Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs

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Supplemental Methods:

Experimental Animal Protocols

Beagle dogs – *Study 1 (conscious dogs)* Fully conscious dogs were continuously infused for 8 h with stroma-free Hb in normal saline (40 mg/ml Hb) via a peripheral intravenous line. Plasma total heme concentrations (free Hb + Hp-bound Hb) were determined by spectrophotometry every 30 min, and the infusion rates adjusted to reach the target plasma heme concentration of 100-150 μM (**Figure 2A)**. Blood pressure was intermittently measured with an indirect oscillometric device (SDI Vet/BP 6000; SDI). Prior to BP recording, dogs were placed in left lateral recumbency and allowed to acclimatize to the surroundings for at least 10 minutes, and the initial BP readings were discarded. For data analysis, the mean of 10 measurements was used. Urine samples were collected from each dog by cystocentesis after 4 and 8 h of Hb infusion, respectively.

Study 2 (anesthetized dogs) Anesthesia was induced with Fentanyl (5 mcg/kg, Sintenyl, Sintetica SA) Midazolam (0.2 mg/kg, Dormicum, Roche Pharma) and Propofol (3-5 mg/kg, Propofol 1% MCT Fresenius), and maintained with isoflurane in 0.1 L/kg/min of O_2. The O_2 flow was reduced to 0.02 L/kg/min when an appropriate end-tidal isoflurane concentration was achieved. During the whole anesthetic episode, warming therapy was provided with a forced-air patient warming system (Bair Hugger, Model 505) to prevent hypothermia. Arterial blood pressure was measured via a 20G intraarterial catheter placed in the right femoral artery. Cardiac output was measured intermittently (baseline, mid-Hb infusion, end-Hb infusion) with Swan-Ganz flow-directed thermodilution catheters (Baxter Healthcare Corporation). Hb (HbA_0 or $\alpha\alpha$–DBBF-Hb) was diluted in normal saline to 40 mg/ml, and infused via a peripheral intravenous line. Infusion rates were as follows: 30 ml/h (0-30 min), 60 ml/h (30-60 min), 90 ml/h (60-90 min), and 120 ml/h (90-120 min). Total plasma heme was measured every 10 min by spectrophotometry. Equal plasma heme concentrations over time of the different experimental groups are shown in **Figure 2B**. Due to the short total exposure times (120 min) in this protocol group, it was not necessary to adjust infusion rates.

Guinea pigs - Fully conscious and freely moving guinea pigs were evaluated as a secondary species to confirm the experimental observations in dogs. Guinea pigs were exchange-transfused at 30% of their blood volume (approximately 5 mL) with HES or with a 1:1 mixture of Hb in HES or Hp to achieve a maximal plasma concentration of ~300 μM. This dose was selected to mimic the maximum acute

exposure conditions in the dog studies, where mean blood pressure and vascular resistance increases in Hb-transfused dogs occurred over a plasma concentration range of 100 to 400 μM. Systolic (SAP), mean (MAP) and diastolic (DAP) arterial blood pressures were continuously monitored at baseline and until 60 min after transfusion. At 24 h, post-dosage animals were anesthetized, the femoral veins cut, and cold saline perfused via the arterial catheter to remove blood. Kidneys were dissected, cut in half, frozen immediately in liquid nitrogen, and stored at -80ºC or fixed in 10% formalin. Guinea pigs typically excreted urine containing Hb within a 2 h period after the start of exchange-transfusion. Urine samples were collected over this time-period, centrifuged, and stored at -80 °C prior to analysis. Additionally, blood (0.2 mL per sampling) was obtained at baseline, immediately after transfusion, and at 4 and 24 h post-transfusion.

To further evaluate the influence of Hp complex formation on the systemic circulation, guinea pigs (n=6) were sequentially administered doses of αα–DBBF, a non-Hp binding chemically modified Hb(1), in the presence or absence of Hp. Guinea pigs were dosed sequentially with αα-DBBF Hb up to a cumulative dose of 64 mg in (1) HES or (2) Hp (1:1 Hp: αα–DBBF) to a total volume of 0.5 mL per dose. The response is reported as the average MAP change recorded within the first 10 min after each dosing.

Renal non-heme Iron and Lipid Peroxidation

Renal iron - Non-heme ferric iron was detected using Perl's method, followed by DAB intensification (2). Briefly, paraffin-embedded kidney sections were de-waxed in xylene, and rehydrated in graded ethanol and deionized water. Sections were incubated with equal volumes of 5% potassium ferrocyanide (Perls) for 45 min at room temperature, and rinsed in deionized water. Next, sections were incubated with 0.3% hydrogen peroxide (H_2O_2) and 0.01 M sodium azide in methanol for 30 min at room temperature, followed by rinsing in 0.1 M phosphate buffer, pH 7.4, incubated with DAB-H_2O_2 (Sigma) for 4 min, washed in deionized water, and lightly counterstained with Gill's II hematoxylin.

