Effect of acute hyaluronidase treatment of the glycocalyx on tracer-based whole body vascular volume estimates in mice

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Submitted 6 July 2012; accepted in final form 24 February 2013


The endothelial glycocalyx forms a hyaluronan-containing interface between the flowing blood and the endothelium throughout the body. By comparing the systemic distribution of a small glycocalyx-accessible tracer vs. a large circulating plasma tracer, the size-selective barrier properties of the glycocalyx have recently been utilized to estimate whole body glycocalyx volumes in humans and animals, but a comprehensive validation of this approach has been lacking at the moment. In the present study, we compared, in anesthetized, ventilated C57Bl/6 mice, the whole body distribution of small (40 kDa) dextrans (Texas Red labeled; Dex40) vs. that of intermediate (70 kDa) and large (500 kDa) dextrans (both FITC labeled; Dex70 and Dex500, respectively) using tracer dilution and vs. that of circulating plasma, as derived from the dilution of fluorescein-labeled red cells and large-vessel hematocrit. The contribution of the glycocalyx was evaluated by intravenous infusion of a bolus of the enzyme hyaluronidase. In saline-treated control mice, distribution volume (in ml) differed between tracers (P < 0.05; ANOVA) in the following order: Dex40 (0.97 ± 0.04) > Dex70 (0.90 ± 0.04) > Dex500 (0.81 ± 0.10) > plasma (0.71 ± 0.02), resulting in an inaccessible vascular volume, i.e., compared with the distribution volume of Dex40, of 0.03 ± 0.01, 0.15 ± 0.04, and 0.31 ± 0.05 ml for Dex70, Dex500, and plasma, respectively. In hyaluronidase-treated mice, Dex70 and Dex40 volumes were not different from each other, and inaccessible vascular volumes for Dex500 (0.03 ± 0.03) and plasma (0.14 ± 0.05) were smaller (P < 0.05) than those in control animals. Clearance of Dex70 and Dex500 from the circulation was enhanced (P < 0.05) in hyaluronidase-treated vs. control mice. These results indicate that the glycocalyx contributes to size-dependent differences in whole body vascular distribution of plasma solutes in mice. Whole body vascular volume measurements based on the differential distribution of glycocalyx-selective tracers appear appropriate for the detection of generalized glycocalyx degradation in experimental animals and humans.

glycocalyx; hyaluronidase; plasma volume; indicator-dilution; dextrans

THE ENDOTHELIAL GLYCOCALYX forms a highly hydrated mesh of polysaccharide structures and adsorbed plasma proteins on the luminal side of all blood vessels (23, 37). It forms the true interface between the flowing blood and the endothelium and has been indicated to play an important role in homeostasis of the vascular wall (31). Experimental microscopy studies in cremaster muscle microcirculation have indicated an important role for the glycocalyx in governing the intravascular distribution of circulating plasma and solutes (8, 26, 35, 36). These studies showed that the glycocalyx under resting conditions significantly limits access of circulating blood toward the endothelium, and that permeation of dextrans into the glycocalyx depends on their size and charge, such that neutral 40-kDa dextrans (Dex40) appeared to have largely unimpaired access to the entire intravascular volume, whereas access of larger dextrans [e.g., 70 kDa (Dex70) and 500 kDa (Dex500)] was significantly hindered by the glycocalyx (8, 32, 35). These features of the glycocalyx have, in the last few years, been applied to derive estimates of systemic glycocalyx volume and barrier properties in experimental animals (1, 16, 33, 38) and in humans (17, 19), and to study the effect of various cardiovascular risk factors on the glycocalyx. In hamster and mice, Dex70 and larger dextrans were used as circulating intravascular tracer, and, agreeing with the intravital microscopy studies, both their whole body distribution volume and vascular clearance were smaller than those of Dex40, which was merely considered to reflect total anatomic vascular volume (1, 33, 38). Systemic distribution volume and vascular clearance of Dex70 compared with Dex40 were found to be decreased during acute hyperglycemic conditions (38), indicating a reduction in systemic glycocalyx volume and systemic glycocalyx barrier properties under these conditions. In humans, Nieuwdorp and coworkers (19, 20) derived circulating plasma volume from the dilution of labeled red blood cells (RBCs) and large-vessel hematocrit and compared this to the distribution volume of Dex40. These authors obtained relatively large, inaccessible vascular volumes for plasma, i.e., compared with the Dex40 volume, of ~1.7 liters in healthy subjects, which were decreased to ~0.5 liter during 6-h hyperglycemia in the same subjects (20). Also, type 1 diabetics (19) and patients with familial hypercholesterolemia (17) were found to have a reduced systemic glycocalyx volume using this method, suggesting generalized loss of glycocalyx in these conditions.

