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EFFECTS OF SPONTANEOUS HEMOLYSIS, PURIFIED, AND CHEMICALLY  
MODIFIED HEMOGLOBIN ON ENDOTHELIUM-DEPENDENT VASODILATION AND  
BASELINE CORONARY HINDRANCE IN RABBIT HEARTS PERFUSED WITH AND  
WITHOUT RED CELLS

BY

W.M. VOGEL, G. CASSIDY, C.S. APSTEIN, AND C.R. VALERI

NAVAL BLOOD RESEARCH LABORATORY  
BOSTON UNIVERSITY SCHOOL OF MEDICINE  
615 ALBANY STREET  
BOSTON, MA 02118

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increase of perfusion pressure  $56 \pm 6$  mm Hg for unmodified Hb prepared by ultrafiltration,  $32 \pm 2$  mm Hg for HPLC-purified Hb Ao, and  $21 \pm 4$  mm Hg for Hb crosslinked with bis 3,5-dibromosalicyl fumarate (DBBF-Hb). Endothelium-dependent coronary dilation was tested in hearts perfused with: a) red cells with supernatant Hb concentrations of 60-650 mg/dl due to spontaneous hemolysis), b) red cells with  $1.6 \pm 0.4$  g/dl Hb Ao, and c) 5.5 g/dl DBBF-Hb without red cells. With spontaneous hemolysis, higher Hb supernatant concentrations were associated with increased baseline coronary hindrance and decreased endothelium-dependent dilator responses to methacholine. Hb Ao and DBBF-Hb were significantly less potent than Hb from spontaneous hemolysis with greater methacholine responses and lower baseline hindrance in relation to supernatant Hb concentration. In red cell perfused hearts, with and without Hb Ao, baseline coronary hindrance was inversely related to methacholine dilator response ( $r = -0.93$ ). We conclude that purification and crosslinking of Hb can decrease coronary constrictor activity by decreasing the inhibition of endothelium-dependent dilator tone.

## ABSTRACT

Hemoglobin (Hb) solutions being developed as red cell substitutes may have vaso-constrictor activity. We tested whether different coronary constrictor activities of certain Hb solutions were due to differential inhibition of endothelium-dependent vasodilation. In buffer-perfused rabbit hearts with constant coronary flow, Hb increased coronary resistance as indicated by increased perfusion pressure. Purification or chemical modification of Hb significantly decreased coronary vasoconstriction. The maximum increase of perfusion pressure  $56 \pm 6$  mmHg for unmodified Hb prepared by ultrafiltration,  $32 \pm 2$  mmHg for HPLC-purified Hb Ao, and  $21 \pm 4$  mmHg for Hb crosslinked with bis 3,5-dibromosalicyl fumarate (DBBF-Hb). Endothelium-dependent coronary dilation was tested in hearts perfused with: a) red cells with supernatant Hb concentrations of 60 - 650 mg/dl due to spontaneous hemolysis), b) red cells with  $1.6 \pm 0.4$  g/dl Hb Ao, and c) 5.5 g/dl DBBF-Hb without red cells. With spontaneous hemolysis, higher Hb supernatant concentrations were associated with increased baseline coronary hindrance and decreased endothelium-dependent dilator responses to methacholine. Hb Ao and DBBF-Hb were significantly less potent than Hb from spontaneous hemolysis, with greater methacholine responses and lower baseline hindrance in relation to supernatant Hb concentration. In red-cell-perfused hearts, with and without Hb Ao, baseline coronary hindrance was inversely related to methacholine dilator response ( $r = -0.93$ ). We conclude that purification and crosslinking of Hb can decrease coronary constrictor activity by decreasing the inhibition of endothelium-dependent dilator tone.

**KEYWORDS:** hemoglobin, endothelium-dependent-relaxing-factor (EDRF), coronary hindrance, vasoconstriction, blood substitutes.

## INTRODUCTION

Hemoglobin solutions being developed as therapeutic red-cell substitutes (15) can elicit vasoconstriction in various vascular beds (11, 17, 25, 28). This vasoconstrictor activity can be altered by different purification techniques or chemical modifications of hemoglobin (16, 25-27). Hemoglobin inhibits vasodilation mediated by the endothelium-dependent relaxing factor (EDRF), nitric oxide (14, 19). Thus, hemoglobin may constrict particular vascular beds by inhibiting dilation due to basal release of nitric oxide. A few studies suggest the presence of EDRF dilator tone in the coronary circulation (1, 23). But other mechanisms may predominate; hemoglobin-induced constriction of porcine coronary artery rings was not related to inhibition of EDRF (4). We examined whether differences in coronary constrictor activity of particular hemoglobin solutions was due to differential inhibition of EDRF. We measured endothelium-dependent coronary dilator responses to methacholine in rabbit hearts perfused with red cells or hemoglobin solution. Because acellular hemoglobin solutions can have lower viscosity than a red-cell perfusate, viscosity was measured and vascular tone was expressed as hindrance (resistance/viscosity). We compared spontaneous hemolysis, HPLC-purified hemoglobin A (Hb-Ao), and hemoglobin crosslinked with bis 3,5-dibromosalicyl fumarate (DBBF-Hb). Hb-Ao and DBBF-Hb were studied because both have less coronary constrictor activity than conventionally prepared hemoglobin (18). The results suggest that purification and crosslinking of hemoglobin can decrease coronary constrictor activity by decreasing the inhibition of endothelium-dependent dilator tone.

