EFFECTS OF STROMA-FREE HEMOGLOBIN SOLUTIONS ON PULMONARY VASCULAR RESISTANCE AND MEDIATOR RELEASE IN THE ISOLATED PERFUSED RABBIT LUNG

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ABSTRACT—The aim of this study was to evaluate the effect of an ultrapure bovine stroma-free hemoglobin (SFH) on pulmonary vascular resistance and mediator release and to analyze potential mechanisms of action in the isolated perfused rabbit lung model. Repetitive bolus applications of small amounts of bovine SFH were examined which resulted in a reproducible acute increase of pulmonary vascular resistance of approx. 9 mmHg (controls, n = 6). It was tested whether the platelet-activating factor (PAF) antagonist WEB 2086 (50 μM; n = 6), the cyclooxygenase blocker diclofenac (10 μg/ml; n = 6), the iron-chelating agent deferoxamine (500 μg/ml, n = 6) and the radical scavenger catalase (5000 U/ml; n = 6) exert a protective effect on vasoconstrictor response to SFH. The pressure increase was completely suppressed in the lungs pretreated with WEB 2086, whereas diclofenac, deferoxamine and catalase failed to inhibit the vasoconstriction due to SFH. No significant differences in either TXB_2 generation or in histamine release were found in the WEB 2086 group compared with untreated lungs. The results point towards the crucial role of PAF in mediation of vasoconstrictor side effects due to SFH.

INTRODUCTION

Growing interest has been focused on the development of oxygen carrying fluids as blood substitutes to avoid serious complications of homologous blood transfusion and to guarantee an unlimited availability. Stroma-free hemoglobin solutions, in particular crosslinked bovine hemoglobin and liposome-encapsulated hemoglobin, represent significant research efforts in this direction (1–3). Until now, the purification of hemoglobin from phospholipids and endotoxins and the crosslinking of the molecule with elimination of hemoglobin dimers seem to be the major determinants of the toxicity of hemoglobin solutions (4–6). However, despite high degrees of purification, complete elimination of toxic side effects could not be achieved (7).

Although a great number of *in vivo* and *in vitro* studies dealing with various hemoglobin preparations exists, the mechanisms associated with serious cardiovascular and humoral side effects following administration of such hemoglobin solutions are still not clarified. An important concern has become the vasoconstrictor effects observed in the systemic as well as in the coronary (8, 9) and cerebral circulation (10–12). Since the frequently described vasoactive potential of free hemoglobin solutions is controversially discussed in literature, no definite conclusion can be drawn. It is still unclear to what extent vasoconstrictor potency is due to the hemoglobin molecule itself or to other constituents of the hemoglobin preparations. In order to elucidate the complex pathophysiological reactions to stroma free hemoglobin solutions, this study was designed to investigate selectively the effects of an ultrapure, polymerized bovine hemoglobin preparation on pulmonary vascular resistance and mediator release and to evaluate possible underlying mechanisms. The experiments were performed in the isolated perfused rabbit lung model, using the PAF-antagonist WEB 2086, the radical scavenger enzyme catalase and the cyclooxygenase-blocker diclofenac, to study a potential involvement of PAF, eicosanoids or toxic oxygen radicals in observed vasoconstriction. Furthermore, the effect of the iron-chelating agent deferoxamine and the correlation with methemoglobin concentrations were tested. In additional pilot experiments, freshly prepared human, rabbit and bovine SFH were used to exclude species or product dependent effects.

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MATERIALS AND METHODS

The lung model

The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail (13, 14). Standard breed rabbits of either sex weighing 3150 ± 186 g (mean ± S.D.) were anesthetized with pentobarbital sodium (60–80 mg/kg) and anticoagulated with heparin-sodium 1000 IE/kg body weight. The isolated lungs were suspended from an electronic weight balance (Hottinger, Baldwin Meβtechnik Type U1, Darmstadt) in a temperature controlled (37°C) and humidified chamber, and were perfused with cell- and plasma-free Krebs-Henseleit-hydroxyethyl-starch buffer solution (KHHB) at a constant flow of 200 ml/min in a recirculating system (circulating volume 200 ml). Ventilation was performed with 4% CO_2 in air (frequency 25/min, tidal volume 30 ml, positive endexpiratory pressure 0.5–1.0 cm H_2O). The pulmonary arterial pressure (PAP), airway pressure (AP) and weight of the isolated lung were recorded continuously by means of pressure and weight transducers. Due to a constant perfusion flow, alterations of perfusion pressure directly reflect alterations of pulmonary vascular resistance. Intermittently samples of perfusate were taken for measurements of pO_2, pCO_2, O_2-saturation (ABL 330, Radi-