Appreciation of these data requires adequate validation of the tracer-based measurement of systemic glycocalyx volume. A useable approach hereto is the evaluation of its sensitivity to targeted glycocalyx degradation. This was previously done for the large- vs. small-dextran approach using single-bolus administration of the enzyme hyaluronidase, which increased systemic distribution volume and vascular clearance of Dex70 compared with Dex40 (1, 33). The effect of an acute enzymatic glycocalyx challenge on the whole body inaccessible vascular volume for plasma using dilution of labeled RBCs vs. small dextrans has, however, so far not been validated, although a reduction in systemic glycocalyx volume in dyslipidemic mice was reported after 4-wk intravenous infusion of active hyaluronidase vs. inac-
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In mice, the systemic glycocalyx volume was recently critically discussed (18). The concerns raised related mainly to the estimation of the initial distribution volume of Dex40 due to rapid renal excretion of small-molecular components within the injected sample, as well as to the validity of the plasma volume estimation from RBC volume and large-vessel hematocrit, since it had been generally recognized in the literature that the derived volume using this approach is lower than the volume estimated by the more common approach of dilution of an albumin-based plasma tracer. This disparity between both plasma volume estimations may, however, be explained by the presence of the glycocalyx, when considering that commonly used plasma tracers, such as albumin, may not only distribute into the circulating part of the plasma, but access (part of) the glycocalyx as well (9). In line herewith, human studies have indicated that use of hydroxyethyl starch (molecular mass ~200 kDa) as plasma tracer revealed distribution volumes not only that were smaller than found for albumin, but that were actually very similar to those based on the distribution of RBCs and large-vessel hematocrit (14, 28), suggesting that tracers need to have a certain size and conformation to be confined to the circulating space only. Accordingly, it is to be expected that the volume distribution of plasma tracers of large enough size would be near equal to that of circulating plasma, and that, during conditions of a degraded glycocalyx, smaller distribution differences would be observed between tracers that have access to the glycocalyx domain vs. tracers that are confined to the circulating part of the plasma only.

The aim of the present study was to evaluate the tracer-based systemic glycocalyx volume measurement with respect to the following: 1) its sensitivity to targeted glycocalyx degradation; 2) the influence of the size of the used tracer; and 3) the contribution of the renal clearance of Dex40. Therefore, we tested in mice the effect of an acute hyaluronidase challenge (1, 2, 5, 10, 15, 20, and 30 min after start of the tracer infusion) on the systemic distribution of different dextrans (Dex40, Dex70, and Dex500), as well as of circulating plasma as glyocalyx-inaccessible tracer was compared with that of simultaneously infused Texas Red-labeled Dex40 (Invitrogen-Molecular Probes) (33, 38). The following tracers were evaluated: 1) circulating plasma as derived from the dilution of fluorescein-labeled RBCs and large-vessel hematocrit (16, 19, 20); and 2) FITC-labeled Dex70 and Dex500 (Sigma-Aldrich). Previous microscopy studies in cremaster capillaries showed that these dextrans were significantly hindered by the endothelial glycocalyx, whereas Dex40 appeared to have unlimited access to the entire intravascular space (8, 32, 35).

METHODS

The study involved experiments in mice. The main part of the study was performed on anesthetized C57Bl/6 mice; additional experiments were performed in conscious Swiss mice. All procedures and protocols were approved by the Animal Care and Use committees of the Academic Medical Center in Amsterdam and of Maastricht University.

Anesthetized Mice: General Surgery and Experimental Protocol

Experiments were performed on C57Bl/6 female mice [n = 35, body weight (BW) 23.0 ± 0.4 g; Charles River Europe] that received standard chow and water ad libitum until the day before the experiment. Mice were overnight fasted and were anesthetized with an intraperitoneal injection of ketamine hydrochloride (125 mg/kg), medetomidine (0.2 mg/kg), and atropine (0.5 mg/kg) (33, 38). Tracheotomy was performed and mechanical ventilation was started by connecting the trachea tube to a pressure-controlled ventilator (SAR-830/P; CWE). Animals were ventilated with a gas mixture of 1:1 O2/N2. Respiration rate was set at 90 breaths/min with a peak inspiratory pressure of 18 cmH2O and a positive end-expiratory pressure of 2 cmH2O. Anesthesia was maintained by continuous intraperitoneal infusion at a rate of 10 ml·kg−1·h−1 of ketamine (3.5 mg/ml), medetomidine (20 μg/ml), and atropine (7.5 μg/ml). The right carotid artery and jugular vein were cannulated for monitoring systemic blood pressure and heart rate and administration of enzyme/saline and tracers, respectively. Depth of anesthesia was checked according to stability of blood pressure and lack of toe pinch reflex, and infusion rate of anesthetics was adjusted, if necessary. Esophageal temperature was maintained at ~37°C by placement of the animal on a temperature-controlled heating pad and radiant heat. At the end of the experimental procedures, the mouse was given an overdose of ketamine.