Lipid peroxidation immunohistochemistry - Mouse monoclonal antibodies against 4-hydroxynonenal-modifed proteins (HNEJ-2) (Oxis International) were employed to assess lipid peroxidation in kidney sections. Sections were dewaxed and rehydrated in graded alcohol and distilled water. Antigen retrieval was performed by heat treatment in a microwave oven for 20 min in 10 mM sodium citrate buffer (pH 6.0), cooled for 30 min at room temperature, and rinsed with deionized water and PBS. Sections were blocked with 3% normal horse serum for 1 h at room temperature before incubation with primary antibodies against 4-HNE (1 μg/ml) overnight at 4°C. Slides were incubated with 3% H_2O_2 in PBS-T for 15 min at room temperature to quench endogenous peroxidase activity. H_2O_2 treatment was performed after primary antibody incubation to avoid detecting any potential oxidative damage caused by H_2O_2 in the quenching step. The signal was developed by peroxidase-conjugated avidin-biotin complexes (Vectastain Elite ABC, Vector Labs) and diaminobenzidine (SigmaFast™ DAB, Sigma). For quantitative image analysis the total intensity of the positively stained areas has been measured after setting respective color thresholds using SigmaScan Pro 5.0 (Systat Inc) as described (3).

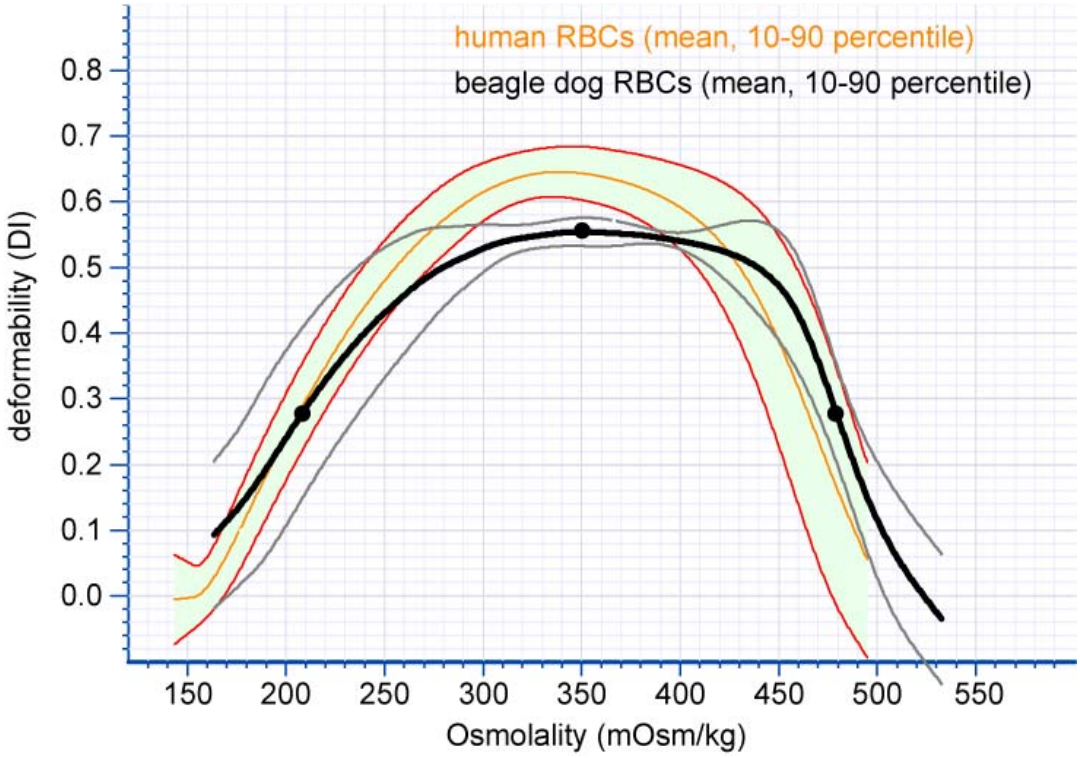
Effects of haptoglobin on biophysical properties and ligand interactions of hemoglobin