ometer Copenhagen), oncotic pressure (Onkometer BMT 921, Dr. Karl Thomae GmbH, Biberach) as well as for determination of histamine, thromboxane A_2 and prostacyclin concentrations. Initially the lungs were perfused with KHHB-solution, using low flow rates in the opened circulatory system to remove remaining blood in the vascular bed. The perfusion fluid was then exchanged for fresh buffer via two separate perfusion circuits two minutes after the beginning of the extracorporeal circulation and 30 min later, after the flow was increased to 200 ml/min. The KHHB perfusion was able to maintain the integrity of the microcirculation for more than five hours in our model, verified by ultrastructural studies. Homogenous capillary organ perfusion and absence of structural endothelial damage (e.g. vacuolization, mitochondrial disintegration or hydropic swelling of endothelial cells) could be confirmed by light- and electronmicroscopical controls. No relevant alterations in vascular tone (< ± 2 mmHg), permeability (weight increase <1.5 g) or mediator (histamine, thromboxane) release occurred during this observation period. Entry criteria for acceptance in the present study was that the lungs had a homogenous white appearance without signs of hemostasis or edema formation (weight gain 0 g/min), and without changes in vascular resistance (≤ ± 1 mmHg) during the 30 min equilibration period.

Experimental protocol

30 lung preparations were randomly assigned to five groups of six each. Following a 30 min equilibration period, the first perfusate sample was drawn for measurements of baseline values. Thereafter, ultrapure bovine SFH was applied repetitively, as a bolus of 1 ml yielding in a final concentration of 0.055 g/dl in the perfusion fluid. Subsequently samples of perfusate were taken at 5 and 15 min post SFH injection to investigate mediator release and vascular reactions. Perfusion with SFH was maintained for 15 min. Subsequently, the perfusion fluid was completely exchanged for fresh KHHB solution followed by a 20 min equilibration period in which PAP returned to a stable baseline level before the renewed SFH application. The first SFH administration was identical in all groups, whereas the second dose was given in the presence or absence of inhibitor substances. Six preparations in which SFH was given three times in absence of any inhibitor served as controls. The same protocol was carried out in experiments, in which additionally either WEB 2086 (50 μM, n = 6) or diclofenac (10 μg/ml, n = 6) was given into the perfusate 10 min. prior to the second injection of SFH. In further experimental set-ups, the SFH induced vasoactive effects were examined in the presence of catalase (5000 U/ml, n = 6) or deferoxamine (500 μg/ml, n = 6) respectively.

Additional pilot experiments, using the same protocol as described, were performed, however, freshly prepared rabbit (n = 3), human (n = 3), or bovine stroma-free hemoglobin (n = 3) as well as methemoglobin prepared from rabbit blood (n = 3) was injected into the pulmonary artery instead of the ultrapure bovine SFH. To exclude effects due to our artificial perfusion fluid, SFH application was also tested when 30 ml autologous blood (n = 3) or equivalent amounts of autologous plasma (n = 3) were added to the perfusate.

Radioimmunoassay of TXB_2 and 6-keto $PGF_{1\alpha}$

Thromboxane B_2 (TXB_2) and 6-keto-$PGF_{1\alpha}$ were assayed serologically from 100 μl of recirculating KHHB as stable hydrolysis products of thromboxane A_2 and prostacyclin by radioimmunoassay according to a method described by Peskar (15). Radioactivity was quantified with a Philips PW 4700 liquid scintillation counter. Results were obtained by standard constructed dose-response curves. The cross-reactivity of TXB_2 antiserum with prostaglandin D_2 was 2.7%, and 0.05% with 6-keto-$PGF_{1\alpha}$, PGE_2, PGE_1, $PGF_{1\alpha}$, 13,14-dihydro-15-keto PGE_2, and 13,14-dihydro-15-keto $PGE_{2\alpha}$, respectively. The cross-reactivity of 6-keto-$PGF_{1\alpha}$ antiserum was 0.05% with TXB_2 and the above mentioned prostaglandins.

Radioimmunoassay of histamine

Histamine was measured by means of a commercially available solid phase radioimmunoassay test kit (see Materials). The acylated histamine in the sample and the acylated [^{125}I]histamine compete for the limited monoclonal antibody bindingsides, which were coated on the surface of the test tubes. Cross-reactivities of the monoclonal antibodies used in the test kit with the following substances were determined: acylated histamine (100%), acylated *t*-methylhistamine (69 × 10^{-4}%), acylated histidine (4 × 10^{-4}%), acylated serotonin (<10^{-4}%), cimetidine (6 × 10^{-4}%), ranitidine (<10^{-4}%), histamine (5 × 10^{-4}%).

