Short-term hyperglycemia increases endothelial glyocalyx permeability and acutely decreases lineal density of capillaries with flowing red blood cells

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Zuurbier, Coert J., Cihan Demirci, Anneke Koeman, Hans Vink, and Can Ince. Short-term hyperglycemia increases endothelial glyocalyx permeability and acutely decreases lineal density of capillaries with flowing red blood cells. J Appl Physiol 99: 1471–1476, 2005. First published July 14, 2005; doi:10.1152/japplphysiol.00436.2005.—Hyperglycemia is becoming recognized as an important risk factor for microvascular dysfunction. We hypothesized that short-term hyperglycemia, either on the scale of hours or weeks, alters the barrier function and the volume of the endothelial glyocalyx and decreases functional capillary density and deformability of the red blood cells (RBCs). All experiments were performed in anesthetized, mechanically ventilated, C57BL/6 mice that were either normoglycemic, acutely hyperglycemic (25 mM) for 60 min due to infusion of glucose, or hyperglycemic (25 mM) for 2–4 wk (db/db mice). The glyocalyx was probed using 40-kDa Texas red dextran, which is known to permeate the glyocalyx, and 70-kDa FITC dextran, which has impaired access to the glyocalyx in healthy animals. Clearance of the dye from the blood was measured. An orthogonal polarization spectral imaging technique was used to visualize the number of capillaries with flowing RBCs of the dorsal flexor muscle. The data indicate that short-term hyperglycemia causes a rapid decrease of the ability of the glyocalyx to exclude 70-kDa dextran. No change in the vascular permeation of 40-kDa dextran was observed. Glyocalyx volume was not affected by short-term hyperglycemia. In addition, 1 h of hyperglycemia resulted in a 38% decrease of the lineal density of capillaries with flowing RBCs. This decreased lineal density was not observed in the 2- to 4-wk hyperglycemia model. Short-term hyperglycemia was without any effect on the deformability of the RBCs. The data indicate that the described increased vascular permeability with hyperglycemia can be ascribed to an increased permeability of the glyocalyx, identifying the glyocalyx as a potential early target of hyperglycemia.

vascular permeability; diabetes; skeletal muscle; db/db mice

HYPERGLYCEMIA, either in its acute form, such as which may occur in hospitalized critically ill patients, or in its chronic form, such as is present in patients with diabetes mellitus, is associated with increased rates of morbidity and mortality and diminished clinical outcome. The central importance of a vascular pathology underlying the hyperglycemia-associated poor prognosis is becoming increasingly evident. Characteristics of hyperglycemia-induced vasopathy include decreased endothelium-dependent vasodilation, increased capillary permeability for large proteins such as albumin, swelling of endothelial cells, decreased capillary dimensions and diameters, and thickening of the basal lamina (5, 13, 20, 23, 33, 41). The endothelial cell glyocalyx, a 0.2- to 0.5-μm matrix lining the luminal surface of all blood vessels, is a significant factor in microvascular regulation by its action on volume and permeability of capillaries (1, 17, 18, 29, 39). Recent evidence demonstrated a role of the glyocalyx in models of ischemia-reperfusion injury, inflammation, and altered lipoprotein levels (26, 28, 38). To our knowledge, no study has examined whether hyperglycemia affects the glyocalyx. This is surprising since it is known that oxidative stress affects the glyocalyx (25), and leukocyte adherence is increased after glyocalyx injury (26). Because hyperglycemia is associated with oxidative stress and increased adherence of leukocytes, changes in the functional properties of the glyocalyx with hyperglycemia would be expected. In this study, we therefore tested the hypothesis that hyperglycemia results in changes in the barrier function and/or the volume of the glyocalyx.