After instrumentation and a 15-min equilibration period, mice were given a bolus (0.05 ml in 1 min through jugular vein) of saline alone (control group) or hyaluronidase (35 units, Type IV-S; Sigma-Aldrich) in saline (33). Next, after a 60-min incubation period, a bolus injection of mixed tracers was administered, and their dilution in blood measured for 30 min (16, 33, 38); the choice for a 60-min incubation period was based on reported observations that glycocalyx exclusion was maximally impaired 45 min to 1 h after administration of the enzyme (1, 8).

In each experiment, the distribution volume of an indicated glycocalyx-inaccessible tracer was compared with that of simultaneously infused Texas Red-labeled Dex40 (Invitrogen-Molecular Probes) (33, 38). The following tracers were evaluated: 1) circulating plasma as derived from the dilution of fluorescein-labeled RBCs and large-vessel hematocrit (16, 19, 20); and 2) FITC-labeled Dex70 and Dex500 (Sigma-Aldrich). Previous microscopy studies in cremaster capillaries showed that these dextrans were significantly hindered by the endothelial glycocalyx, whereas Dex40 appeared to have unlimited access to the entire intravascular space (8, 32, 35).

RBCs vs. Dex40. Blood (~1 ml) was collected from a donor mouse by cardiac puncture and centrifuged, and the RBCs labeled with sodium fluorescein (250 mg/ml) for 5 min. After washing, the labeled cells were resuspended in saline to the initial volume. Two min before infusion, 0.1 ml of the labeled blood was mixed with an equal volume of Dex40 (15 mg/ml), and 0.1 ml of this tracer mix was infused in the animal in 1 min. Blood samples (5 μl) were collected in heparinized capillary tubes through tail bleeding at time t = ~5 (pre), 2, 3, 4, and 5 min after start of the infusion for determination of the fraction of labeled RBCs (16, 19, 20). In addition, 30-μl samples were collected in ~30 s at t = ~5 (pre), 2, 5, 10, 15, 20, and 30 min for determination of Dex40 concentrations (16, 33, 38).

Dex70/Dex500 vs. Dex40. Dextran mix (0.1 ml) (2.5 mg/ml FITC-labeled dextran + 10 mg/ml Dex40 in phosphate-buffered saline) was manually infused in the jugular vein in 1 min, and blood was subsequently sampled (30 μl) through tail bleeding at t = ~5 (pre), 2, 5, 10, 15, 20, and 30 min after start of the tracer infusion (33, 38). In each mouse, urine production was assessed by collecting visibly excreted urine during the duration of the experiment in a capillary tube, together with the remaining urine content in the bladder right after the mouse had been euthanized (at t = 35 min) (38). In addition, kidneys, heart, and hindlimb muscles were collected after the experiment and blotted, and their wet weight measured. Tissues were stored at 70°C for 3 days and then weighed again for obtaining dry weight.

Conscious Mice: General Surgery and Experimental Protocol

To evaluate the contribution of the anesthetic conditions on the measurements, additional experiments were performed in cannulated, conscious female Swiss mice (n = 6, BW = 34.5 ± 0.4 g; Charles River Europe). The Swiss strain rather than the C57Bl/6 mouse was
chosen for these measurements, because the feasibility of these type of experiments was shown to be greater in these larger animals (10–12), while systemic hemodynamics appear very comparable between Swiss and C57Bl/6 mice, both in the conscious state (11, 15), as well as during anesthesia (11, 39). Mice were instrumented with chronic catheters in right femoral artery and right jugular vein, as described in detail previously (10, 12). Catheters were guided and exteriorized for ~2 cm to the neck of the animal and plugged with metal pins. After catheter implantation, mice were allowed to recover for at least 3 days before the experiment was performed.

