Attenuation of postischemic reperfusion injury in striated skin muscle by diaspirin-cross-linked Hb

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The development of hemoglobin-based oxygen carriers as red blood cell (RBC) substitutes has been hampered by unwanted side effects such as nephrotoxicity, complement activation, and risk of transmission of infectious diseases. These impediments have recently been eliminated through significant technical improvements in the production and purification procedures of stroma-free hemoglobin, on the one hand, and chemical modification procedures of the hemoglobin molecule, on the other hand.

Diaspirin-cross-linked hemoglobin (DCLHb) is a stroma-free hemoglobin solution characterized by an intramolecular cross-link between the $\alpha$-chains (2). DCLHb has been shown to function as an oxygen carrier, particularly under conditions of ischemia-reperfusion (I/R) (3, 13). However, there is concern that hemoglobin-based oxygen carriers could enhance formation of oxygen free radicals, particularly during reperfusion of postischemic tissues (5). One of the potential mechanisms is the release of free iron from the prosthetic heme group, thus promoting the formation of hydroxyl radicals via the Haber-Weiss reaction by acting as a Fenton reagent (24). This would lead to an enhancement of lipid peroxidation and leukocyte-endothelial cell interaction, activation of the inflammatory cascade, release of cytosolic enzymes, and destruction of postischemic tissues (12).

To date, there is no in vivo evidence to suggest that an oxygen-carrying blood substitute is a potential source of free radical-induced leukocyte activation and adhesion associated with reperfusion injury. The aim of this study therefore was to analyze the effects of DCLHb as an oxygen-carrying blood substitute and as a hemoglobin-based therapeutic on the local microcirculation and tissue PO$_2$ after severe ischemia followed by reperfusion.

Materials and Methods

Hemoglobin solution. DCLHb [99$\alpha$-bis(3,5-dibromosalicyl) fumarate hemoglobin, lot no. HBX-A92–268–42793] was provided by Baxter Healthcare (Deerfield, IL). The characteristics of the hemoglobin solution have been described previously (20).

Animal model. Syrian golden hamsters weighing 50–70 g (Charles River, Sulzfeld, Germany) were kept under acclimated conditions with free access to tap water and pellet food ad libitum. Experiments were approved by the local ethics committee and performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (DHHS, revised 1985). The dorsal skinfold chamber in awake Syrian golden hamsters (19) was used for microcirculation and local tissue PO$_2$. The chamber implantation was performed 48–72 h before the experiment under ketamine-xylazine anesthesia (130 and 20 mg/kg body wt i.p., respectively). Polyethylene catheters (Portex, Hythe, UK) were inserted into the jugular vein and carotid artery for monitoring of mean arterial pressure (MAP), heart rate (HR), and arterial blood gases.

Experimental protocol. For measurement of tissue PO$_2$ and intravital microscopic analysis, animals were randomly assigned to three different treatment groups. After a recovery period of at least 48 h, baseline values were recorded. Animals were subjected to a period of 4 h of pressure-induced ischemia, which was applied using an adjustable screw clamp with a transparent silicon pad, as previously described (19).

Animals of group A (n = 7) received 5 ml/kg body wt iv of isotonic saline (0.9% NaCl) 15 min before reperfusion; ani-
mals of group B (n = 8) received the same dose of 6% Dextran 60 (Dx-60; 60 kDa; Schiwa, Germany) as an isoncotic control; and animals of group C (n = 8) were treated with 5 ml/kg body wt. 10% DCLHb. Measurements were made before ischemia and at 0.5, 2, and 24 h of reperfusion.