Alterations of the glyocalyx induced by hyperglycemia may be associated with changes in the functional properties of the capillaries. Although many studies can be found that have characterized capillary morphology by histological techniques, few studies have reported on how short-term hyperglycemia affects the number of capillaries with blood flow, i.e., functional capillary density. One recent study (2) demonstrated decreased functional vascular density using a dorsal skinfold chamber in 20-wk-old Type 2 diabetic mice. Kindig et al. (20) reported no change in the lineal density of flowing capillaries in skeletal muscle in an insulin-deficient, Type 1 diabetic rat model of ~8-wk diabetic condition. However, due to decreased red blood cell (RBC) flux, it was concluded that skeletal muscle O2 delivery was markedly reduced (20). In the present study, we addressed the question of whether short-term (hours to 2–4 wk) hyperglycemia, without a concomitant insulin deficiency, affects the lineal density of capillaries with flowing RBCs. To this end, we applied orthogonal polarizing spectroscopy (OPS; Refs. 12, 35) imaging of skeletal muscle to observe intravital capillary RBC flow. OPS imaging uses green polarized light to illuminate the area of interest, which is reflected by the background and absorbed by hemoglobin, thereby producing high-contrast images of that part of the microcirculation that contains moving RBCs. In previous studies, we and others have shown that small vessels (<20 μm) are often the first to show abnormal behavior in divergent pathologies before alterations in global measurements such as venous O2 tension and mean arterial pressure become obvious (6, 31, 36).

Finally, deformability of RBC is another likely target of increased blood glucose levels, and it has been postulated that lower RBC deformability may contribute to diabetes-induced alterations of the microcirculation (30). However, studies have...
mostly examined RBC deformability by a filtration technique, which has been criticized to be lacking selectivity, sensitivity, and reproducibility (16). In this study, we evaluated RBC deformability with a laser-assisted optical rotational cell analyzer (LORCA) (15) to examine whether short-term hyperglycemia affects RBC deformability.

In summary, we hypothesize that short-term hyperglycemia without low levels of insulin, on a scale of either hours and/or weeks, results in alteration of glycocalyx permeability and/or volume, diminished perfusion of the microcirculation, and decreased deformability of RBCs. We tested this hypothesis by determining 1) glycocalyx permeability and volume by fluorescent-labeled dextrans of different molecular masses, which allow glycocalyx characterization (27, 28, 40); 2) lineal density of capillaries with flowing RBCs, visualized by the OPS-technique; and 3) stress-strain relations of RBC using LORCA.

All experiments were performed in a recently developed, anesthetized and mechanically ventilated mouse model with stable hemodynamics (44).

METHODS

Preparation. Animals were treated according to the guidelines of the Declaration of Helsinki, and all procedures were in accordance with the requirements of the Animal Ethics Commission of the University of Amsterdam. Preparation of the animals was as described previously (44). In short, after the induction of anesthesia with ketamine (125 mg/kg)-medetomidine (0.2 mg/kg)-atropine (0.5 mg/kg), a tracheotomy was performed and mechanical ventilation started. Ventilation parameters were as reported (44), resulting in optimal blood pressures at a arterial $PCO_2$ of 30 Torr, arterial $PO_2$ of 170 Torr, and pH of 7.3. Anesthesia was maintained by continuous intraperitoneal infusion at a rate of 0.1 ml·10$^{-1}$·g$^{-1}$·h$^{-1}$ (3.5 mg/ml ketamine + 20 $\mu$g/ml medetomidine + 7.5 $\mu$g/ml atropine). The carotid artery was cannulated for blood pressure and heart rate recording, and the vena jugularis was cannulated for fluid and fluorescent-labeled dextran administration. Temperature was controlled at 37°C using rectal temperature monitoring, a temperature-controlled heating pad, and an infrared lamp. The dorsal flexor muscles of the right hind leg were made accessible for OPS imaging through an incision of the skin. Only during the imaging period (2 × 5 min) was the skin carefully retracted from the muscle. For the largest part of the experiment, the skin was overlaying the muscle to prevent extensive drying of the muscle. The dorsal flexor muscles of the mouse consist of 70–80% of type IIB fibers, with the remaining part consisting of type IIA and type I fibers (11).