## METHODS

Isolated heart. Male New Zealand rabbits (1.5-2.0 kg) were anesthetized with iv pentobarbital and anticoagulated with iv heparin. Hearts were isolated and perfused at a constant coronary flow rate via the cannulated

aorta with non-recirculated Krebs buffer as previously described (25-27). The perfusate contained (in mM): 118 NaCl, 25 NaHCO<sub>3</sub>, 4.7 KCl, 2.0 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 0.4 Na EDTA, 5.5 glucose, and 1.0 Na lactate. The perfusate was gassed with 95% O<sub>2</sub> - 5% CO<sub>2</sub> and passed through a bubble trap and a 1.6 μM glass fiber filter (Whatman GF/A) just above the heart. Perfusion pressure was measured by a transducer attached to the aortic cannula. Hearts were paced and temperature was kept at 37 C. Hearts developed pressure against a water-filled balloon in the left ventricular chamber; ventricular pressure was measured by a transducer attached to the cannulated balloon. Coronary venous effluent was collected from the cannulated pulmonary artery. For perfusion with resuspended red cells or concentrated hemoglobin solutions a 20 μM transfusion filter (Fenwal PDF-20) was used and 200 ml of recirculated perfusate was oxygenated with a coil of thin-walled silicone tubing (2).

Red-cell perfusate. Fresh heparinized cow blood was centrifuged at 5 C for 15 min at 1000 x g; plasma and buffy coat were removed; red cells were suspended in Krebs buffer with 60 mg/L gentamicin and spun again for 15 min at 1000 x g; washing was repeated two more times. Packed red cells were stored at 4 C, washed daily, for up to 5 days. Red cells were resuspended in Krebs buffer with 4 g/dl bovine albumin (fraction V, fatty acid free, ICN Biochemicals), 2 mg/l gentamicin, and no EDTA. Ionized Ca<sup>++</sup> was adjusted to 1.2 mM.

Hemoglobin solutions. Hemoglobin solutions were prepared from outdated human red cells. Hemoglobin A<sub>0</sub> purified by anion exchange HPLC (8) was obtained from Dr. M.A. Marini, Letterman Army Institute of Research, Presidio of San Francisco, CA. Hemoglobin crosslinked with 3,5 bis dibromo salicyl fumarate (DBBF-Hb) (7) and heat pasturized (10) was prepared by Baxter Healthcare Corp., Round Lake, IL. Hemoglobin partially purified by ultrafiltration (12) was obtained from Dr. R. Condie, Department of Surgery, University of Minnesota, Minneapolis, MN. Electrolytes were adjusted to

that of the Krebs buffer and solutions were filtered through 0.2  $\mu\text{m}$  membrane filters (Gelman, Acrodisc). Hemoglobin solutions were stored at 4 C under sterile conditions.

Blood chemistry and viscosity. Samples were analyzed for  $\text{P}_{\text{O}_2}$ ,  $\text{P}_{\text{CO}_2}$ , and pH, with a blood-gas analyzer (Instrumentation Laboratories model IL-813). Oxyhemoglobin, and methemoglobin were measured with an IL 282 CO-Oximeter. Supernatant hemoglobin was measured spectrophotometrically (5). Concentrations of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$ , were measured by ion-selective electrodes (NOVA biomedical, model 6). Viscosity was measured at 37 C by the flow rate of a sample through a porous bed viscometer (21). Viscosity is reported in relative units by dividing the time for a volume of sample to flow through the bed by the time needed for an equal volume of water. Porous bed viscometers were obtained from Dr. E.W. Merrill, Department of Chemical Engineering, Massachusetts Institute of Technology.