Tissue oxygen measurement. Measurement of local tissue PO2 in the chambered tissue was performed using a Clark-type oxygen multwire platinum electrode (MDO electrode, Eschweiler, Kiel, Germany) as described by Kessler and Lübbers (see Ref. 11). The electrode was connected to a 15-channel computer-assisted amplifier (MIB, Steindorf, Germany). At the preset time points of investigation, the cover glass of the transparent chamber was gently removed, and the tissue was superfused with isotonic saline solution (Braun, Melsungen, Germany) at room temperature. The MDO electrode was placed on the tissue with a microstep motor as previously described (20). The MDO electrode contains eight single platinum wires in an asymmetric distribution, each measuring individual tissue PO2 values within an approximately spherical volume of 25 µm3. An integrated temperature probe allowed continuous measurement of local tissue temperature for on-line correction of the tissue PO2 values. A period of 5–6 min was needed for collection of 100–120 single-tissue PO2 values, which was achieved by moving the electrode via a step motor. Tissue PO2 was determined within the underlying tissue according to the experimental protocol at 0.5, 2, and 24 h of reperfusion. Tissue PO2 values were grouped in classes of 5 mmHg and plotted vs. percent frequency of occurrence (see Fig. 4). Spatial distribution of tissue PO2 values allows the assessment of variations of tissue PO2, ranging from values of zero to values representing arteriolar tissue PO2 in a highly reproducible manner (16).

Intravital microscopy. Analysis of the microvascular parameters in the striated skin muscle of the hamster was performed with an intravital microscope (Zeiss, Jena, Germany) connected to a computer-controlled stepping motor for exact repeated measurements of the identical vessel segments. For visualization of leukocyte-endothelial cell interactions, leukocytes were stained by intravenous injection of 0.5% rhodamine 6G (0.15 mg/kg body wt, Sigma Chemicals, Deisenhofen, Germany). Animals were injected with 15 ml/kg body wt of an intravenous bolus of FITC-Dx (150 kDa; Pharmacia, Uppsala, Sweden) for analysis of the macromolecular leakage of microvessels and the RBC velocity. Video images were recorded on videotapes and evaluated off-line by a computer-assisted microcirculation analysis system (CapImage, Dr. H. Zeintl, Heidelberg, Germany). Parameters were assessed as follows. 1) Functional capillary density (FCD) was defined as the length of RBC-perfused capillaries in the striated skin muscle per observation area (cm/cm2). 2) Leukocyte-endothelial cell interactions were determined by quantitative analysis of the number of nonadherent leukocytes passing per minute (NAL/min), the fraction of rolling leukocytes (%), and the number of firmly sticking leukocytes per endothelial surface (stickers/mm2). The rolling fraction was calculated using the formula:

\[
\text{rolling fraction} = \frac{\text{rolling cells}}{\text{rolling cells} + \text{NAL}} \times 100\%
\]

where rolling cells were defined as the number of leukocytes intermittently attaching to the vessel wall of postcapillary venules (rollers/min). 3) RBC velocity was measured in capillaries and in postcapillary venules (mm/s). 4) Through extravasation of the fluorescence marker FITC-Dx, a semi-quantitative analysis of permeability changes of postcapillary venules was assessed by calculation of the quotient of extravasal vs. intravascular fluorescence intensity.

Histomorphology. For electron microscopy tissue sections were preserved by superfusion and simultaneous intra-arterial perfusion of the animals with Karnovsky's solution (paraformaldehyde + 25% glutaraldehyde + Soerensen buffer, Sigma Chemicals).

Statistics. Data were tested for normal distribution by ANOVA (Kolmogorov-Smirnov test). Because of the limited number of animals per treatment group, nonparametric tests were used. For analysis between groups, the Kruskal-Wallis test followed by the Mann-Whitney U test was used. For analysis within groups the Friedman and Wilcoxon tests were used. Data were corrected using the Bonferroni-Holm procedure. Differences were considered statistically significant at P < 0.05. Despite nonparametric distribution, data are presented as arithmetic means ± SE.

RESULTS

Systemic parameters. HR and MAP remained unchanged in the NaCl- and Dx-60 treated groups. MAP increased after infusion of DCLHb by 21% above baseline at 0.5 h of reperfusion, and this higher blood pressure was significantly different from the value in 0.9% NaCl-treated and Dx-60-treated animals (Table 1). No significant changes of HR were observed among treatment groups.