Experimental protocol. Three different groups of mice (male) were studied. The wild-type mice were obtained from Harlan, Holland, whereas the $db/db$ mice were obtained from Harlan, UK. All groups received Ringer lactate at an infusion rate of 0.3 ml·10$^{-1}$·g$^{-1}$·h$^{-1}$ from 0 to 15 min, of 0.2 ml·10$^{-1}$·g$^{-1}$·h$^{-1}$ from 15 to 30 min, and of 0.1 ml·10$^{-1}$·g$^{-1}$·h$^{-1}$ from 30 to 90 min of the protocol. The control normoglycemia group (C57BL/6; 21 ± 1 g; 7–9 wk; $n = 7$) and the chronic hyperglycemia groups (C57BL/6; 21 ± 1 g; 7–9 wk; $n = 7$) were oriented across the monitor screen, the line perpendicular to the muscle fiber direction varied between 0.6 and 0.9 mm in length. Lineal density of capillaries with flowing RBCs is expressed as number of capillaries per millimeter (20, 21). It should be noted that the OPS technique does not discern whether alterations in lineal density is due to changes in diameters of muscle fiber and/or capillary or changes in proportion of capillaries with remaining blood flow.

RBC deformability was also determined at the start of the experiment and at 90 min of the protocol. Blood (25 $\mu$l) was sampled by tail bleeding, dissolved in PBS containing 140 mM polyvinylpyrrolidone, and analyzed by the LORCA for determination of the shear stress-strain relation (15).

To assess whole body fluid shifts, urine production was measured by weighing the urine collected during the experiment in a capillary tube, together with the bladder content at the end of the experiment. Total blood volume was calculated from hematocrit and total plasma volume. Hydration status of organs was estimated from determination of wet weight of blotted heart and kidney at the end of the experiment and their dry weight after 2 days of storage at 70°C.

Glycocalyx determination. At 95 min of the protocol, 0.1 ml of the dextran solution (10 mg/ml 70-kDa FITC dextran + 2.5 mg/ml 40-kDa Texas red dextran in 1% albumin-MOPS solution) was manually infused through the vena jugularis within 1 min. Blood was subsequently sampled in 50-$\mu$l heparinized capillaries through tail bleeding at time = 97, 100, 105, 110, 115, and 125 min. Hematocrit was determined after capillary centrifugation, and capillary plasma was collected and stored at −20°C until analysis.

Concentration-time curves for both dextrans were determined using fluorescence measurements at 490/535 nm for 70-kDa FITC dextran and at 595/615 nm for 40-kDa Texas red dextran in 96-well plate with a Victor spectrometer. Numerous studies have shown that the 70-kDa dextran does not penetrate the glycocalyx and is therefore retained in the vasculature, whereas the 40-kDa dextran penetrates the glycocalyx within minutes and may leave the vasculature (27, 28, 40). For permeability testing of the glycocalyx, use was made of this different behavior of both dextrans by examining the dextran concentration, normalized to the doses given, for the first 30 min after administration. For volume determination, the concentration-time curves were fitted for each experiment separately with a monoexponential function, and the concentration was determined at time = 95.5 min (30 s after start of bolus injection) by extrapolation. From the concentration and the known amount of infused dextran, the distribution volume for dextran 40 and 70 was calculated for each experiment. Note that averaged volume estimations cannot be derived from the averaged time-concentration curves (Fig. 2) because glycocalyx volume is inversely proportional to dextran concentration. Based on our previous observation that the 40-kDa Texas red dextran equilibrates quickly with the glycocalyx (40), we use this dextran to obtain an estimate on total plasma volume (circulating plasma + glycocalyx), whereas, with the observation that the 70-kDa FITC dextran does not penetrate the glycocalyx (40), this dextran is used to obtain an estimate of circulating plasma volume only. Consequently, the glycocalyx volume can be calculated from the difference in distribution volume of both dextrans.