Experimental protocols. Coronary constrictor activity was assessed in buffer-perfused hearts as previously described (25-27). Coronary flow was constant at a rate initially producing a perfusion pressure of 70 mmHg. Hemoglobin solutions (7 g/dl) were infused into the perfusion line with a syringe pump to achieve concentrations of 10 to 200 mg/dl in the perfusate. With coronary flow constant, vasoconstriction was observed as increased perfusion pressure. Each infusion rate was maintained for 1 to 2 minutes until perfusion pressure approached a plateau. These hemoglobin concentrations had no effect on left ventricular developed pressure. Solutions were tested in separate groups of hearts. Data were analysed by analysis of variance for repeated measures (3).

Endothelium-dependent dilation was assessed in separate experiments because little endothelium-dependent dilation was obtained in buffer-perfused hearts. Nine hearts were perfused with red cells at a hematocrit of 35%.

Hearts were paced just above their intrinsic rates and balloon volume was adjusted to produce a left ventricular diastolic pressure of zero. Coronary flow was adjusted to maintain perfusion pressure at 100 mmHg during 20 minutes of equilibration and held constant thereafter. Perfusate samples were collected for measurements of hematocrit, supernatant hemoglobin, viscosity, blood gases, total hemoglobin, and oxygen content. Vasodilator responses to methacholine were then determined. Stock solutions of 0.1 and 1.0 mg/dl methacholine bromide (Sigma) were prepared fresh daily in Krebs buffer. Bolus injection of methacholine (0.01 to 0.05 ml) caused coronary dilation manifested as a transient decrease of perfusion pressure lasting 1 to 2 minutes. Injected dose was increased from 10 to 500 ng until a maximum response was obtained. Methacholine responses are reported as the percent decrease of perfusion pressure at 250 ng, which produced nearly maximal responses. Due to hemolysis, supernatant hemoglobin ranged from 60 to 650 mg/dl. Methacholine responses and baseline coronary hindrance were plotted as functions of supernatant hemoglobin concentration.

In 5 of those hearts with supernatant hemoglobin less than 0.2 g/dl methacholine responses were retested in the presence of hemoglobin A<sub>0</sub>. Hemoglobin A<sub>0</sub> (7 g/dl, 11 ± 3% methemoglobin) equilibrated with room air at 37 C was infused into the perfusion line for ten minutes at 1/10 the total coronary flow rate, which was held constant. During the last five minutes of the infusion methacholine responses were tested and perfusate samples were collected for hematocrit, and supernatant hemoglobin. Changes in hemodynamic values during addition of hemoglobin A<sub>0</sub> were evaluated by paired t test.

A separate group of 5 hearts was perfused in the absence of red cells with 7 g/dl DBBF-Hb. Because methemoglobin concentration was 21.3 ± 0.2 % reduced (ferrous) hemoglobin was 5.5 g/dl. The protocol described for red-cell-perfused hearts was followed for these hearts. Coronary hemodynamic

values were compared by Student's unpaired t test to those observed in 7 of the red-cell-perfused hearts with supernatant hemoglobin less than 0.2 g/dl.

Methacholine response and baseline coronary hindrance were analysed as functions of hemoglobin concentration; the relationships obtained with Hb-Ao, DBBF-Hb or spontaneous hemolysis were compared by analysis of covariance. To account for small variations in perfusate composition (eg. increased K<sup>+</sup> due to hemolysis) multiple linear regression was used to determine whether hemoglobin preparations differed in their effects on coronary hindrance and methacholine response independent of pH, K<sup>+</sup>, Na<sup>+</sup>, and Ca<sup>++</sup> concentrations.

Statistics. Values are presented as the mean  $\pm$  SE. A probability less than 0.05 was considered significant for the various statistical tests used.

## RESULTS

Coronary constrictor activity of three hemoglobin solutions is shown in figure 1. Each solution caused dose-related increases in coronary vascular resistance, as evidenced by increased perfusion pressure with constant coronary flow. The constrictor activity was greatest for the ultrafiltered lysate, less for Hb-Ao, and least for DBBF-Hb. All groups differed significantly from each other at the highest concentration tested.

Effects of Hb-Ao in red-cell-perfused hearts are summarized in table 1. Dilution of the red-cell perfusate with acellular Hb-Ao caused small but significant decreases in hematocrit and, thus, viscosity of the perfusate. A 22% increase of perfusion pressure with constant coronary flow indicates increased coronary resistance and hindrance. Addition of Hb-Ao decreased the dilator response to methacholine by half.