Materials

A cell- and plasma-free perfusion medium was used in the present study in order to avoid the complex interactions with different circulating cells, which may mask direct effects of the added hemoglobin solutions on the vessel wall. The perfusate consisted of a Krebs-Henseleit buffer solution with additional poly(0–2-hydroxyethyl)-starch (Haessteril 10%, Fresenius AG) in order to maintain a colloid oncotic pressure between 23–25 mmHg yielding in final concentrations of: starch 50 g/liter; Na^+ 138 mmol/liter; K^+ 4.5 mmol/liter; Mg^{2+} 1.33 mmol/liter; Cl^- 135 mmol/liter; Ca^{2+} 2.38 mmol/liter; glucose 12 mmol/liter; HCO_3^- 12 mmol/liter. The osmolality was approximately 330 mosm/kg (Mikro-Osmometer, Roebling Meßtechnik, Berlin). The pH of the buffer solution was adjusted to 7.4 with 1 M $NaHCO_3$. Effects due to endotoxin contamination of the plasma-free perfusate can be excluded in our model as assessed in previous experiments. No hemodynamic reactions, thromboxane generation or histamine release were observed following endotoxin addition to the perfusate in the absence of plasma complement components.

Stroma-free hemoglobin solutions

A commercially available bovine ultrapure stroma-free hemoglobin solution was preferred for the comparative inhibitor studies because of its standardized preparation and purification with the following specifications: hemoglobin 11 ± 2 g/dl (incudes hemoglobin and methemoglobin), methemoglobin < 10%, pH 7.8 ± 0.4, osmolarity 290 ± 10 mosm/kg, sodium 145 ± 10 mmol/liter, chloride 140 ± 10 mmol/liter, potassium 4.0 ± 1.0 mmol/liter, endotoxin < .05 EU/ml (limulus amebocyte lysate chromogenic assay), free glutaraldehyde < 10 ng/ml, polymerized hemoglobin with MW > 500,000: ≤ 10% of total protein, polymerized hemoglobins with MW ≤ 68,000: ≤ 50% of total protein.

Human hemoglobin was obtained from freshly extracted heparinized blood from healthy donors. After centrifugation at 1500 g for 10 min, the red blood cells were concentrated after removal of plasma and buffy coat, resuspended, and again centrifuged and washed three times with sterile physiological saline. Subsequently the red cells were lysed by addition of sterile destilled water and treatment with ultrasonics (Labsonic 2000, B. Braun, Melsungen, Germany) for 5 min. Stromal and other solid fragments were removed by centrifugation (2000 G for 15 min) and filtration through sterile gauze. The supernatant was diluted with KHHB to achieve hemoglobin concentrations between 9–13 g/dl (OSM2, Radiometer, Copenhagen, Denmark), equivalent to the used ultrapure bovine SFH. 1 ml of the prepared lysate was injected into the arterial line as described in the experimental protocol. Methemoglobin content was < 5% of total hemoglobin.

Rabbit hemoglobin solution was prepared from freshly drawn autologous rabbit blood as described above.

Bovine blood was extracted for the preparation of unmodified free *bovine* hemoglobin solution. To produce methemoglobin, 1 ml of $K_3Fe(CN)_6$ (Merck, Darmstadt, Germany) solution (1 g/dl) was added to 20 ml hemoglobin solution.

The selective PAF-antagonist WEB 2086 ($C_{22}H_{22}ClN_8O_2S$, MW 455.97) was a generous gift from Boehringer Ingelheim (Ingelheim, Germany). The high potency of WEB 2086 was assessed in *in vitro* experiments (for review see Ref. 16). The molar concentrations ratio of agonist (PAF) to antagonist (WEB 2086) was very close to 1, suggesting an affinity to PAF binding sites similar to that of PAF itself (17). Since we are not able to measure PAF concentrations in the perfusion fluid, a higher WEB 2086 dose (50 μM approx. 1.52 mg/kg body weight respectively) was chosen above the upper range of those doses confirmed to be effective in various animal models, e.g. guinea pigs, rats (0.01–0.5 mg/kg i.v.) (18, 19).

Diclofenac sodium (Voltaren) and Deferoxaminmesilat (Desferal) were purchased from Ciba-Geigy (Wehr, Germany). Catalase from bovine liver from Sigma Chemicals (St Louis, USA). Based on dose response studies, catalase was given in a concentration which has been shown to completely inhibit free radical generation in our model (unpublished data). In order to make sure that the concentration of deferoxamine in the perfusion fluid was sufficient to inhibit free radical generation, the substance was used in a dose exceeding the approved concentrations. With regards to the iron chelating capacity on molar basis, the applied dose of deferoxamine was 30 times higher than the iron supplied with the hemoglobin solution.