Microcirculatory parameters. Venular and arteriolar diameters are listed in Table 2. There were no significant changes in arteriolar diameters after reperfusion of the posts ischemic muscle, whereas initially reperfusion led to an increase in venular diameter in all three groups.

RBC velocity was significantly reduced in postcapillary venules of 0.9% NaCl-treated and DCLHb-treated animals from 0.73 ± 0.06 and 0.86 ± 0.07 mm/s at baseline to 0.28 ± 0.05 (P < 0.05 vs. baseline) and 0.53 ± 0.11 mm/s (P < 0.05 vs. baseline) at 0.5 h of reperfusion, respectively. RBC velocity was significantly reduced in the early reperfusion period in Dx-treated animals to 82% of control values (Fig. 1).

Table 1. HR and MAP of awake Syrian golden hamsters before and after reperfusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time Points</th>
<th>0.5 h</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>HR</td>
<td>405 ± 38</td>
<td>384 ± 22</td>
<td>395 ± 18</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>113 ± 6</td>
<td>107 ± 6</td>
<td>111 ± 9</td>
</tr>
<tr>
<td>Dx-60</td>
<td>HR</td>
<td>411 ± 51</td>
<td>400 ± 16</td>
<td>411 ± 31</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>107 ± 7</td>
<td>102 ± 11</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>DCLHb</td>
<td>HR</td>
<td>416 ± 29</td>
<td>363 ± 29</td>
<td>385 ± 61</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>106 ± 3</td>
<td>129 ± 14*</td>
<td>125 ± 17*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 8 hamsters/treatment group. Heart rates (HR) are in beats/min; mean arterial pressures (MAP) are in mmHg. DCLHb, diaspirin-cross-linked hemoglobin. *P < 0.05 vs. 0.9% NaCl and Dextran-60 (Dx-60; 60 kDa) by Mann-Whitney U test.
prominent in the NaCl-treated group, in which the rolling fraction rose from 17.1 ± 1.3 to 48.6 ± 1.7% and leukocyte sticking increased by 20-fold from 17 ± 1 to 354 ± 58 mm⁻² after 2 h of reperfusion. These changes were significantly attenuated in the two treatment groups. It is noteworthy that the reduction of leukocyte rolling and sticking was more pronounced after treatment with DCLHb compared with Dx-60. Leakage of the fluorescent marker FITC-Dx (150 kDa) was significantly reduced at 24 h of reperfusion in DCLHb-treated animals compared with Dx-60 (Fig. 1).

In 0.9% NaCl-treated animals, a significant decrease of FCD was noted from 148 ± 6 to 129 ± 14 cm²/cm². The decline of FCD was less pronounced in animals treated with DCLHb, leading to a nonsignificant decrease of FCD compared with baseline.

Local tissue Po₂. No significant changes were found in any treatment group with respect to local tissue Po₂ (Fig. 3). However, there was a slight reduction in mean tissue Po₂ values by 16% (from 18.2 ± 1.9 mmHg at baseline to 15.3 ± 5.3 mmHg at 0.5 h of reperfusion) in the 0.9% NaCl control, which was accompanied by a left shift of the Po₂ distribution, i.e., frequency of reading (Fig. 4). Measurements of tissue Po₂ after pretreatment with Dx-60 and DCLHb showed that in both experimental groups tissue Po₂ was effectively restored on reperfusion (Fig. 4). Analysis of tissue Po₂ distribution indicated a left shift in tissue Po₂ values to hypoxic values (0–10 mmHg) in 0.9% NaCl-treated animals only, whereas such a shift was not observed in DCLHb or Dx-60 treatment groups (Fig. 4).