The mouse remained in its own cage during the measurements; generally, the animal assumed a resting, nonmoving position in the corner of its cage. For sampling blood without substantial disturbance of the animal, the following procedure was used. The arterial line was gently clamped with a hemostat, the metal plug was removed, and a 1-ml syringe with a small needle was inserted into the catheter outlet. After the hemostat was released, ~0.1 ml of blood was collected, accounting for the dead volume of the catheter. Next, the line was clamped again, and the syringe replaced by a capillary tube. The hemostat was released until ~30 µl of blood had entered the tube and then was closed again. Finally, the initial ~0.1 ml of collected blood was re-infused, after which the line was flushed with heparinized saline, and replugged with the metal pin. The venous catheter was used to administer the tracers. The catheter was clamped with a hemostat, the plug was removed, and the syringe with the tracer mix inserted into the outlet of the catheter. After release of the hemostat, the tracer mix was infused in ~1 min. After the line was flushed with heparinized saline, it was closed with the pin for the remainder of the experiment. One-half of the mice (n = 3) received the Dex500/Dex40 tracer mix, and the other one-half received the mix of labeled RBCs and Dex40. In these experiments, only control measurements were performed, and no hyaluronidase was administered. Furthermore, urine and tissues were not collected.

Tracer Analysis

RBCs. Labeled RBCs were measured using a FACScan analyzer (FACScalibur; Becton Dickinson, Mountain View, CA), with at least 100,000 cells being counted to measure the circulating fraction of labeled cells (19, 20). Data were analyzed using Cellquest (Becton Dickinson, San Jose, CA). The circulating plasma volume was calculated as [(1 – Ht) × Vrcs]/Ht, where Vrcs is the circulating RBC volume [(1/circulating fraction of labeled RBCs) × volume of labeled cells injected], and Ht is the large-vessel hematocrit (16, 19, 20). The fraction of labeled cells at t = 2, 3, 4, and 5 min was averaged and used as circulating fraction; unlabeled erythrocytes obtained before the injection (t = 0 min) served as negative controls.

Dextran. Capillaries were centrifuged, hematocrit was determined, and plasma collected and stored at −20°C until fluorescence analysis. In each sample, fluorescence was measured at 490/535 nm (excitation/emission) for the FITC-labeled dextrans (Dex70 and Dex500) and at 595/615 nm for Dex40 with a spectrophotometer (VICTOR; PerkinElmer), and dextran concentrations were calculated in reference to defined dilutions of the infused tracer mix in plasma from donor mice (16, 33, 38). For all dextrans, plasma concentrations were normalized to the amount injected, and the time-concentration curve was fitted with a monoexponential function (20, 33, 38). In addition, because Dex40 has been suggested to rapidly egress from the circulation, linear extrapolation of the concentration between t = 2 and t = 5 min was also done for this dextran (see Fig. 1A). The initial distribution volume of each dextran was determined from the extrapolated dilution at the start of tracer injection. Vascular clearance was defined as the percentage decrease in tracer concentration at the end of the experiment (t = 30 min) compared with the extrapolated concentration at the start of tracer injection (t = 0 min) (33).

Urine samples were stored at −20°C until analysis, when dextran concentrations were calculated in reference to defined dilutions of the infused tracer mix in urine from donor mice. The percentage dextran recovery in the urine in an experiment was determined from the total volume of urine sampled and its dextran concentration, normalized to the amount injected.

Statistics

Summary data are reported as means ± SE, with n referring to the number of animals studied. Differences in tracer behavior (distribution volume, vascular clearance, recovery in urine) in anesthetized mice were analyzed using one-way analyses of variance; post hoc comparisons were performed using Tukey tests. Differences in Dex40 distribution volume obtained when using linear vs. exponential extrapolation to t = 0 concentration were pairwise tested (t-test). Pearson correlation analysis was performed to evaluate whether systemic hematocrit as well as Dex40 volume and clearance were related to blood pressure. Effects of hyaluronidase were compared with control (saline) mice using unpaired t-tests, and differences between conscious and anesthetized animals were compared using unpaired t-tests as well. Results were considered statistically significant with P ≤ 0.05.
back-fitting of the concentrations at the initial Dex40 concentration in each experiment by linear components in the infused Dex40 sample, we also estimated concentration due to rapid renal excretion of the small molecular would lead to an underestimation of the initial Dex40 concentration. Since it had been suggested that backward-extrapolation of the control and hyaluronidase-treated mice (Table 1).