Rabbit anti-TXB_2 and rabbit anti-6-keto-$PGF_{1\alpha}$ were purchased from Paesel (Frankfurt, Germany), ^{3}H-labeled TXB_2 and ^{3}H-labeled-6-keto-$PGF_{1\alpha}$ from New England Nuclear (Dreieich, Germany), precipitating goat antirabbit antibodies from Calbiochem-Behring (Frankfurt, Germany). A radioimmunoassay

test kit from Immunotech (Hermann Biermann GmbH, Bad Nauheim, Germany) was used for the determination of histamine.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Differences between groups were tested by one-way analysis of variance (ANOVA) followed by a Scheffe's multiple comparison procedure. Significance was accepted at $p < .05$.

This study was approved by the Animal Subject Protection Committee of the University of Giessen. The care and handling of animals were in accordance with the NIH guidelines for the use of experimental animals.

RESULTS

Baseline values of PAP between 7.8 and 9.1 mmHg and airway pressure between 4 and 5.5 mmHg were similar in all groups and in agreement with previous studies reported by our group (14). The baseline PAP was considered as zero in each lung for comparisons of further changes in PAP due to SFH application. Thus, the following values express changes from baseline. The results are summarized in Table 1, showing an acute pressure increase between 5 and 9 mmHg at 5 min following the first SFH injection in all groups. The sustained increase in pulmonary vascular resistance was interrupted after 15 min by washing out the SFH and exchanging the whole perfusion fluid for fresh buffer (CP). After another 20 min equilibration period (time point 40 min), approximate baseline levels were achieved. The acute vasoconstrictor response was reproducible or even enhanced after repetitive SFH injection in the controls. In contrast to this, the pressure increase in the lungs pretreated with the PAF antagonist WEB 2086 was completely suppressed ($p < .0001$), whereas the response to SFH in absence of WEB 2086 (3. SFH injection after 80 min) was similar to the controls. Fig. 1 represents original tracings of PAP showing the time course of the constrictor effects in control (*A*) and WEB 2086-treated (*B*) lungs.

To determine the role of eicosanoids in mediating the described reaction, the cyclooxygenase blocker diclofenac was added to the perfusate 10 min prior to SFH. However, the applied dose was shown to completely suppress TXB_2 generation as assessed by undetectable amounts in the radioimmunoassay, but pressure response to SFH was not inhibited. Only a slight

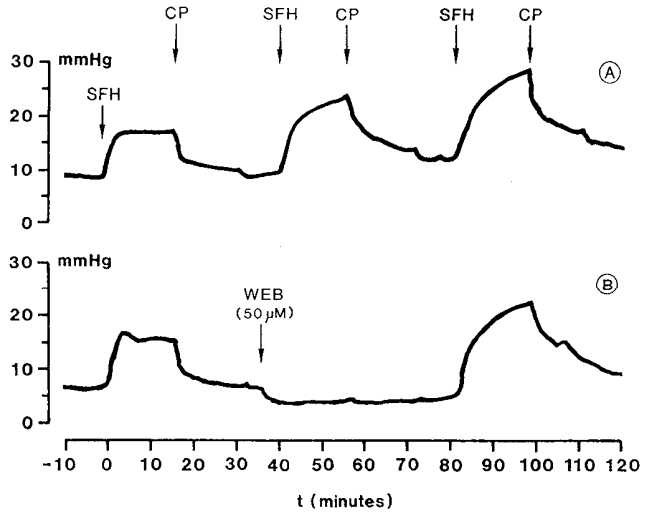

FIG. 1. **Effect of repetitive bolus application of 1 ml of SFH on pulmonary arterial pressure.** *A*, representative tracing of a control lung. SFH-induced pressure increase was reproducible after change of perfusate (*CP*) and renewed SFH-injection. *B*, the same protocol was used but WEB 2086 (50 µM) was injected 10 min prior to the second SFH application. WEB 2086 completely prevented the pressure reaction due to SFH, whereas the third SFH injection in absence of WEB 2086 (after CP) evoked a similar vasoconstrictor response as in control.

but not significant attenuation of the pressure reaction ($p = .057$), compared to controls, could be observed in the diclofenac treated lungs.

The potential toxicity of oxygen radicals, generated by autoxidation of hemoglobin, was tested by application of the scavenger enzyme catalase 10 min before SFH injection. Catalase, however, failed to protect the lungs from the increase in vascular resistance due to SFH. Furthermore, the influence of the iron-chelating agent deferoxamine on SFH-induced pressure response was examined, but preadministration of deferoxamine did not prevent the increase in PAP.