Effects of perfusion with DBBF-Hb are summarized in table 2. The DBBF-Hb solution had less total hemoglobin and lower viscosity than the red-cell perfusate. At a perfusion pressure of 100 mmHg, coronary flow with DBBF-Hb was 2.5 times that in red-cell-perfused hearts; coronary resistance was less

with DBBF-Hb, but there was no difference in hindrance. Thus, DBBF-Hb had no effect on coronary vasomotion at this concentration, but increased coronary flow due to lower viscosity. The increased coronary flow with DBBF-Hb was sufficient to compensate for a lower total hemoglobin content and  $O_2$  content;  $O_2$  delivery (arterial  $O_2$  content  $\times$  coronary flow), myocardial  $O_2$  consumption, and  $O_2$  extraction were similar in hearts perfused with red cells or DBBF-Hb. Dilator responses to methacholine were still obtained with 5.5 g/dl DBBF-Hb, but they were less than in hearts perfused with red cells.

Methacholine responses and baseline hindrance are shown in figure 2 as functions of supernatant hemoglobin concentration. In red-cell-perfused hearts higher hemoglobin concentrations due to spontaneous hemolysis were associated with decreased methacholine responses and increased baseline hindrance. Methacholine responses and baseline hindrance in the presence of Hb-A or DBBF-Hb did not fall along the same regression line as hemoglobin released by hemolysis, but were shifted rightward to higher hemoglobin concentrations ( $P < 0.05$  by analysis of covariance). Thus, Hb-Ao and DBBF-Hb were less potent vasoconstrictors and inhibitors of EDRF than hemoglobin released by hemolysis. Multiple linear regression indicates that these differences were independent of variations in pH or electrolytes, but higher  $Na^+$  concentration was associated with increased baseline hindrance ( $P < 0.05$ ) and higher  $Ca^{++}$  concentration was associated with increased methacholine responses ( $P < 0.03$ ).

The relation between methacholine response and baseline coronary hindrance in red-cell-perfused hearts is shown in figure 3. There was a close inverse relation, such that hearts with smaller methacholine responses had greater baseline coronary hindrance independent of the type or amount of hemoglobin in the perfusate. This suggests that both of these hemoglobins increased coronary hindrance by inhibiting a baseline endothelium-dependent dilator tone. In hearts perfused with DBBF-Hb without red cells an opposite

linear relationship was observed; hearts with lower baseline hindrance and greater coronary flow had smaller methacholine responses.

#### DISCUSSION

In buffer-perfused hearts, coronary constrictor activities of three hemoglobin solutions were compared. As reported by others (18), both Hb-Ao and DBBF-Hb had less coronary constrictor activity than an unmodified, stroma-free hemoglobin solution prepared by ultrafiltration. This accords with our previous observations that stringent purification or glutaraldehyde crosslinking decreases the coronary constrictor activity of hemoglobin (25-27). We tested the possibility that decreased coronary constrictor activity of the purified and modified solutions was associated with decreased inhibition of endothelium-dependent dilation. Muscarinic agonists such as acetylcholine and methacholine are prototypical endothelium-dependent vasodilators (14, 19). Hemoglobin released by spontaneous hemolysis was a more potent inhibitor endothelium-dependent dilation than either Hb-Ao or DBBF-Hb. Methacholine responses were virtually eliminated by 0.4 - 0.6 g/dl hemoglobin from hemolysis but could still be elicited in the presence of 1.6 g/dl Hb-Ao and 5.5 g/dl DBBF-Hb. Hemoglobin released by hemolysis was also more potent than Hb-Ao at increasing coronary hindrance. Concentrated DBBF-Hb had no measurable constrictor effect; coronary hindrance of hearts perfused with 5.5 g/dl DBBF-Hb without red cells did not differ significantly from red-cell-perfused hearts with low supernatant hemoglobin concentration.

From the relation in figure 2, methacholine responses would be inhibited by 50% at 300 mg/dl hemolysate hemoglobin. This is higher than the range of  $10^{-6}$  to  $10^{-5}$  M hemoglobin (6.8 - 68 mg/dl) which completely inhibits endothelium-dependent relaxations in vascular strips or buffer-perfused hearts (13, 19, 23). Resistance to inhibition by hemoglobin might result from better preservation of endothelial cell structure and function with the red-cell-

albumin perfusate than with crystalloid buffer. We previously observed that higher hemoglobin concentrations were needed to cause vasoconstriction in blood than in buffer-perfused hearts (25).

The strong correlation between methacholine response and baseline coronary hindrance in red-cell-perfused hearts implies a basal endothelium-dependent dilator tone. Hemoglobin or N-monomethyl arginine (NMMA), an inhibitor of NO formation, have been used by others to block endothelium-dependent dilation in buffer-perfused hearts (1, 23); increased coronary resistance after inhibition of EDRF was taken as evidence of endothelium-dependent dilator tone. Our results extend these observations to hearts perfused with red cells at physiological coronary flow rates, in contrast to the high coronary flow rates of buffer-perfused hearts (25). This distinction is important because increased flow rate stimulates release of EDRF (29).