RESULTS

Anesthetized Mice

There were no differences in blood pressure, heart rate, hematocrit, urine production, and tissue hydration between control and hyaluronidase-treated mice (Table 1). Linear vs. exponential extrapolation of Dex40 concentration. Since it had been suggested that backward-extrapolation of the entire time-concentration curve using a monoexponential fit would lead to an underestimation of the initial Dex40 concentration due to rapid renal excretion of the small molecular components in the infused Dex40 sample, we also estimated the initial Dex40 concentration in each experiment by linear back-fitting of the concentrations at \( t = 2 \) and \( t = 5 \) min only (Fig. 1A). Linear back-extrapolation did indeed result in higher initial Dex40 concentrations compared with the exponential backward-extrapolation in both groups of mice, and hence Dex40 volumes of distribution were smaller when derived from the linear vs. the exponential fit (Fig. 1B). The difference in calculated Dex40 volume between both approaches was 0.038 ± 0.013 ml in control mice and 0.072 ± 0.011 ml in hyaluronidase-treated mice (\( P = 0.052 \)). Given this ambiguity in estimation of initial Dex40 concentration, we wanted to ensure that our measure of systemic glycocalyx volume would not be biased by an overestimation of the Dex40 volume and, therefore, decided to use the linear extrapolation procedure for derivation of the initial Dex40 volume in all experiments. Rapid loss of dextran molecules by renal excretion seems not relevant for plasma Dex70 and Dex500 concentrations (Fig. 1A), and initial concentrations of these dextrans were approximated by backward-extrapolation of the monoexponential fit, as done in our laboratory’s previous experiments (33, 38).

Systemic volumes of distribution of plasma and dextrans. Systemic distribution volumes of the various tracers are shown in Fig. 2. RBC volume was derived from the dilution of fluorescein-labeled RBCs and, together with hematocrit, revealed a circulating blood volume of 1.24 ± 0.06 ml in control mice, which, when corrected for BW, corresponds with a volume of 55.3 ± 1.6 ml/kg BW. RBC and total blood volume were not different in hyaluronidase-treated mice. Analysis of variance revealed a difference (\( P < 0.0025 \)) in distribution volume for the four tracers (circuiting plasma, Dex500, Dex70, Dex40) in saline-treated mice, but not in the hyaluronidase-treated animals. Individual distribution volumes of the different tracers were, however, not statistically different in the hyaluronidase-treated mice compared with the control group.

Comparison of volume of circulating tracer and Dex40. In each experiment, the distribution volume of circulating plasma and Dex70 and Dex500 was compared with that of simultaneously infused Dex40 to derive whole body vascular volumes that are not accessible to plasma or large dextrans. These volumes are presented in Fig. 3. In line with the increase in distribution volume from plasma-Dex500-Dex70-Dex40 (Fig. 2), the volume difference with Dex40 was largest for plasma, 0.31 ± 0.05 ml (14.0 ± 2.5 ml/kg BW). The nonaccessible volume for Dex500 was about one-half of that for plasma (0.15 ± 0.04 ml; 6.8 ± 1.8 ml/kg BW), and almost negligible for Dex70 (0.03 ± 0.01 ml). In hyaluronidase-treated animals, the nonaccessible vascular volumes for plasma and Dex500 were reduced compared with con-

Table 1. Hemodynamic and fluid characteristics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Hematocrit, %</th>
<th>Urine, μl</th>
<th>Dry-to-Wet Weight (Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td>Saline</td>
<td>59.7 ± 2.3</td>
<td>273 ± 6</td>
<td>44.4 ± 1</td>
<td>194 ± 48</td>
<td>0.260 ± 0.004</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>58.8 ± 1.8</td>
<td>277 ± 10</td>
<td>44.7 ± 1</td>
<td>208 ± 23</td>
<td>0.253 ± 0.006</td>
</tr>
</tbody>
</table>

Values are means ± SE; total no. of animals: n = 35 (saline; n = 18, hyaluronidase; n = 17). Blood pressure and heart rate were obtained in n = 10 saline and n = 12 hyaluronidase-treated animals. Blood pressure, heart rate, and hematocrit were averaged for the duration of the experiment (time \( t = -5 \) until \( t = 30 \) min). No significant correlation was observed between blood pressure and systemic hematocrit.
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Correlation analysis revealed that both the amount of Dex40 cleared from the circulation, as well as the amount recovered in the urine, were related to the systemic blood pressure during the experiment (Fig. 4B); there was no correlation between blood pressure and the distribution volume of Dex40, however (data not shown). On average for all control and hyaluronidase-treated animals, the ratio of the amount of Dex40 found in urine compared with the amount lost from the circulation was 0.53 ± 0.05.