Comparing all groups, the inhibition of the pressure response, which differed significantly ($p < .0001$) from all other groups, could be exclusively demonstrated in the WEB 2086 treated

TABLE 1. **Changes in pulmonary arterial pressure from baseline in the different groups**

		mmHg				
Intervention	Time (min)	Controls (n = 6)	WEB 2086 (n = 6)	Diclofenac (n = 6)	Catalase (n = 6)	Deferoxamine (n = 6)
1) SFH	0	0	0	0	0	0
	5	6.8 ± 2.9	9.1 ± 3.2	7.2 ± 4.0	5.6 ± 1.2	8.7 ± 4.1
	15	6.3 ± 2.7	9.2 ± 4.4	8.7 ± 6.7	5.8 ± 1.5	9.2 ± 6.0
CP	20	1.9 ± 1.1	1.8 ± 1.9	2.8 ± 3.9	1.1 ± 1.2	1.4 ± 1.4
Inhibitors	30	0.8 ± 0.6	0.1 ± 1.2	1.6 ± 2.8	0.1 ± 0.3	0.2 ± 0.9
2) SFH	40	0.4 ± 0.5	−1.9 ± 0.9	0.5 ± 1.5	0.2 ± 0.3	0.5 ± 1.3
	45	8.8 ± 2.7	−1.2 ± 1.2*	6.2 ± 4.0	5.4 ± 1.5	9.8 ± 5.4
	55	12.2 ± 4.5	0.1 ± 2.4*	7.8 ± 4.7	7.2 ± 3.2	16.4 ± 8.1
CP	60	5.3 ± 1.5	−0.7 ± 2.3	2.6 ± 3.1	2.4 ± 2.3	8.5 ± 3.9
3) SFH	80	1.8 ± 1.2	−0.4 ± 0.9	0.2 ± 1.1	2.4 ± 1.9	7.7 ± 2.9
	85	10.7 ± 2.9	6.9 ± 4.4	4.3 ± 2.4	8.2 ± 3.9	12.9 ± 5.2
	95	16.2 ± 3.9	11.6 ± 6.0	6.1 ± 2.6	12.7 ± 4.5	14.2 ± 6.28

SFH was given three times: 1) SFH in absence of inhibitors; 2) SFH after change of perfusate (CP) and pretreatment with inhibitors (except controls); 3) SFH after CP and again without inhibitors.
* $p < .0001$ versus all other groups (ANOVA).

lungs. There were no significant differences by analysis of variance among catalase, deferoxamine and diclofenac treated and control lungs with respect to the constrictor effect of SFH. Permeability in terms of weight increase as monitored in our model, was not essentially influenced during the observation period. Mean maximal weight increase at the end of the experiment at time point 100 min was between 0.5 and 2.3 g from baseline.

Analysis of perfusate samples revealed an immediate increase in circulating histamine and a slight increase in TXB_2 following the first injection of SFH in the controls (Fig. 2). Baseline values were reobtained after change of perfusion fluid. The repeated SFH injections evoked no further increase in the histamine concentration. This pattern of histamine release due to SFH was not significantly altered in the WEB treated lungs. The slight increase in TXB_2 and 6-keto-$PGF_{1\alpha}$ (data not shown), detectable 15 min post SFH, was reproducible after each SFH injection and was almost identical in control and WEB 2086 group. Although preadministration of WEB 2086 was able to suppress the pressure reaction due to SFH, no inhibitory effect on the mediator release could be found. Oxygen saturation, pO_2 and pCO_2 did not significantly change throughout the observation period.

In pilot experiments, the effect of SFH was examined in the presence of blood and plasma components and compared to the cell and plasma-free KHHB perfusion. Similar increases in PAP were obtained in those lungs in which either 30 ml rabbit blood or plasma was added to the perfusate. To exclude possible species specific effects or reactions dependent on the methemoglobin content, freshly prepared rabbit, bovine, and human hemoglobin as well as methemoglobin solutions (methemoglobin content >99% of total hemoglobin) were investigated in additional experiments. Despite varying methemoglobin content, a similar vasoconstrictor response in terms of pressure increase could be demonstrated after administration of rabbit (6–9.8 mmHg), human (4.2–5.2 mmHg), bovine (4.3–7.6 mmHg) SFH, and methemoglobin (7.1–7.6 mmHg) in our model.