Oxygen equilibrium studies – Oxygen binding studies for Hb and Hb-Hp were carried out in a Hemox Analyzer (TCS Scientific). The samples were equilibrated with pure nitrogen gas and reoxygenated with air. The oxygen tension was measured using a Clark oxygen electrode (Model 5331 oxygen probe, Yellow Springs Instrument). The oxygen saturation of Hb was monitored via a built-in dual-wavelength spectrophotometer. A typical experiment was conducted with a Hb concentration between 60 and 75 μM (heme) at 37°C, and each experiment was repeated three times. The final solution (4 ml) contained 4 Al of the Hayashi enzymatic reduction system to maintain the metHb content to a minimum level(4). Oxygen equilibrium curves (OECs) were obtained and analyzed yielding P50 (the partial pressure of oxygen at which Hb is 50% saturated), and n50, the Hill coefficient for oxygen binding. The data analysis was performed by nonlinear least-squares curve fitting of the Adair equations derived from the Hemox Analyzer software (P50 Plus, Version 1.2).

Nitric Oxide Reaction Kinetics - The kinetics of NO oxidation of the oxy forms of Hb and Hb-Hp also known as "NO dioxygenation" were measured in the stopped-flow spectrophotometer. Prior to the mixing of the reactants, NO stock solutions (~2 mM) were prepared by washing the gas through a deoxygenated solution of 1 M NaOH before saturating with a deoxygenated 0.05 M bis-Tris buffer, pH 7.0, in a gastight serum bottle at room temperature. This stock solution was then transferred to a gastight syringe for appropriate dilutions with deoxygenated bis-Tris buffer. Solutions of air-equilibrated Hb were mixed against anaerobic solutions of NO, and the conversion of oxy-Hb to ferric Hb was monitored by absorbance changes at 420 nm. The value of the bimolecular rate constant for this reaction is known to be extremely large, on the order of 1×10^7 $M^{-1}s^{-1}$, so the concentration of NO after mixing was kept low (25 μM) to minimize loss of the reaction in the dead time of the instrument. Under these conditions, the rate of autoxidation reaction of NO with dissolved oxygen is negligible compared with the rate of reaction with $HbFe^{2+}$. The concentration of Hb in this experiment was kept at 0.5 μM to use a pseudo-first-order approximation(5).

Autoxidation kinetics Hb - solutions (20 μM) were incubated at 37°C in sealed cuvette in air equilibrated 50 mM Chelex-treated phosphate buffer with or without antioxidant enzymes, i.e. catalase (414 U/ml) and superoxide dismutase (4.6 U/ml). Both Hb and Hb-Hp were reduced to maximum levels of the ferrous forms prior to autoxidation experiments as described earlier (6). Dithionite was added to reduce Hbs, and subsequently removed by Sephadex G-25 chromatography. The levels of Fe^{2+} Hb and Hb-Hp were verified spectrophotometrically. The absorbance spectra between 450 and 700 nm were recorded over time in a temperature-controlled photodiode array spectrophotometer (HP-8453). The proportions of Hb species (oxy, met, and hemichromes) were calculated from each spectrum by multicomponent analysis based on their extinction coefficients(6). The oxyHb percentage changes due to the spontaneous oxidation of Hbs were plotted as a function of time. The autoxidation rate constants were obtained by nonlinear least-squares curve fitting using single or double exponential equations in SigmaPlot software.

Supplement Figure 1:



Supplement Figure 1: Comparison of human (n=60) and beagle dog (n=4) RBC fragility.

The RBC fragility has been determined by osmotic gradient Ektacytometry as follows:

Osmotic gradient Ektacytometry, called Osmoscan, was performed as described previously with an Ektacytometer (Technikon, Bayer, Germany) (7-9). The principle of measurement is to record the deformability of erythrocytes experiencing constant shear stress by laser diffractrometry in a continuously changing environment. To achieve a reasonable shear stress a high viscosity of environmental solution is needed, in our case we used a 20% dextran ~ 70.000 da (*Carl Roth GmbH, Germany*) solution**.** This solution was buffered with 10 mM $NaKHPO_4$ to eliminate the effect of pH on deformability (10). Also 17 mM Glucose was added to increase the survival time of the erythrocytes. To increase the stability of the solutions 0.4 g/l of sodium triazide were added. To vary the osmolality of this solution, we used sodium chloride. Because the Technicon Ektacytometer uses conductivity as indicator for osmolality, it was necessary to calibrate the system before measurement. This was done by measuring the solution mentioned above with different amounts of sodium chloride added simultaneously in the Ektacytometer's conductivity meter and a cryoscopic Osmometer (Gonotec Osmomat 030). 500 l whole blood, collected in a standard 10ml *BD K2E Vacutainer*® (18.0 mg of K_2EDTA), was mixed with 3 ml of equimolal dextrane solution and directly inserted into the Ektacytometer for measurement. The blood was always processed wihtin maximal 4-5 hours after withdrawal, all samples where measured twice with almost a perfect correlation.

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