Additional experiments were performed in Swiss mice with cannulated femoral artery and jugular vein. In three animals, the distribution of Dex500 and Dex40 was measured, and in the other three the combination of labeled RBCs and Dex40 was administered. Systemic hematocrit in the conscious animals was 45.7 ± 2.1% (P = nonsignificant compared with anesthetized mice), and the following volumes of distribution (in ml) were found: 0.78 ± 0.11 (RBC), 0.99 ± 0.08 (plasma), and 1.35 ± 0.10 (Dex500). For Dex40, a distribution volume of 1.64 ± 0.18 ml was obtained when using the linear back fitting of the concentrations at t = 2 and 5 min only, and 1.63 ± 0.11 ml when using the monoeponential fit for all time points (P = nonsignificant). When correcting for the larger BWs of the Swiss mice, comparable distribution volumes were found between the two experiments (Fig. 5). As a result, the volume difference between Dex40 and Dex500 (8.5 ± 3.6 ml/kg BW; n = 3) and between Dex40 and circulating plasma (18.4 ± 4.5 ml/kg BW; n = 3) were comparable to those in the anesthetized animals that had received saline. The amount of Dex40 cleared from the circulation at the end of the measurements was 89.9 ± 2.3% of the amount injected, which was higher than found in the control mice under anesthesia (P < 0.05).

DISCUSSION

Based on intravital microscopic observations of rodent cremaster muscle microcirculation, indicating distinct intravascular domains for RBCs and macromolecules as a result of the barrier properties of the endothelial glycocalyx, systemic glycocalyx volumes have recently been estimated in humans and...
The present study aimed at a validation of previously reported tracer-based measurements of systemic glycocalyx volume. In these studies in experimental animals and humans, a range of glycocalyx volumes was presented, and the origin for this disparity could be related to the different plasma tracers that were used, as well as to the differences in experimental conditions, including the use of anesthesia. Furthermore, the methodology was recently critically discussed, while the contribution of the glycocalyx to the measurement had not been evaluated for all tracers. In the present study, we, therefore, determined distribution volumes of a selection of tracers in anesthetized, as well as conscious mice, and challenged the glycocalyx using the enzyme hyaluronidase.

Rationale of the Study

The present study aimed at a validation of previously reported tracer-based measurements of systemic glycocalyx volume. In these studies in experimental animals and humans, a range of glycocalyx volumes was presented, and the origin for this disparity could be related to the different plasma tracers that were used, as well as to the differences in experimental conditions, including the use of anesthesia. Furthermore, the methodology was recently critically discussed, while the contribution of the glycocalyx to the measurement had not been evaluated for all tracers. In the present study, we, therefore, determined distribution volumes of a selection of tracers in anesthetized, as well as conscious mice, and challenged the glycocalyx using the enzyme hyaluronidase. In short, the following novel findings were obtained. 1) Comparison of the distribution volume of RBC-derived plasma volume vs. that of Dex40 in mice gives a measure of systemic glycocalyx volume, which is comparable to that previously reported in humans. 2) This measure appears hardly influenced by an uncertainty in Dex40 volume estimation as a result of clearance by the kidneys. 3) This measure is sensitive to acute glycocalyx degradation. 4) This measure is larger than the glycocalyx volume obtained by Dex500 and Dex70 as plasma tracer.

Methodological Aspects: Clearance of Small Dextrans

The methodology used to derive whole body vascular volumes in the present and previous studies (19, 33, 38) is based on the tracer dilution technique. The choice of the tracers was inferred from intravital microscopic observations of intravascular tracer distribution in cremaster capillaries (8, 32, 35) and in isolated mesenteric arteries (30), which revealed the glycocalyx to exclude RBCs and to constitute a significant barrier for FITC-labeled dextran of Dex70 and larger, without apparent hindrance of Texas Red-labeled Dex40. In line herewith, Dex40 distribution volume was shown to exceed that of circulating plasma and large molecular mass dextrans in the present (Fig. 2) and previous studies (1, 16, 33, 38). A major concern has been raised, however, regarding the estimation of the initial volume of distribution of Dex40 due to its rapid clearance from the circulation (18), as discussed in the following paragraph.