Histomorphology. Analysis of the electron microscopic cross-sections revealed destruction of striated muscle architecture, with focal myolysis and formation of vacuoles and edema within the muscle fibers as signs of ischemic tissue damage at 24 h of reperfusion in the 0.9% NaCl control group (Fig. 5A). The swelling of endothelial cells in capillaries was accompanied by formation of vacuoles within these cells. A prominent feature of these sections was obstruction of capillaries by RBC. Treatment with Dx-60 attenuated the disintegration of the muscle architecture and reduced vacuolar degeneration, but there was still massive endothelial swelling in capillaries, causing near complete obstruction of the capillary lumina (Fig. 5B). The postischemic tissue damage was most effectively reduced in DCLHb-treated animals (Fig. 5C). Only rare formation of vacuoles and myolysis and virtually no endothelial swelling in capillaries was found when compared with Dx-60 or 0.9% NaCl treatment.

### Table 2. Diameters of postcapillary venules and arterioles in striated skin muscle preparation of awake Syrian golden hamsters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time Points</th>
<th>Time of reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>0.5 h</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>Venules</td>
<td>39.5 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>Arterioles</td>
<td>59.0 ± 7.7</td>
</tr>
<tr>
<td>Dx-60</td>
<td>Venules</td>
<td>38.7 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>Arterioles</td>
<td>51.0 ± 9.3</td>
</tr>
<tr>
<td>DCLHb</td>
<td>Venules</td>
<td>39.5 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Arterioles</td>
<td>56.2 ± 12.8</td>
</tr>
</tbody>
</table>

Values are diameters (means ± SD) in units of µm; n = 8 hamsters/treatment group. Ratio of arterioles to venules: 0.9% NaCl, 0.356; Dx-60, 0.542; DCLHb, 0.495.
DISCUSSION

The findings of the present study provide the first in vivo evidence that the hemoglobin-based oxygen carrier DCLHb reduces leukocyte-endothelial cell interactions and macromolecular leakage in venules of striated skin muscle after 4 h of pressure-induced ischemia followed by reperfusion. DCLHb improved both functional capillary density and local tissue oxygenation of striated skin muscle after I/R; these findings were corroborated by electron microscopic demonstration of a reduction of postischemic reperfusion injury.

The dorsal skinfold chamber preparation in the awake Syrian golden hamster is a suitable model to study the pathophysiology of I/R without unwanted side effects of anesthetic drugs on the microcirculation and local tissue oxygenation (19). The model permits assessment of the therapeutic effects of pharmacological agents on postischemic reperfusion injury. The tissue $P_{O_2}$ distribution histogram obtained from the hamster dorsal skinfold has been shown to be highly reproducible and of predictive value for I/R injury (20). Four hours of pressure-induced ischemia followed by reperfusion was identified as the crucial ischemia time for induction of severe tissue damage in striated skin muscle (9). Therefore, the model is well suited to study the therapeutic efficacy of pharmacological agents for the treatment of I/R injury or, conversely, the properties of drugs with the potential to exacerbate reperfusion injury.

Various studies have proven the oxygen-carrying and volume-substituting properties of DCLHb when used as a RBC substitute. DCLHb is effective in restoring macro- and microcirculatory parameters (18) as well as tissue oxygenation (20, 22) after severe hemorrhagic shock. Despite the favorable effects of DCLHb in the...
treatment of hemorrhagic shock, a condition characterized by partial global ischemia (12), the potential property of hemoglobin-based oxygen carriers to catalyze lipid peroxidation (24) is of major concern, particularly under conditions of I/R.

Motterlini et al. (17) studied the effects of stroma-free hemoglobin solutions on the oxidative-stress response of vascular endothelial cells in vitro. HbA0 as well as αα-cross-linked Hb were capable of inducing hydroxyl radical formation. Faassen et al. (5) have shown in vitro that purified hemoglobin induces lipid peroxidation; however, this effect was dependent on temperature and duration of storage of hemoglobin (5). Dawidson et al. (4) demonstrated deleterious effects of unmodified stroma-free hemoglobin on the intestine after resuscitation of rats. The authors proposed the release of free iron ions from the prosthetic heme with ensuing generation of the cytotoxic hydroxyl radical as the underlying mechanism (24) through further activation of leukocyte-endothelial cell interactions, which contribute to impairment of microvascular perfusion and tissue oxygenation in the target organ. Although not proven in vivo, these mechanisms may lead to the exacerbation of reperfusion injury in postischemic tissue.