DISCUSSION

Growing interest is focused on the development of blood substitutes to resolve the problem of availability and to reduce disease transmission associated with the use of human blood. Although purified stroma-free hemoglobin solutions represent one promising research effort, several effects still remain unclear including the vasoactive potential observed in *in vitro* and *in vivo* studies. The aim of this study was to evaluate the effect of an ultrapure hemoglobin solution on pulmonary resistance and mediator release and to analyze potential mechanisms of action. For this purpose experiments were performed in the iso-

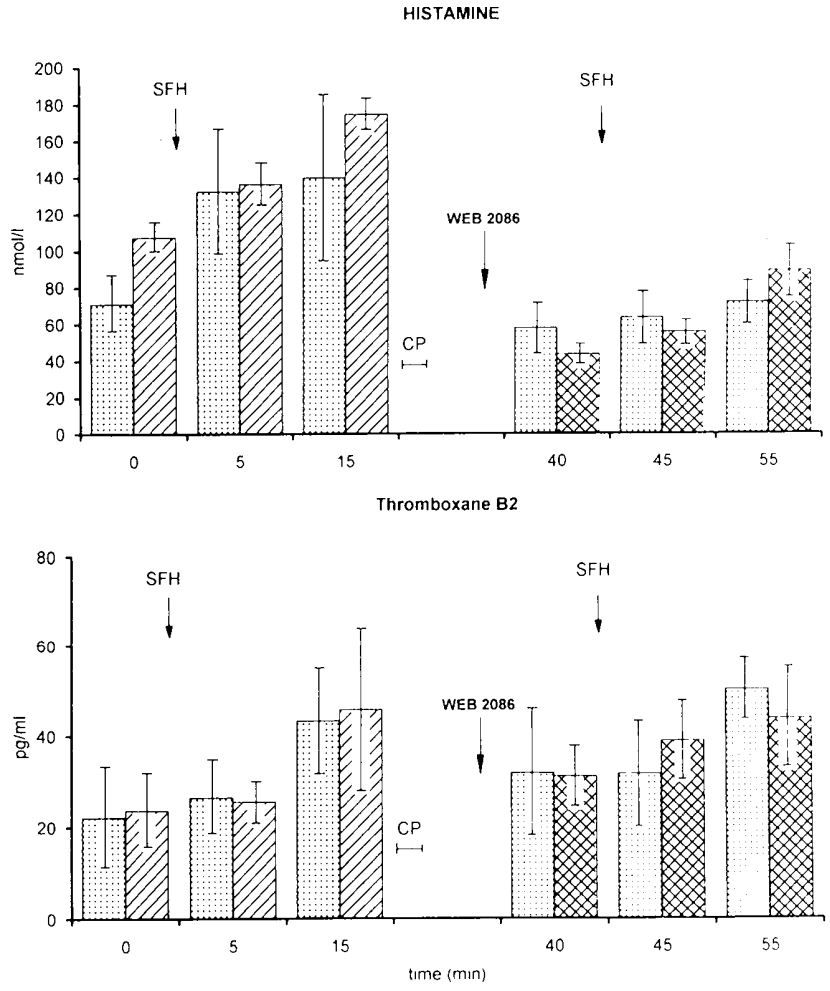

FIG. 2. **Histamine release and TXB_2 generation in control (n = 6) and WEB 2086 treated group (n = 6).** The time points 0 and 40 represent baseline values before SFH injection. Mean values ± S.E. are presented 5 and 15 min following the first SFH application without inhibitor, and after the second SFH injection (45 and 55 min) in controls and after pretreatment with WEB 2086 (50 μM). Histamine concentrations increased similarly after the first SFH injection in both groups, whereas no further increase occurred after the second application. TXB_2 concentration increased slightly after the first and second SFH injection in both groups. The pattern of either histamine release or TXB_2 generation did not differ significantly between control and WEB 2086 group.

lated rabbit lung, which allows the investigation of basic pathomechanisms involved in such complex reactions and the elucidation of the multiple interactions between blood components, endothelium and organ tissue.

Taken together, the findings of the present study provide evidence that stroma-free hemoglobin evokes a significant increase in pulmonary vascular resistance when injected intravenously, independently of the originating species and of the purification method. Our results are supported by previous *in vivo* and *in vitro* studies that indicated vasoconstrictor activity induced by various modified hemoglobin solutions. Gilroy et al. (20) demonstrated vasoconstrictor activity of stroma-free and polymerized pyridoxilated hemoglobin solutions in a rabbit ear artery preparation. Using a primate model, Moss et al. (21) showed that infusion of stroma-free hemoglobin tended to elevate blood pressure without any change in cardiac output or heart rate. Furthermore, coronary (8, 9) and cerebral vasoconstriction (e. g. after subarachnoid hemorrhage) caused by human hemoglobin has been reported in previous *in vitro* and *in vivo* studies (11, 12, 22, 23). In the current experiments, a similar vasoconstrictor potential even after small doses of various free hemoglobin preparations such as bovine, rabbit and freshly prepared human hemoglobin could be demonstrated. The use of the ultrapure preparation in our study implicate that the vasoconstrictor effect cannot be attributed to insufficient purification, but it seems likely to be due to hemoglobin itself. Consistent with this concept, Macdonald et al. (6) found that a minor, non dose dependent, vasoconstrictor activity remains in the isolated perfused rabbit heart after purification of human stroma-free hemoglobin solution to a single hemoglobin component. In contrast to this, Rabinovici (24) did not observe cardiovascular, hematological and humoral side effects following free hemoglobin infusion but after injection of liposome-encapsulated hemoglobin (LEH). LEH application evoked transient biological responses, such as hypertension, tachycardia, thrombocytopenia hemoconcentration and elevation of plasma TXA_2 in conscious rats. He suggested that the hemoglobin/phospholipid interactions might account for the side effects, possibly through interactions with blood elements and the resultant production of PAF and TXA_2. In order to test this humoral hypothesis, he pretreated rats with the selective PAF antagonist BN 50739 and subjected the animals to LEH administration while monitoring cardiovascular, hematological and biochemical indices. In agreement with our results, the PAF-antagonist BN 50739 (10 mg/kg) injected 30 min prior to LEH application, completely prevented the hemodynamic reactions and hemoconcentration. The reasons for these deviating results after application of free hemoglobin in comparison with LEH remain obscure.