In red-cell-perfused hearts, the relationship between methacholine response and baseline coronary hindrance was the same for hemoglobin released by hemolysis and for Hb-A<sub>0</sub>. This suggests that both increased hindrance by the same mechanism (i.e. inhibition of EDRF) despite the lesser potency of Hb-A<sub>0</sub>. The lesser potency of Hb-A<sub>0</sub> as a vasoconstrictor and inhibitor of EDRF might be due to removal of a lipophilic contaminant. An unidentified lipophilic contaminant of albumin can cause severe coronary constriction (16); lysophosphatidyl choline (20) and oxidized lipoproteins (22) inhibit endothelium-dependent dilation; and lipids are difficult to remove from hemoglobin without special procedures (9).

DBBF-Hb was only a weak inhibitor of endothelium-dependent coronary dilation. This accords with the observation that renal vasodilator responses to acetylcholine could be elicited in the presence of 400 mg/dl DBBF-Hb (16). Crosslinking of hemoglobin might decrease its access to the space between endothelium and vascular smooth muscle, or alter its interaction with NO. No

data directly supports either possibility. Hemoglobin can apparently inhibit EDRF-mediated dilation without penetrating the endothelial layer (13) and capillary permeability of intramolecularly crosslinked hemoglobin did not differ significantly from that of unmodified hemoglobin (6). These observations favor the possibility that crosslinking alters the interaction between NO and hemoglobin. In hearts perfused with DBBF-Hb, methacholine responses were smaller in hearts with the highest flow rates, the exact opposite of the relation observed in hearts perfused with red cells. High coronary flow rate stimulates release of EDRF (29). It is possible that the high coronary flow rates induced by the low viscosity DBBF-Hb perfusate increased baseline endothelium-dependent dilator tone and limited the further release of EDRF which could be elicited by methacholine.

In summary, purification or modification of hemoglobin resulted in decreased coronary constrictor activity associated with decreased inhibition of endothelium-dependent dilation. Because there appears to be substantial dilator tone due to basal release of NO in humans (24), minimal ability to inhibit endothelium-dependent dilation may be a desirable property of hemoglobin-based red-cell substitutes.

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Table 1.

Effects of hemoglobin Ao infusion in red-cell-perfused hearts.

	Red Cells Alone	Red Cells + Hb-Ao	Change	P
Supernatant Hb (g/dl)	$0.11 \pm 0.02$	$1.73 \pm 0.38$	$1.61 \pm 0.38$	< .02
Hematocrit (percent)	$35 \pm 1$	$30 \pm 1$	$-5 \pm 1$	< .01
Viscosity (relative units)	$3.3 \pm 0.1$	$2.8 \pm 0.1$	$-0.5 \pm 0.1$	< .01
Coronary Flow (ml/min/g)	$1.27 \pm 0.14$	$1.24 \pm 0.16$	$-0.03 \pm 0.15$	ns
Perfusion Pressure (mmHg)	$98 \pm 4$	$120 \pm 5$	$22 \pm 3$	< .005
Coronary Resistance (mmHg/ml/min/g)	$81 \pm 11$	$103 \pm 13$	$22 \pm 8$	< .05
Coronary Hindrance (mmHg/ml/min/g)	$25 \pm 3$	$36 \pm 4$	$11 \pm 3$	< .02
MeCh Response (% decrease CPP)	$15.3 \pm 0.9$	$6.8 \pm 0.5$	$8.5 \pm 0.8$	< .001

Values are mean  $\pm$  SE for 5 hearts before (red cells alone) and during infusion of hemoglobin. Statistical significance of differences was determined by Student's paired t test. Viscosity is in relative units with viscosity of water at 37 C = 1. Dilator response to 250 ng intracoronary methacholine (MeCh) is reported as % decrease of coronary perfusion pressure (CPP). Hindrance = resistance divided by viscosity.

Table 2.

Comparison of hearts perfused with red cells or acellular DBBF-Hb solution.