The fraction of labeled RBCs was constant for the FACS samples taken at \( t = 2, 3, 4, \) and 5 min after tracer injection (data not shown), suggesting mixing to be complete at the first sampling time point, \( t = 2 \) min. For the dextran, subsequent to \( t = 2 \) min, samples were taken at \( t = 5, 10, 15, 20, \) and 30 min to enable adequate appraisal of the dextran clearance kinetics (33, 38). Systemic clearance was considerable for Dex40, around 60% in 30 min in the anesthetized mice and 90% in the conscious mice, and more than one-half of this appeared in the urine in this time period (Fig. 4), illustrating that elimination of this tracer by the kidneys is prominent. Since dextran samples are recognized to typically have a rather broad molecular mass distribution, it has been anticipated that particularly the smallest polymers in the injected Dex40 sample are rapidly cleared by the kidneys because they are able to pass the glomerular barrier relatively easily (2, 34), thereby leaving the larger molecules to exchange more slowly at later times (18). The higher initial Dex40 concentration when approximated by linear backward-fitting of the concentrations at \( t = 2 \) and \( t = 5 \) min vs. by extrapolation of the entire time-concentration curve using the monoexponential fit (Fig. 1A) indeed seems to suggest an enhanced Dex40 clearance in the first 5 min after injection compared with the later time points in our experiments. The consequence for the approximation of initial Dex40 volume was, however, small, such that the linear approach was associated with a 3.6 and 7.5% smaller Dex40 volume compared with the monoexponential approach in the control and hyaluronidase-treated anesthetized mice, respectively (Fig. 1B), while in the conscious mice the difference seemed even less due to the larger Dex 40 clearance at later times during the experiment. Vascular clearance was much smaller for the FITC-labeled of Dex70 and Dex500 and may represent slow equilibration of the tracer with the glycocalyx domain (35) rather than renal clearance of these large molecules, as exemplified by the low retrieval of these dextran in the urine (Fig. 4A).
Choice of Plasma Tracers

Circulating plasma volume was derived from the dilution of fluorescein-labeled RBCs and large-vessel hematocrit (16, 19, 20). The relative vascular volume, which was not accessible to circulating plasma in the mice, compared well with those in the human studies [Figs. 2 and 3; 0.3 ml in 1-ml Dex40 = 35% in mice; 1.7 liters in 4.7-liter Dex40 = 36% in humans (20)]. Although the absolute value of this volume has been suggested to be an underestimate of the true glycocalyx volume because the Dex40 partition coefficient between plasma and glycocalyx fluid is likely less than one (18), the quantity appears to agree well with reported glycocalyx dimensions in the microcirculation. Thus, assuming that 25% of the total blood volume in the mice (1.25 ml) is located in the microcirculation, a glycocalyx volume of 0.3 ml would reduce functional perfused microvascular volume by 50%, which, in a capillary with an anatomic diameter of 5 μm, would be brought about by a blood-excluding glycocalyx with a thickness of ~0.7 μm. Glycocalyx dimensions of 1 μm were estimated by Pries and coworkers for explaining the discrepancy between experimental estimates of apparent viscosity or flow resistance in microvessels in vitro vs. in vivo (22, 24), while exclusion zones of 0.4–0.5 μm in cremaster capillaries have been reported for Dex70 and Dex500 with intravital microscopy (8, 35), suggesting that these dextrans may have substantial access into the glycocalyx domain compared with plasma. Indeed, the plasma volume obtained appeared lower than that for Dex500 and Dex70, which is in line with experimental studies in mice (e.g., Refs. 7, 25), in which plasma volume was estimated by dilution of labeled albumin or a tracer that binds to albumin. The findings also support clinical observations showing that tracer-based estimations of plasma volume generally reveal greater volumes of distribution than those based on the dilution of labeled RBCs and large-vessel hematocrit (18). While traditionally the difference has been contributed to an overestimation of whole body hematocrit when using large-vessel hematocrit (e.g., Ref. 25) due to a difference in distribution pattern between RBCs and plasma in the microcirculation as a result of the Fahraeus effect, this perception of a variant distribution of circulating RBCs and plasma in the microcirculation has been seriously challenged by intravital microscopic measurements in cremaster muscle microcirculation by Duling and coworkers (3–6, 36). In these studies, discharge hematocrit in microvessels was demonstrated to be quite similar to large-vessel hematocrit, suggesting that circulating RBCs and plasma may actually be distributed uniformly across microvascular networks (4, 5). Moreover, it was indicated that significant exclusion of circulating RBCs and plasma by the glycocalyx may underlie the low tube hematocrit in microvessels under resting conditions (13). As a consequence, the disparity between tracer-based estimations of plasma volume vs. those based on the dilution of labeled RBCs and large-vessel hematocrit has been suggested to reflect the fact that assumed plasma tracers do not only distribute into the circulating part of the plasma, but can access (part of) the glycocalyx as well, resulting in an overestimation of plasma volume (9, 36). Indeed, evidence for rapid transient interactions of albumin or albumin-binding tracers and the glycocalyx has been presented in the past (9, 21, 27). The data in Fig. 3 suggest that Dex70, which have a comparable size as albumin, yet are considered to be inert macromolecules, had significant access to the glycocalyx domain. Furthermore, this accessibility for Dex70 appeared much larger than that for the Dex500, contrasting to intravital microscopic observations showing that Dex70 and Dex500 were excluded by the glycocalyx to essentially the same extent (8, 35). The suggestion that also the Dex500 had access to part of the glycocalyx in our experiments appears, however, not consistent with a previous study in humans in which the distribution volume of hydroxyethyl starch with a molecular mass of ~200 kDa was found to be actually nearly similar to that of circulating plasma based on RBC distribution (14). Besides the possibility of species differences in barrier properties of the glycocalyx, the relative large deformability of dextrans may perhaps have permitted significant permeation of these polysaccharides into the glycocalyx in our experiments (34).