In order to clarify the underlying mechanisms of the complex pathophysiological response to stroma-free hemoglobin solutions, different inhibitor substances were tested, including the PAF antagonist WEB 2086. To determine the role of eicosanoids in mediating the described reactions, the cyclooxygenase blocker diclofenac was injected prior to hemoglobin solution. Although the applied dose completely suppressed TXA_2 and prostacyclin generation (undetectable amounts of their stable metabolites in the radioimmunoassay), the increase in pulmonary resistance, however, was not inhibited. In comparison with controls (without inhibitors), only an insignificant small reduction of the pressure response could be noticed. These results are in accordance with those reported by Vogel (8) on the buffer perfused isolated rabbit heart. Likewise, addition of ibuprofen to the perfusion fluid had no effect on the vasoconstrictor response to human hemoglobin. Otherwise, Toda et al. (12) provided evidence that cerebroarterial constriction elicited by oxyhemoglobin in monkeys and dogs *in vivo* and *in vitro* appear to result from the release of vasoconstrictor prostaglandins, but not TXA_2. Different sensitivity and reactions to SFH, due to the highly differentiated coronary, cerebral or pulmonary vascular beds, may explain the partially conflicting results.

Another source of toxicity of hemoglobin solutions represent the tendency of hemoglobin to autoxidate and generate oxygen free radicals (25–27). It was feared that without the protective erythrocytic environment, hemoglobin, which is dissolved into the circulating plasma, would undergo easy and unimpeded oxidation and become a continuous source of oxygen free radicals. To test this hypothesis, the scavenger enzyme catalase was given before hemoglobin application, but without significant results. Likewise, superoxide dismutase plus catalase did not prevent cerebral vasospasm caused by oxyhemoglobin in monkeys (28). Another pathway of radical formation is represented by the iron in the hemoglobin molecule. If traces of Fe^{2+} are freed, superoxide and H_2O_2 can react together to produce the highly toxic hydroxyl radical ($OH\cdot$) (Haber-Weiss reaction), which causes lipid peroxidation of the cell membranes and leads to disruption of organelle membranes, release of lysosomal enzymes and perpetuation of injury (29, 30). It has been proposed that the iron-chelating agent, deferoxamine, may abridge this series of events (31). In fact, we found that deferoxamine did not afford protection against SFH-induced unfavorable effects. The lack of any protective effect of either catalase or deferoxamine suggests, however, that the autoxidation of hemoglobin is not a major problem leading to vascular reaction in our cell and plasma-free perfused lung model, even in the absence of protective systems against autoxidation in the plasma. To find out whether free hemoglobin evokes its vasoconstrictor response also in the presence of the endogenous inhibitor and scavenger systems of blood and plasma, rabbit blood or plasma was added to the perfusate prior to injection of SFH. Since a similar response on pulmonary resistance was detectable, a possible artificial effect dependent on the experimental setting could be excluded. This was also confirmed by Vogel (8), who observed equivalent effects due to rabbit and human SFH in isolated rabbit hearts, perfused with either Krebs-Henseleit buffer or whole blood. These findings as well as the results of the experiments in which methemoglobin was tested support the thesis that free radical generation is not mainly responsible for the vasoconstrictor effect. In other studies (5, 32), however, evidence is provided that oxygen free radicals are involved in the pathogenesis of systemic and cerebrovascular toxicity. On the one hand, the different results might be explained by the use of differently prepared hemoglobin solutions with possibly varying content of endotoxins or phospholipids increasing O_2-radical production. On the other hand, they might be explained

by the complex response evoked in the animal by systemic application, as compared to the selective reaction in the isolated lung model.