	Red Cells No added Hb (n=7)	DBBF Hb No Red Cells (n=5)	Difference	P
Total Hb (g/dl)	11.6 ± 0.4	5.5 ± 0.02	6.0 ± 0.5	< 0.001
Supernatant Hb (g/dl)	0.1 ± 0.01	5.5 ± 0.02	5.4 ± 0.03	< 0.001
Hematocrit (percent)	33 ± 1	0	33 ± 1	< 0.001
Viscosity (relative units)	3.1 ± 0.1	1.1 ± 0.02	2.0 ± 0.1	< 0.001
Coronary Flow (ml/min/g)	1.7 ± 0.2	4.3 ± 1.0	2.6 ± 0.9	< 0.015
Coronary Resistance (mmHg/ml/min/g)	61 ± 5	27 ± 5	34 ± 7	< 0.001
Coronary Hinderance (mmHg/ml/min/g)	20 ± 1	25 ± 5	5 ± 4	ns
MeCh Response (% decrease CPP)	17 ± 1	9 ± 2	8 ± 2	< 0.005
Arterial O <sub>2</sub> (volume %)	15.4 ± 0.4	7.9 ± 0.1	7.5 ± 0.5	< 0.001
Venous O <sub>2</sub> (volume %)	12.5 ± 0.5	7.0 ± 0.1	5.5 ± 0.6	< 0.001
O <sub>2</sub> Consumption (ml/min/100g)	4.8 ± 0.6	3.9 ± 0.5	0.9 ± 0.8	ns
O <sub>2</sub> delivery (ml/min/100g)	26 ± 2	34 ± 8	8 ± 7	ns
O <sub>2</sub> extraction	18 ± 3	12 ± 2	6 ± 4	ns

Values are mean ± SE. Statistical significance of differences was determined by Student's t test. P values > 0.05 are indicated as not significant (ns). O<sub>2</sub> delivery = arterial O<sub>2</sub> content x coronary flow. Viscosity is in relative units with viscosity of water at 37 C = 1. Dilator response to 250 ng intracoronary methacholine (MeCh) is reported as % decrease of coronary perfusion pressure (CPP). Hinderance = resistance divided by viscosity.

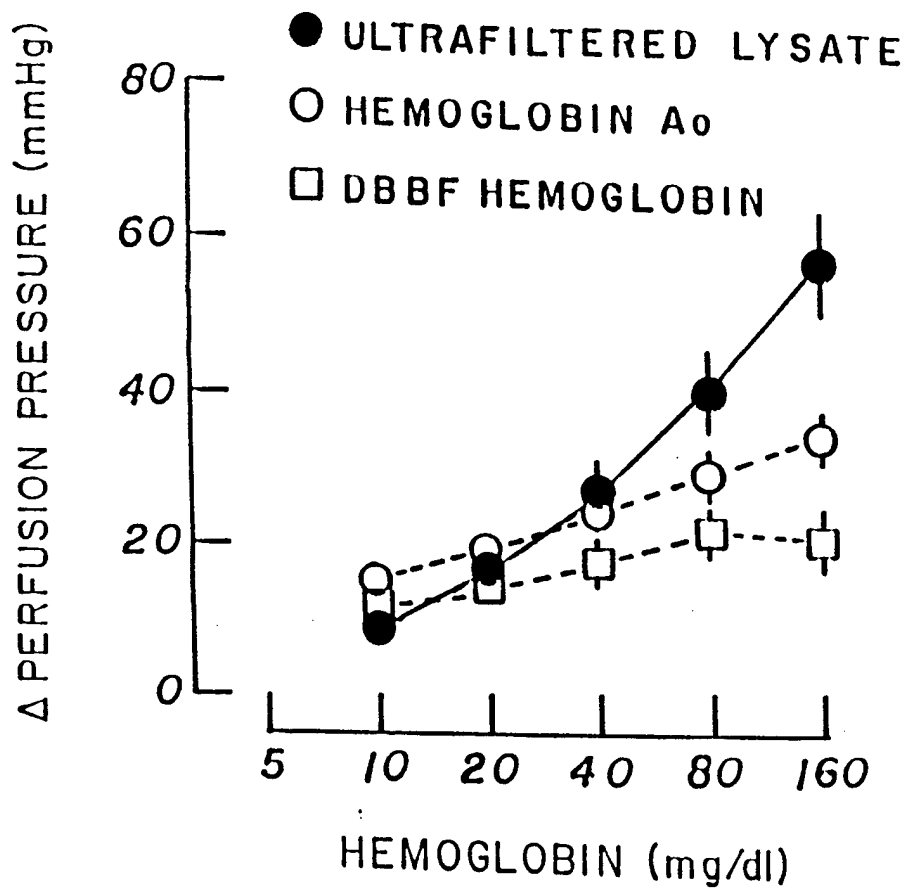


Figure 1. Coronary constrictor effect of hemoglobin solutions in buffer-perfused hearts. Vasoconstriction is expressed as increase in perfusion pressure at constant coronary flow rate (baseline perfusion pressure = 70 mmHg). Different hemoglobin (Hb) solutions were tested at various concentrations in 3 groups of hearts. Increase of perfusion pressure is plotted as a function of hemoglobin concentration on a log scale. Analysis of variance for repeated measures indicates significantly increased perfusion pressure with increasing Hb concentration in each group, with a significant interaction between groups and concentration (i.e. the slopes differ). All groups differed significantly from each other in the increase of perfusion pressure at 160 mg/dl Hb. Values are means  $\pm$  SEM for: lysate (n = 14), Hb-A<sub>0</sub> (n = 7), DBBF-Hb (n = 5).