The apparent profound permeation of large dextrans in the experiments described in Figs. 2 and 3 was not explained by the use of anesthesia, as indicated by the additional data that were obtained in the conscious mice and which showed a similar difference in distribution volume for Dex500 and circulating plasma (Fig. 5). Since the ketamine-medetomidine mix has been shown to have potent cardiodepressive effects (39), causing the relatively low blood pressures and heart rates during the experiments that were performed under anesthesia (Table 1), we wanted to make sure that these hemodynamic conditions would not interfere with our measurements. Previous experiments in this conscious mouse model reported blood pressures of ~100 mmHg and heart rates of ~500–600 beats/min (11, 12, 15), underscoring the influence of anesthesia on the hemodynamic conditions. These different conditions did not, however, affect systemic hematocrit and the distribution volumes for the different tracers (Fig. 5). Only the clearance of Dex40 from the circulation was found to be higher in the conscious animals compared with the anesthetized animals. Since Dex40 clearance and excretion were both related to blood pressure (Fig. 4B), the higher Dex40 clearance in the conscious animals seems well accounted for by the higher blood pressures in these animals.

Sensitivity to Glycocalyx Degradation

Hyaluronidase treatment appeared to be associated with an almost equal distribution of the dextrans in the vascular system (Figs. 2 and 3), suggesting that the size-dependent barrier properties of the glycocalyx were greatly lost in the case of glycocalyx degradation. In contrast, Cabrales and coworkers (1) did also observe an effect of hyaluronidase on systemic Dex70 distribution, but not on that of their large dextran tested, which had a molecular mass of 2,000 kDa. The current findings appear to deviate from intravital microscopic findings of Henry and Duling (8), who showed a reduction in exclusion for FITC-labeled Dex70, but not Dex500, after a bolus of hyaluronidase in hamsters, and illustrate that measurements of tracer exclusion in capillaries using intravital microscopy do not necessarily relate to actual tracer distributions in the whole body circulation. The distribution volume of circulating plasma was, however, still substantially smaller than that of Dex40 in the hyaluronidase-treated animals (Fig. 3), which can be explained by the fact that degradation of the glycocalyx was incomplete using this approach (1, 8). Nevertheless, the estimated inaccessible volume for plasma was reduced to ~50% of control, supporting the involvement of the glycocalyx in the distribution difference between plasma and Dex40. In agreement with our laboratory’s previous studies (33, 38), glycocalyx degradation was also associated with an increased...
vascular clearance of both Dex70 and Dex500, but not Dex40, suggesting enhanced movement of these tracers across the vascular wall as a result of the impairment in barrier properties of the glycocalyx.

**Conclusion**

In the present study in anesthetized mice, measurements of whole body vascular volume using tracer dilution of fluorescently labeled RBCs and dextran substance intravital microscopic observations of macro- and microvessels, showing that the glycocalyx acts as a molecular filter governing the intravascular distribution of plasma solutes. Comparison of the distribution space of Dex500 and circulating plasma vs. that of the presumed glycocalyx-accessible tracer Dex40 resulted in substantial inaccessible volumes for large dextran and circulating blood, which were both reduced by >50% after single intravenous infusion of hyaluronidase, illustrating that the volume estimates using these tracers are sensitive to acute glycocalyx damage. Because the glycocalyx has been inferred to play an important role in regulation of vascular homeostasis, the described measurements of whole body glycocalyx volume and barrier properties appear, together with microscopic observations of glycocalyx dimensions, to be valuable for detection of glycocalyx damage and vascular vulnerability during atherogenic conditions in experimental animals, as well as humans.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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