Since pretreatment with the selective PAF-antagonist WEB 2086 completely inhibited the vasoconstrictor response to bovine hemoglobin solution, it can be presumed that the generation of PAF plays an important role in mediating the observed side effects. WEB 2086, however, did not protect against SFH-induced histamine release. Hence, these responses are probably not due to PAF formation. The lack of TXA_2 involvement in mediating increased pulmonary vascular resistance, as assessed by almost unchanged TXB_2 levels as well as the failure to attenuate the vasoconstrictor response by diclofenac, points to a direct effect of PAF on the vascular reaction or other mechanisms not examined. Imbalances between constrictor and dilatator mediators regulating vascular tone, in particular an interference of SFH solutions with the vasodilator substance nitric oxide (NO) seems to be of significance. Since NO is supposed to play an important role as a physiological vasodilator in conductance and resistance vessels, consideration on how NO may be inactivated in the circulation is given in recent studies (for review see Ref. 33). It has been suggested that NO liberated from endothelium is inactivated by hemoglobin (HbO_2) via conversion to methemoglobin and nitrate. *In vitro* studies indicated that the ferrous heme of the hemoglobin act as electron donor to be converted to ferric heme in this reaction (34). With regards to the hemoglobin induced vasoconstriction Martin et al. 1986 (35) reported that bovine hemoglobin induced coronary vasoconstriction indirectly by interfering with vasodilation, associated with spontaneously released endothelium-derived relaxing factor NO, possibly by interacting with guanylate cyclase activity in vascular smooth muscle cells (36). Accordingly, hemoglobin-induced endothelium-dependent inhibition of vascular smooth muscle relaxation by binding NO has also been hypothesized to participate in the pathogenesis of cerebral vasospasm (10). In contrast to this Toda et al. (37) concluded from their data that hemoglobin-induced cerebral vasoconstriction occurs independent of the inhibition of spontaneously released NO. Likewise Nakagomi (38) demonstrated an unaltered increase in vascular pressure after removal of the endothelium which is thought to be the major source of NO. Moreover, Beny et al. (39) showed in the pig coronary artery that hemoglobin causes both endothelium-dependent and endothelium-independent contraction, however independently of an inhibition of NO effects. Contrary to this, recent studies confirmed the hypothesis that hemoglobin inhibits endothelium dependent relaxation in the coronary and cerebral circulation (40, 41). From the current data, no final conclusions can be drawn regarding the pathogenic role of NO in the hemoglobin induced vasoconstriction. Another interesting aspect concerning the pathomechanism of the vasoconstriction seems to be the release of the extremely potent vasoconstrictor endothelin (42, 43). There is accumulating evidence that endothelin-1, may also account for the severe cerebral vasospasm associated with subarachnoid hemorrhage (44, 45). Ohlstein et al. (46) recently found that oxyhemoglobin can directly stimulate endothelin biosynthesis in cultured endothelial cells. Whether endothelin-induced vasoconstriction is caused by direct effects on vascular smooth muscle cells or via PAF and eicosanoid formation is still unclear. Several studies point towards the involvement of thromboxane A_2 and PAF in the pathogenesis of endothelin induced vasoconstriction (47, 48). Barnard et al. (49) demonstrated that endothelin induced an increase in pulmonary arterial pressure in rabbit, dog and rat lungs which was attenuated by cyclooxygenase inhibitor ibuprofen. Similarly pretreatment with PAF-antagonist reduced vascular smooth muscle cell contraction due to endothelin (50). Further investigations on the interactions between endothelium-derived nitric oxide, endothelin, and lipid mediators are warranted to elucidate this complex mechanism.

It should be noted that despite the use of a standardized ultrapure hemoglobin solution a batch-to-batch variability concerning the percentage of methemoglobin, P_{50}, and sterility, has to be considered. Furthermore, potential time dependent processes might alter the composition of the hemoglobin and could result in inconsistent biological responses. Therefore, we investigated methemoglobin, different charges of SFH as well as freshly prepared bovine, human and rabbit hemoglobin. In spite of varying percentages of methemoglobin, no significant correlation could be determined between constrictor activity and methemoglobin content.

In summary, bolus injection of small amounts of SFH resulted in an immediate increase in vascular resistance, independently of originating species of hemoglobin, which could be completely prevented by PAF antagonist. However, the examination of other mechanisms which may account for the vasoconstrictor potential of free hemoglobin preparations, such as the release of TXA_2, generation of oxygen radicals or oxidation of the iron in the hemoglobin molecule, as assessed by pretreatment with specific inhibitors, revealed no major influence on SFH-induced vascular response in this study. The current results point towards the crucial role of PAF in mediating vasoconstrictor side effects in response to application of free hemoglobin. Further studies are required to elucidate the pathophysiological mechanisms leading to the vasoconstrictor potential of stroma-free hemoglobin solutions and to improve the biological compatibility before clinical use.

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