What goes up must come down: glucose variability and glucose control in diabetes and critical illness
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Chapter 3

Mild hyperglycaemia disturbs vascular homeostasis in humans

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Abstract

Hyperglycaemia induces oxidative stress, disturbs endothelial function, damages the endothelial glycocalyx and causes a prothrombotic shift in coagulation and fibrinolysis. Little is known about the exact blood glucose level necessary to start these processes. The aim of this study was to determine at which level of glycaemia these changes occur. A stepwise hyperglycaemic clamp was performed in eleven healthy human males at blood glucose (BG) levels of 6, 8 and 10 mmol/l for two hours each while suppressing endogenous insulin release. Oxidative stress, assessed by malondialdehyde, showed a gradual increase highly correlating with BG. Coagulation, assessed by prothrombin fragments F1+2, significantly increased at 6 mmol/l and was followed by an increase in both plasmin-antiplasmin complexes and d-dimer levels at 8 mmol/l, indicating fibrinolysis activation. The endothelial glycocalyx, measured by hyaluronic acid levels, showed no relevant change during the clamp. Hyaluronidase showed a gradual decrease indicating increased hyaluronidase substrate binding by shedding of glycocalyx constituents, significant at 10 mmol/l. There was no indication of a cumulative effect of glycaemia over time on any of the parameters. These data indicate that oxidative stress as well as coagulation activation already starts at near normal BG levels, while endothelial glycocalyx changes occur at 10 mmol/l.
Introduction

Patients with diabetes mellitus are at high risk of developing cardiovascular disease. A combination of accelerated atherosclerosis and a shift towards a pro-coagulant state leads to atherothrombotic events in nearly two thirds of all patients with diabetes. Hyperglycaemia, its defining feature, has been shown to cause both endothelial dysfunction, a precursor of atherosclerosis, and activation of the coagulation system. Endothelial dysfunction is paired with damage to the endothelial glycocalyx, the protective layer of proteoglycans and glycosaminoglycans lining the luminal side of all blood vessels. Hyperglycaemia induced disruption of the endothelial glycocalyx results in a pro-atherogenic state, characterised by increased vascular permeability, coagulation activation and increased cellular adhesion and migration. The formation of reactive oxygen species (ROS) is an important mechanism by which hyperglycaemia leads to endothelial (glycocalyx) damage, since high doses of anti-oxidants are able to attenuate this damage. Furthermore, hyperglycaemia induced ROS formation may also affect the coagulation system, by influencing gene transcription of coagulation and fibrinolytic factors.

It is unclear, however, at which glucose level these vascular changes first occur and whether this is an on-off phenomenon with a threshold or a continuous relationship. This distinction is also of importance given the recent debate about the impact of glycaemic variability on the development of complications in patients with diabetes. Some have argued that a high variation in blood glucose levels throughout the day has a greater impact on pro-atherogenic processes than a stable high glucose. If so, a threshold phenomenon should exist for these processes, since a dose-dependent effect would lead to comparable outcome when the mean blood glucose levels are equal.

Most studies investigating the effects of hyperglycaemia on vascular homeostasis have described effects of a glucose level of 10 mmol/l or higher, but epidemiological studies suggest that vascular damage actually starts at near normal glucose levels. Even in the lower glucose ranges a linear relationship between HbA1c, fasting plasma glucose and vascular complications of diabetes was demonstrated in patients with both type 1 and type 2 diabetes. Moreover, impaired glucose tolerance and impaired fasting glucose, both representing only mildly elevated glucose levels, already carry an increased risk for macrovascular disease.

In the present study we describe the effects of only mildly elevated glucose levels on oxidative stress, the endothelial glycocalyx and the thrombotic system in healthy males, studied by performing a stepwise glucose clamp while suppressing endogenous insulin levels.
Results

Protocol
We investigated whether near-normal glucose levels were associated with endothelial
dysfunction by means of a stepwise hyperglycaemic clamp while suppressing endogenous
insulin production by octreotide infusion. Blood glucose (BG) levels were maintained at
6, 8 and 10 mmol/l successively for 2 hrs per level (Figure 1). We obtained blood samples
every 30 minutes during the clamp. Also, the day after the clamp a fasting blood sample
was obtained to assess the recovery after the glucose load.

Patients
In total, 14 healthy non-smoking Caucasian males with a fasting plasma glucose level
≤5 mmol/l without risk factors for macrovascular disease as measured by BMI, blood
pressure, cholesterol and triglyceride levels were included in the study. Of those, one
dropped out due to febrile illness before the study day and two subjects were excluded
before analysis of the blood samples due to poor performance of the hyperglycaemic
clamp, resulting in 11 subjects who were included in the final analyses. Baseline
characteristics of the included subjects are listed in Table 1.

Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>n = 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>24.3 (3.6)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.6 (1.9)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>115.1 (12.3)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>68.9 (8.5)</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/l</td>
<td>4.7 (0.2)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.3 (0.2)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>4.0 (0.9)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>2.2 (0.9)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.5 (0.3)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.7 (0.4)</td>
</tr>
</tbody>
</table>

Values are expressed as means (SD). BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein

Hyperglycaemic clamp
During the clamp endogenous insulin levels were adequately suppressed. Plasma insulin
levels after 1 hr of octreotide infusion and at the end of the clamp were comparable with
fasting levels in all patients (median <15 pmol/l, maximum 43 pmol/l). Mean glucose levels of all included time points are depicted in Figure 1.

