, we tested the hypothesis that, if -NO causes vasoconstriction in hemolysate-perfused lungs, then CO or cGMP itself (the product of the interaction of CO or -NO with the guanylyl cyclase) should similarly cause vasoconstriction. Pressor responses after administration of the well-recognized vasodilator compound CO or the cell-permeant analog of cGMP have not been previously reported. The lung is not unique in these responses. Recently, we demonstrated hypersensitization to ANG II in isolated, pressurized, renal afferent and efferent arterioles after exposure to hemolysate (6).

Indeed, not only cGMP but also cAMP caused vasoconstriction in hemolysate-containing perfused lung, and this vasoconstriction was inhibited by Ca2+-entry blockers. These data suggest that the cGMP- and cAMP-induced pulmonary vasoconstriction depends on Ca2+ entry into VSMC. Hemolysate increases cytosolic Ca2+ concentration ([Ca2+]i) in cultured basilar artery SMCs through both Ca2+ influx and release from internal stores (30). Clearly, a Ca2+-influx-dependent, intense

![Fig. 3](image)

**Fig. 3.** In these 3 representative tracings, the pressure range of the recorder was set at 0–50 mmHg to accommodate very high pressures, if they occurred with treatment. Hemolysate is added before ANG II injection (far left). After the H (hypoxic) response was determined, lungs are regassed with 21% O2. A: diltiazem treatment prevents pressor response to 8-BrcGMP (10^-4 M). B: pressor responses to ANG II and H are determined (far left). Hemolysate is added, and hypersensitivity to ANG II and H is documented. Addition of diltiazem prevents increase in Ppa that normally accompanies DBcAMP and also attenuates hypersensitive pressor response that normally occurs after addition of ANG II (far right). C: H-7 also reduces pressor effect of 8-BrcGMP.

![Fig. 4](image)

**Fig. 4.** Okadaic acid and Ca2+ antagonists are effective blockers of cyclic nucleotide-induced pulmonary pressor response. Bars, change in Ppa (ΔPpa) in presence of hemolysate for all experiments; n, no of experiments. In 2 lungs, verapamil pretreatment (Ca2+-entry blockade) abolished the 8-BrcAMP-induced pressor response. In each of the lungs, there was documentation of the presence of a cyclic nucleotide-induced pressor response before addition of blocking agent.
pulmonary vasoconstriction triggered by cGMP or cAMP is a paradoxical finding. An unknown factor in RBC may be responsible for these responses. The results in lungs perfused with chemically dissimilar Ca^2+ channel blockers suggest that a Ca^2+ -mediated event participates in 1) paradoxical vasoconstriction in the presence of vasodilators and 2) hypersensitization to low concentrations of ANG II or to hypoxia. The increase in pressure is reproducible (Fig. 4) and can be long lasting (Fig. 3). In addition, sequential bolus injections of cAMP each initiate vasoconstriction that is manifested by elevated perfusion pressure. The vasculature appears to remain hypersensitive once it has been exposed to hemolysate. However, the failure of the pressure to return to control values might indicate underlying structural or biochemical damage to vascular tissues.

The hemolysate preparation also increases [Ca^{2+}] in pulmonary artery VSMCs and in aortic VSMCs (6). Thus, not only the intact lung vasculature but isolated VSMCs respond to hemolysate with altered Ca^{2+} signaling.

With evidence that both cyclic nucleotides caused vasoconstriction in the hemolysate-perfused lung, we examined downstream signaling events involving PK activation and phosphorylation. The nonspecific PK inhibitor H-7 (17) and the phosphatase inhibitor okadaic acid (15) both attenuated cAMP- and cGMP-triggered vasoconstriction in hemolysate-perfused lungs. Pharmacological inhibition of the cyclic nucleotide-induced pulmonary pressor responses—possibly more so by phosphatase inhibitors than by PK inhibitors—points toward a possible effect of hemolysate to alter the phosphorylation state of one or several target proteins involved in VSMC contraction. In this context, it is of interest that either NO or 8-BrcGMP induces elevations of [Ca^{2+}] in and causes contraction of gastrointestinal SMCs during protein kinase A and G inhibition (20). Perhaps a factor in hemolysate alters kinase activity.

We speculate that, because K^+ channels are critically important in lung vascular tone regulation, as has been suggested (2, 3), the paradoxical vasoconstriction in the presence of hemolysate might be explained by an imbalance of phosphatase and kinase activities that lead to altered phosphorylation of a K^+ channel or a regulator protein (22). Again, in this context, it may be of interest that NO-triggered cGMP generation, followed by an increase in Ca^{2+} entry, has been recently described in pancreatic acinar cells (29). In addition, closure of K^+ channels, initiated by an increase in cAMP and followed by an increase in channel protein phosphorylation, has been described in isolated taste-receptor cells (4, 5). Thus cyclic nucleotides, under conditions in which RBCs have undergone lysis, can cause severe vasoconstriction rather than vasodilation. Perhaps, in the presence of a hemolysate factor, VSMCs in vessels of the lung (present study), of the kidney (6), and in culture (6, 20) exhibit responses that begin to resemble those observed in taste-receptor cells.

In conclusion, we suggest that our observations may shed new light on the mechanism of hemolysis or hemorrhage-associated vasoconstriction, which is a serious clinical problem (1, 25), and perhaps also on vasomotor tone control in general. Hemolytic syndromes such as sickle cell disease, hemolytic uremic syndrome (HELLP), and thrombotic thrombocytopenic purpura (TTP) have disparate etiologies, cause anemia, and are triggered either by drugs or by a number of bacterial toxins. The present results suggest that, in addition to the underlying clinical insult, hemolysis itself may be an additional factor responsible for the intense vasoconstriction seen in one or more organ systems. That hemolysate can cause profound pulmonary hypertension was first described in cattle and sheep (28). More recently, it has been shown that VSMCs from rabbit basilar arteries are contracted by hemolysate (30) and that a low-molecular-weight component, possibly in concert with hemoglobin, may be responsible for the intense vasoconstriction observed after subarachnoid hemorrhage (25).

We are presently engaged in efforts to isolate and identify a low-molecular-weight hemolysate factor that
may be responsible for the altered behavior of pulmonary and renal vessels (6). Further work is also required to identify the proteins that exhibit enhanced phosphorylation after exposure to hemolysate in the perfused lung preparation.

This work was supported by a Vascular Academic Award (National Heart, Lung, and Blood Institute K07-HL-02825) and by an award from the Primary Pulmonary Hypertension Cure Foundation, Baltimore, MD.

Address for reprint requests and other correspondence: N. F. Voelkel, Univ of Colorado Medical School, 4200 E. Ninth Ave., C272, Denver, CO 80262 (E-mail: Norbert.Voelkel@UCHSC.edu).

Received 19 May 1998; accepted in final form 5 January 1999.

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