Fig. 4. Histogram of local tissue P02 of striated skin muscle after reperfusion in animals treated with 0.9% NaCl (A), Dx-60 (B), or DCLHb (C). Values are means (x) ± SD. Note left shift of tissue P02 levels in 0.9% NaCl-treated animals to ranges of 0–5 mmHg compared with Dx-60 or DCLHb.
Contrary to observations of these undesirable effects of unmodified stroma-free hemoglobin solutions are recent findings from other investigators using purified and heat-pasteurized DCLHb. In contrast to the work by Dawidson et al. (4), it has been reported that DCLHb is efficacious in restoring intestinal mucosal oxygenation and preserving villous architecture after hemorrhagic shock in rats (6). Cole et al. (3) found a significant reduction of brain injury and cerebral edema formation, as assessed by histomorphology after focal cerebral ischemia in rats previously hemodiluted with DCLHb (3). Cerebral blood flow was significantly higher in DCLHb-treated animals compared with albumin-treated animals. McKenzie et al. (13) investigated the effects of coronary perfusion with DCLHb during angioplasty. Myocardial function and S-T segment depression, early signs of ischemia, were attenuated during temporary occlusion of coronary arteries in swine infused with DCLHb. In a separate model of coronary I/R, McKenzie et al. (13) observed that DCLHb infusion before reperfusion reduced infarct size, decreased reperfusion arrhythmias, and improved regional myocardial function in swine. Pincemail et al. (21) directly studied the generation of free radicals in a model of renal ischemia in rabbits by means of electron-spin resonance spectroscopy. Compared with control, no exacerbation of free radical generation was found after exchange transfusion with DCLHb. These reports are in agreement with our in vivo findings that the modified hemoglobin DCLHb does not enhance postischemic reperfusion injury. Moreover, our data suggest that DCLHb can be considered as a therapeutic agent for treatment of I/R-induced microvascular disturbances. The reduction of leukocyte-endothelial cell interactions, the reduction of macromolecular leakage of FITC-Dx, and the improvement of muscle histomorphological integrity after reperfusion imply a protective action of DCLHb in I/R.

Like other investigators (8), we too have encountered an elevation of MAP after administration of DCLHb. The increase of MAP after the infusion of hemoglobin is partly mediated via scavenging of NO/endothelium-derived relaxing factor, an increase in endothelin release (25), and/or sensitization of catecholamine receptors (8). Despite the pressor effects of DCLHb in vitro (7), no vasoconstriction of the arterioles analyzed (15–80 µm) was observed after administration of DCLHb in this I/R model. Similar results were reported by Sharma and Gulati (26), who showed that administration of DCLHb resulted in an increase in blood flow to the skin, whereas blood flow to the musculoskeletal system remained constant (26). These findings suggest the