Statistical analysis. Values are given as means ± SE. One-way ANOVA with post hoc comparisons among group means were used to evaluate differences ($P < 0.05$) in dependent parameters. Repeated two-way ANOVA for blood pressure and elongation index was
performed, followed by comparisons among means corrected with Bonferroni when a significant main effect was found.

RESULTS

Global measurements. At baseline, blood glucose levels were similar for the normoglycemic (4.3 ± 0.4 mM) and acute hyperglycemic mice (4.3 ± 0.4 mM), whereas the chronic hyperglycemic mice demonstrated severe hyperglycemia with blood glucose levels of 26.4 ± 0.6 mM (Fig. 1A). The glucose infusion protocol brought blood glucose in the acute hyperglycemic group within 30 min to a similar level as observed for the chronic hyperglycemic group, where it was maintained for the remaining part of the experiment. At the start of the experiment, mean arterial blood pressure was higher for the chronic hyperglycemic mouse (95 ± 2 mmHg) compared with control (81 ± 3 mmHg) but became similar during the course of the experiment (Fig. 1B). The rise in blood glucose in the acute hyperglycemic group was associated with an increase in blood pressure of 20 mmHg at the start of hyperglycemia. At 60 min of hyperglycemia, blood pressure was still elevated significantly in the acute hyperglycemic group (84 ± 5 mmHg) compared with the normoglycemic group (72 ± 4 mmHg).

Table 1 reflects the main measurements for understanding of whole body fluid shifts with hyperglycemia. Hematocrit was significantly elevated in the chronic hyperglycemic group, with a nonsignificant trend in increased blood volumes. This increase in fluid volume with hyperglycemia was compensated by a significant dehydration of organs with volumes. This increase in fluid volume with hyperglycemia was compensated by a significant dehydration of organs with volumes. As reflected by the increased dry-to-wet ratio of heart and kidney together.

Vascular measurements. Figure 2 shows time courses of dextran concentrations, normalized to doses given, for the three groups. The 40-kDa dextran curve in the normoglycemic group shows a slow disappearance of dextran from the blood, indicating permeabilization through the vessel wall. This rate of disappearance can be given by the power of the exponential fit of the dextran curves. No significant alteration in this power for the 40-kDa dextran curves were observed when going from the normoglycemic to the hyperglycemic groups, indicating that short-term hyperglycemia is without effect on the vascular permeabilization of 40-kDa dextran. A different picture emerges for the 70-kDa dextran. During the 30-min observation, the concentration of this dextran remains constant in the normoglycemic group, indicating no disappearance and therefore permeabilization of 70-kDa dextran through the glycocalyx. However, in both the acute hyperglycemic group and the chronic hyperglycemic group, 70-kDa dextran concentration decreases, reflected by a significant increase in the power of the exponential fit compared with the normoglycemic group. Despite these changes in glycocalyx permeability with short-term hyperglycemia, the estimated volumes of total plasma (40-kDa dextran), circulating plasma (70-kDa dextran), and glycocalyx were not affected by short-term hyperglycemia (Fig. 3).

Figure 4 depicts the lineal density of capillaries with flowing RBCs at baseline and after 90 min of experimentation for all three groups. At baseline, the number of capillaries with RBC flow was similar for the normoglycemic (34 ± 1 capillaries/mm), the acute hyperglycemic (37 ± 4 capillaries/mm), and the chronic hyperglycemic (36 ± 1 capillaries/mm) groups. However, raising blood glucose levels for 90 min induced a 38% decrease in the lineal density of capillaries with flowing RBCs in the acute hyperglycemic group (23 ± 4 capillaries/mm). No significant changes with 90 min of experimentation in number of capillaries with blood flow were observed for the normoglycemic (33 ± 2 capillaries/mm) and chronic hyperglycemic groups (36 ± 2 capillaries/mm).

RBC deformability. The LORCA determined the elongation of the RBCs as a function of shear stresses between 0.3 and 30 Pa (Fig. 5). At the start of the experiment (Fig. 5A) and at the end of the experiment, statistical analysis revealed no significant effect of short-term hyperglycemia on the deformability of the RBCs.