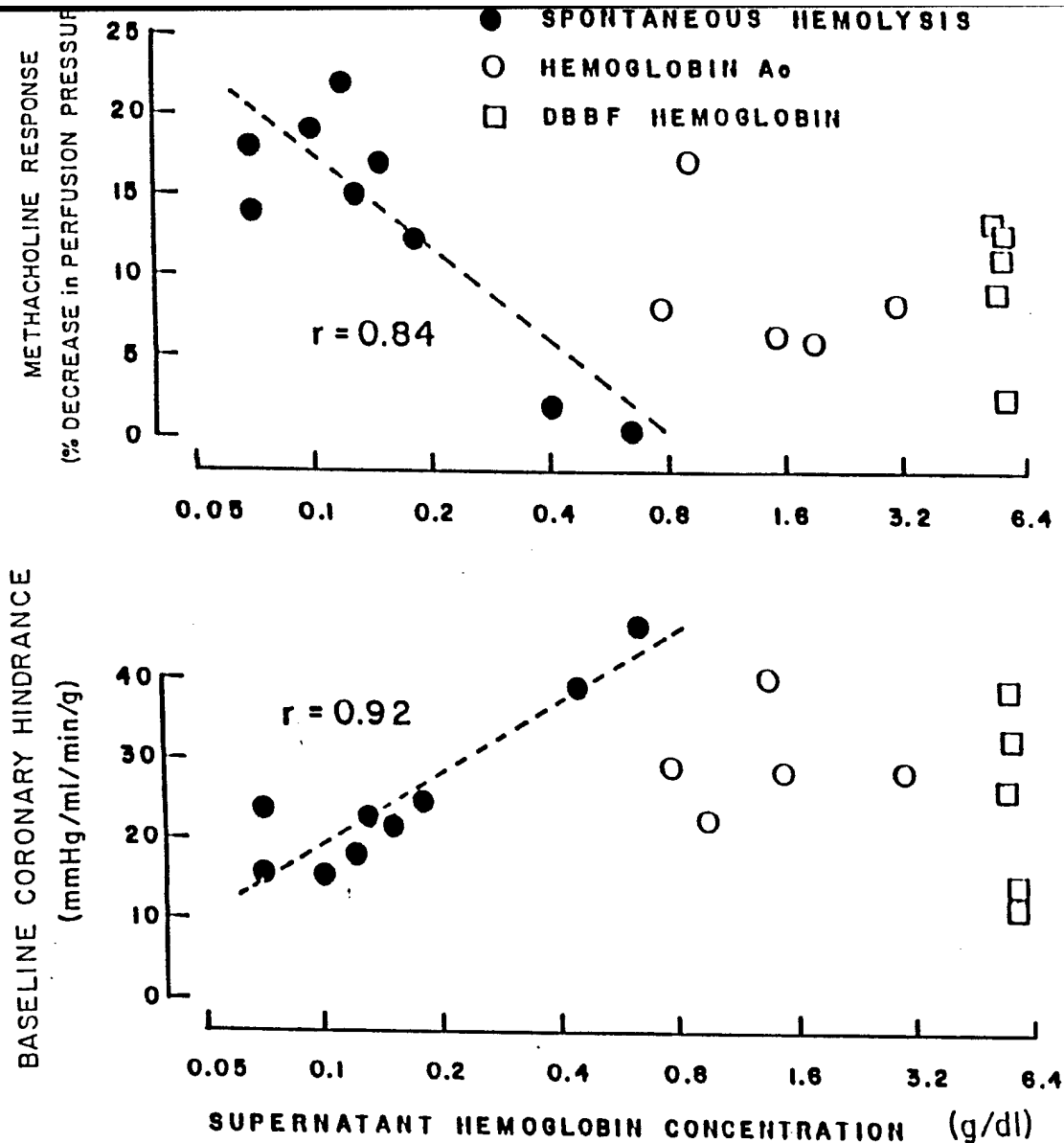


Figure 2. Methacholine responses and baseline coronary hindrance. Dilator responses to methacholine (upper panel) and baseline coronary hindrance (lower panel) are shown in relation to perfusate supernatant hemoglobin concentration on a log scale. Dashed lines indicate linear regression relationships in 9 red-cell-perfused hearts with supernatant Hb from spontaneous hemolysis. When supernatant hemoglobin was increased by addition of Hb-A<sub>0</sub> to the red-cell perfusate or by perfusion with DBBF-Hb without red cells, methacholine responses were significantly greater and baseline hindrance was significantly less relative to hemoglobin concentration than in the spontaneous hemolysis group by analysis of covariance (i.e. the values for Hb A<sub>0</sub> and DBBF-Hb fell off the lines established for spontaneous hemolysis).