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**Fig. 5.** Electron microscopic analysis of postischemic tissue preserved after induction of 4 h of pressure ischemia followed by 24 h of reperfusion in a saline-treated control animal (A), a Dx-60-treated animal (B), and after DCLHb treatment (C) (total magnification 4,400-fold). A: In saline-treated animal marked myolysis and destruction of muscle fibers with edema and formation of vacuoles were visible. Capillaries were stuffed with red blood cells, accounting for an obstruction. B: Infusion of Dx-60 before reperfusion lead to a reduction of myolysis and destruction of muscle architecture compared with saline-treated animals through formation of larger vacuoles within muscle. Prominent endothelial swelling with obstruction of capillary lumen is also shown. C: Cross-sections of DCLHb-treated animals showed a markedly reduced formation of edema within muscle and of endothelial cells, and rare formation of vacuoles within muscle. Section shows reduced ischemic tissue damage compared with saline-treated and Dx-60-treated animals.
existence of regional differences in the vascular response to DCLHb. The initial nonsignificant increase of RBC velocity in our study at 0.5 h after reperfusion in DCLHb-treated animals may be attributed to an initial constriction of arterioles in the early reperfusion phase. A velocity increase of similar degree has been described to occur in the same model also under physiological conditions, with an immediate onset in the first minutes after intravenous injection of DCLHb (18). This effect, however, was transient. Our studies suggest that the vasoconstrictor effect, probably accounting for a longer-lasting increase in MAP, documented for arterioles in vitro does not occur in small arterioles and does not compromise blood flow to the skin under physiological or pathophysiological conditions. Kumar (10) reported that resuscitation with DCLHb improved perfusion of kidney and brain of hemorrhaged rats.

FCD is an indicator of tissue perfusion and correlates with the degree of reperfusion injury, as assessed in different tissues (19). A reduction of FCD, also known as the “no-reflow” phenomenon, is thought to be the pivotal mechanism contributing to I/R injury (15). The present study confirms the beneficial effect of Dx-60 in enhancing FCD under conditions of I/R, as reported by other investigators (14, 19). DCLHb restored microvascular perfusion more efficiently than Dx-60, as shown by results obtained with intravital and electron microscopy. Tsai et al. (29) investigated the effect of ααααα-Hb compared with Dextran-70 (Dx-70) on FCD and oxygen delivery after isovolemic hemodilution to a systemic hematocrit of 30% of control in the hamster dorsal skinfold chamber in the absence of I/R. Their study found a gradual reduction of FCD but no notable differences in oxygen-carrying capacity after hemodilution either with Dx-70 or ααααα-Hb. Recent findings from our own laboratory showed a significant decrease of FCD after isovolemic hemodilution to a hematocrit of 30% with both DCLHb (18) and ultrapurified bovine hemoglobin (1) in the same model. Because FCD describes the length of RBC-perfused capillaries per square centimeter of tissue, the reduction of FCD after isovolemic hemodilution might be attributable to an altered microhematocrit. In contrast to hemodilution, the attenuation of the decrease in FCD after 4 h of ischemia followed by reperfusion might be due to less capillary endothelial swelling as verified by electron microscopic analysis.

Different results obtained by various investigators with respect to the efficacy and toxicity of hemoglobin solutions are likely to be explained by the diversity of the solutions used, owing to the process of production and purification. For instance, because of their biological activity, traces of membrane phospholipids that may be contaminants of Hb solutions are known to elicit toxic effects. In support of this view are results described by Rabinovici et al. (23), who were able to prevent toxic effects of liposome-encapsulated hemoglobin by treating recipients of the compound with a platelet-activating factor (PAF) receptor antagonist (23). PAF-like lipids originating from membrane phospholipids can bind to the PAF receptor (28); therefore the effect of the receptor antagonist may be attributable to competitive inhibition of binding of PAF-like lipids to the PAF receptor rather than PAF itself, thus implicating toxic properties of phospholipids remaining in these hemoglobin solutions.

Our results are not in agreement with the paradigm that oxygen-carrying solutions contribute per se to an enhancement of oxygen free radical formation by virtue of providing more oxygen to the tissues. This notion is also supported by findings from Sirsjo et al. (27), who demonstrated that hyperbaric oxygen treatment results in improved FCD and RBC velocity and reduction of I/R injury in striated skin muscle in the same experimental model. Furthermore, several studies have demonstrated that oxygen-carrying blood substitutes do not have a notoriously deleterious impact on I/R injury, as hypothesized by Sadrzadeh et al. (24) and Faassen et al. (5). On the contrary, our findings and those from other studies using DCLHb indicate that because the different characteristics depend on various production and purification procedures, oxygen-carrying solutions have to be clearly defined with regard to purity and consistency for evaluation as potential RBC substitutes.

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