DISCUSSION

The main findings of this study may be summarized as follows: 1) short-term hyperglycemia specifically increases the permeability of the glycocalyx, without alterations in the permeability of other parts of the vessel wall, 2) the volume of the glycocalyx is unaffected by short-term hyperglycemia, 3) hyperglycemia at a time scale of hours results in a 38% decrease of the lineal density of capillaries with moving RBCs in skeletal muscle, 4) hyperglycemia for 2–4 wk in a model of Type 2 diabetes was without effect on the lineal density of capillaries with flowing RBCs in skeletal muscle, and 5) short-term hyperglycemia does not result in stiffer RBCs as determined by the LORCA technique.

Vascular measurements. The dichotomy that glycocalyx permeability is effected without alterations of glycocalyx volume coincides with enzymatic evidence suggesting that glycocalyx permeability is determined by the hyaluronan component, whereas glycocalyx thickness (volume) is regulated by the proteoglycan component of the glycocalyx (7, 17, 27).
These data suggest that hyperglycemia mainly affects the hyaluronan component of the glycocalyx. Interestingly, this supports the contention that O$_2$ radicals figure prominently in hyperglycemia-detrimental effects (10), because it was shown that radicals specifically degrade hyaluronan (25). The increased glycocalyx permeability with hyperglycemia may also explain the increased leukocyte adhesion with hyperglycemia (19), knowing that glycocalyx shedding increases this adhesion (26).

The observation that increased glycocalyx permeability coincides with decreased lineal density of flowing capillaries during acute hyperglycemia suggests the possibility that alterations in glycocalyx constitution affects flow regulation at the level of capillaries. Other studies have also shown that pathological conditions associated with increased vascular permeability, such as sepsis, result in decreased microvascular perfusion (6, 21, 31). Remarkably, Lam et al. (21) observed almost similar changes in functional capillary density of dorsal flexor muscle with sepsis (from 35 ± 2 capillaries/mm in control to 23 ± 1 capillaries/mm in sepsis conditions) as the changes observed in the present study in this muscle with acute hyperglycemia (from 37 ± 4 capillaries/mm at baseline to 23 ± 4 capillaries/mm in the acute hyperglycemic condition). One likely mechanism that may tie together altered glycocalyx permeability and microvascular flow is endothelium-derived nitric oxide (NO) release. It has been shown that decreasing the glycosaminoglycan component of the glycocalyx completely reduced the shear-induced endothelium-derived NO release (9, 24). Maybe it is this component of NO in the vasculature that, through decreased arteriolar dilation as a consequence of diminished NO, results in diminished microvascular perfusion of the capillaries. The increase in blood pressure with short-term hyperglycemia that was observed in the present study is also indicative of decreased levels of NO. This would correspond well with studies showing that, on pharmacologically increas-

**Table 1. Parameters reflecting fluid shifts in whole animals due to hyperglycemia**

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit, %</th>
<th>Blood Volume, ml</th>
<th>Urine Production, ml</th>
<th>Dry-to-Wet Ratio (Heart:Kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoglycemia</td>
<td>40±2</td>
<td>1.71±0.12</td>
<td>0.19±0.01</td>
<td>0.258±0.004</td>
</tr>
<tr>
<td>Acute hyperglycemia</td>
<td>42±1</td>
<td>2.12±0.22</td>
<td>0.71±0.06*</td>
<td>0.280±0.005*</td>
</tr>
<tr>
<td>Chronic hyperglycemia</td>
<td>46±1*†</td>
<td>2.12±0.13</td>
<td>1.20±0.13*</td>
<td>0.271±0.003*</td>
</tr>
</tbody>
</table>