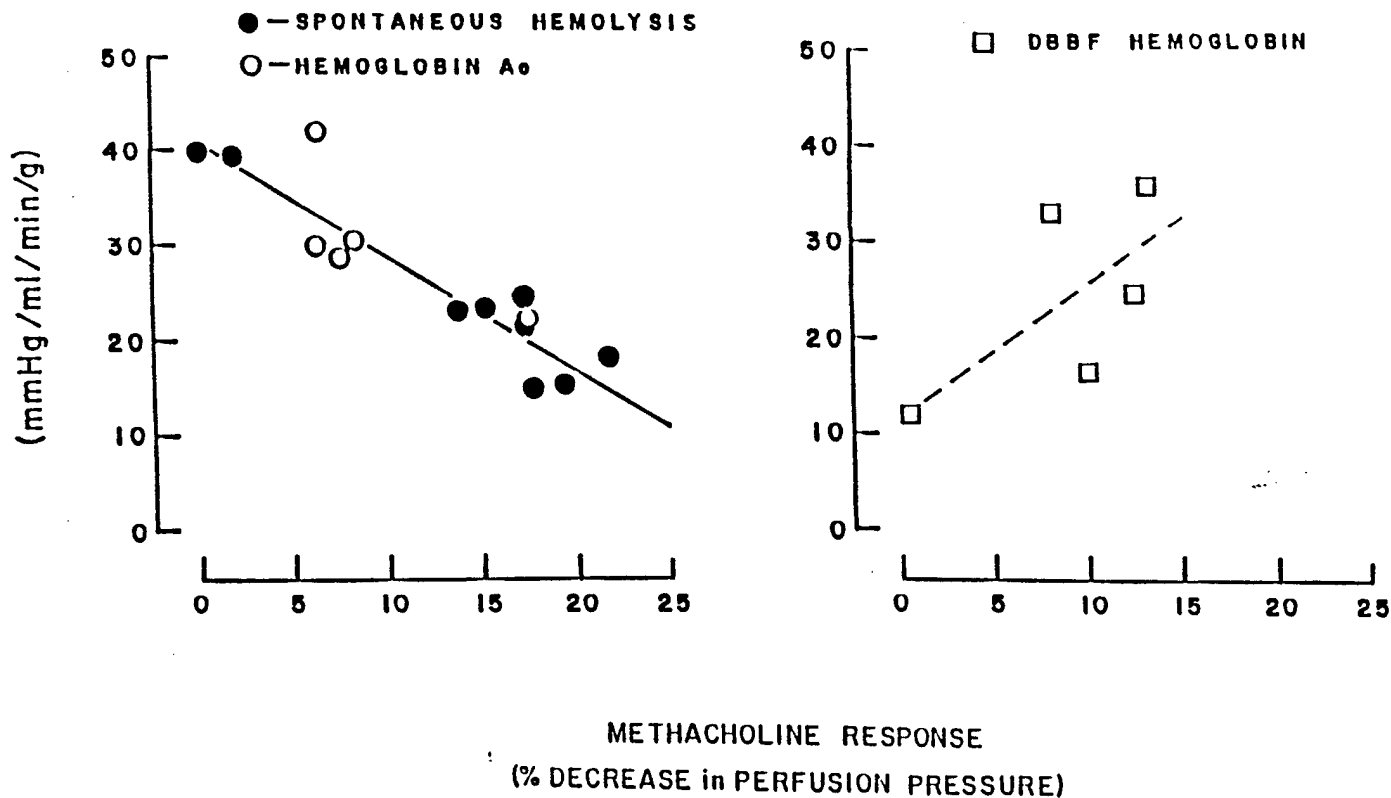


Figure 3. Coronary hindrance as a function of methacholine response. The left panel shows baseline hindrance and dilator response to intracoronary methacholine (250 ng bolus) in 9 red-cell-perfused hearts with supernatant hemoglobin (Hb) from spontaneous hemolysis (0.05 to 0.6 g/dl) in 5 red-cell-perfused hearts with supernatant Hb increased to  $1.7 \pm 0.4$  g/dl by infusion of Hb A<sub>o</sub>. The right panel shows values for hearts perfused with 5.5 g/dl DBBF-Hb without red cells. In red-cell-perfused hearts hindrance was inversely related to methacholine response ( $r = -0.93$ ); an opposite linear relation obtained for hearts perfused with DBBF-Hb without red cells ( $r = 0.65$ ).