Values are means ± SE of hematocrit, blood volume, urine production, and dry-to-wet ratio of heart and kidney for the 3 different groups. *P < 0.05 vs. normoglycemia. †P < 0.05 vs. acute hyperglycemia.
ing NO production, microvascular perfusion in septic patients was improved (6, 36). Obviously, further studies simultaneously examining glycocalyx and microvascular perfusion with special emphasis on NO production are needed to explore this potential important microvascular blood flow regulation. Why hyperglycemia lasting for weeks was not associated with decreased lineal density in this study, whereas the increased glycocalyx permeability was persistent, is not clear. In a study of streptozotocin-induced diabetic rats, the hyperglycemic state was also not associated with an alteration of the lineal density of flowing capillaries (20). Importantly, it was found that the proportion of capillaries maintaining normal flow decreased by 38% but that this decrease was fully compensated for in terms of flowing capillaries (20). Importantly, it was found that the lineal density of capillaries with flowing RBCs was also not associated with an alteration of the lineal density of the muscle atrophy (3, 34). It is possible that, in the present study, a decrease in lineal density of capillaries with flowing RBCs was not observed with hyperglycemia lasting for 2–4 wk because of severe muscle atrophy. Further studies are necessary to answer this question conclusively for Type 2 diabetes.

**RBC deformability.** Although it has been suggested that alterations of RBC deformability resulted in impaired blood flow in diabetes (22, 30), not all studies have found decreased RBC deformability with diabetes (e.g., Refs. 32, 43). In this study, short-term hyperglycemia did not result in stiffer RBCs. This result cannot be ascribed to a selectivity of our analysis technique, because the LORCA specifically analyzes RBC deformability. It has been shown that factors such as low cholesterol levels (8) and short duration (<3 yr) of diabetes (42) may all prevent diabetes-associated decreased RBC deformability. Because our db/db mice have high cholesterol levels (37), the most likely factor why RBCs are not stiffer in our study is that we only studied short-term hyperglycemia. It seems, therefore, that the vascular disturbances described in this study with short-term hyperglycemia cannot be ascribed to changes in RBC deformability.

**Global measurements.** Infusion of hypertonic glucose solution will result in whole body fluid shifts (14). To estimate the fluid shifts occurring in our model with 1–2 h of acute hyperglycemia, blood volume, urine production, and hydration status of organs were measured. For both heart and kidney, a 7.9% decrease in water content was measured. Assuming that this fluid shift results in a 1 ml of water loss, the measured values quite precisely explain the osmotic fluid shifts occurring with 1–2 h of hyperglycemia. Hyperglycemia results in dehydration of organs, with dehydration fluid being directed to the vascular system and increased diuresis. The increased diuresis with acute hyperglycemia was found to be due to increased nephron filtration rate and reduced proximal reabsorption in the kidney (4). It could be argued that these volume shifts may also explain (besides glycocalyx alterations) our 70-kDa dextran concentration-time curves. However, were this the case, then a decreased concentration of the 40-kDa dextran with acute hyperglycemia should be observable. No such changes in the 40-kDa dextran curves were noted, making it unlikely that these volume shifts are the cause of changes in the 70-kDa dextran curves.

In conclusion, acute hyperglycemia results in a decreased lineal density of skeletal muscle capillaries with flowing RBCs, which is restored when hyperglycemia lasts for weeks in a Type 2 model of diabetes. Further detailed studies are necessary to evaluate whether, despite normalization of the lineal density, the O2 supply per unit muscle mass is actually decreased, as observed in streptozotocin-induced hyperglycemic models (20).

Most importantly, the increased disappearance rate from the blood with short-term hyperglycemia of the 70-kDa FITC dextran, a dextran that in the healthy condition does not penetrate the endothelial glycocalyx and therefore does not leave the vasculature, suggests that the well-known increased vascular permeability with hyperglycemia is localized to an increased permeability of the glycocalyx. The glycocalyx may therefore be an early target of hyperglycemia detrimental effects.

**Fig. 5.** Deformability of RBC determined by the laser-assisted optical rotational cell analyzer at start (baseline; time = 0 min; A) and end experiment (time = 90 min; B) for the 3 groups. Deformability is expressed as elongation index as function of shear stress.
HYPERGLYCEMIA INCREASES GLYCOCALYX PERMEABILITY

REFERENCES