![Figure 1 Glucose clamp](image)

**Figure 1 Glucose clamp**
Glucose values obtained during the clamp. Data are expressed as mean (SD) of the previous 30 minutes. Dotted lines represent the time points where glucose infusion was increased to go to the next glucose level.

**Oxidative stress**
Oxidative stress was assessed by quantitative determination of malondialdehyde (MDA) in plasma using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). MDA is a reactive and potentially mutagenic aldehyde which is formed as a result of lipid peroxidation caused by hyperglycaemia induced formation of ROS. Lipid peroxidation is thought to be an important part of the pathogenesis of atherosclerosis and the metabolites are frequently used as biomarkers for oxidative stress.

Plasma MDA levels during the glucose clamp are depicted in Figure 2 and Table 2. Plasma MDA levels did not increase after 1 hr octreotide infusion (median [IQR] 6.6 [6.2-7.7] μmol/l to 6.8 [6.0-8.0] μmol/l, \( P = 0.89 \)). After the start of the glucose infusion, plasma MDA increased gradually accompanying the increase in blood glucose with a strong correlation between MDA and blood glucose levels (\( \rho = 0.82, P <0.001 \), Spearman correlation). Median MDA levels at the 8 mmol/l glucose plateau were significantly higher than after 1 hr octreotide infusion (9.9 [9.3-10.6] μmol/l, \( P = 0.02 \)) and were further increased substantially at the 10 mmol/l plateau (11.8 [10.8-12.5] μmol/l, \( P = 0.01 \)). No cumulative effect of glucose over time during each plateau was observed (Friedman test for repeated measures). The day after the clamp median plasma MDA levels had returned to baseline (6.4 [5.6-6.9] μmol/l, \( P = 0.24 \)).
Table 2 Plasma levels of the parameters of interest during the glucose clamp

<table>
<thead>
<tr>
<th>Marker</th>
<th>Unit</th>
<th>Baseline</th>
<th>t = 1 hr</th>
<th>6 mmol/l</th>
<th>8 mmol/l</th>
<th>10 mmol/l</th>
<th>t = 24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>MDA</td>
<td>μmol/l</td>
<td>6.6 (6.2-7.7)</td>
<td>6.8 (6.0-8.0)</td>
<td>8.0 (7.6-9.6)</td>
<td>9.9 (9.3-10.6)*</td>
<td>11.8 (10.8-12.5)*</td>
<td>6.4 (5.6-6.9)</td>
</tr>
<tr>
<td>F1+2</td>
<td>pmol/l</td>
<td>140 (118-151)</td>
<td>170 (124-474)</td>
<td>508 (275-1714)*</td>
<td>731 (446-1095)*</td>
<td>608 (445-817)*</td>
<td>136 (123-169)</td>
</tr>
<tr>
<td>vWF</td>
<td>%</td>
<td>66 (50-127)</td>
<td>63 (53-113)</td>
<td>59 (39-86)*</td>
<td>58 (39-107)*</td>
<td>55 (42-78)*</td>
<td>76 (58-130)</td>
</tr>
<tr>
<td>ETP</td>
<td>nM.min</td>
<td>1271 (1167-1415)</td>
<td>1237 (1074-1429)</td>
<td>1297 (1082-1425)</td>
<td>1263 (1137-1440)</td>
<td>1296 (1143-1486)*</td>
<td>1328 (1170-1481)*</td>
</tr>
<tr>
<td>Peak thrombin</td>
<td>nM</td>
<td>222 (200-264)</td>
<td>209 (186-244)</td>
<td>208 (180-245)</td>
<td>187 (174-245)</td>
<td>209 (189-243)</td>
<td>226 (211-265)</td>
</tr>
<tr>
<td>PAP</td>
<td>µg/l</td>
<td>366 (268-460)</td>
<td>335 (244-934)</td>
<td>540 (271-854)</td>
<td>603 (398-1060)*</td>
<td>615 (508-711)</td>
<td>426 (277-650)</td>
</tr>
<tr>
<td>d-dimer</td>
<td>mg/l</td>
<td>0.00 (0-0.18)</td>
<td>0.00 (0-0.05)</td>
<td>0.08 (0-0.23)</td>
<td>0.28 (0.05-0.51)*</td>
<td>0.36 (0.04-0.51)*</td>
<td>0.19 (0.0-0.39)*</td>
</tr>
<tr>
<td>HA</td>
<td>ng/ml</td>
<td>49.6 (48.1-50.2)</td>
<td>49.5 (47.9-50.2)</td>
<td>49.9 (48.8-50.9)</td>
<td>49.9 (47.0-51.5)*</td>
<td>50.3 (47.2-51.2)*</td>
<td>51.6 (50.4-54.5)*</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>U/ml</td>
<td>51.6 (43.6-55.3)</td>
<td>49.4 (44.0-58.3)</td>
<td>45.4 (36.3-48.6)</td>
<td>48.3 (35.4-55.2)</td>
<td>36.0 (33.4-41.4)*</td>
<td>49.5 (44.1-53.0)</td>
</tr>
</tbody>
</table>

At each glucose plateau the median (IQR) values of the mean value per patient are depicted. MDA, malondialdehyde; F1+2, prothrombin fragment 1+2; vWF, von Willebrand factor; PAP, plasmin-antiplasmin complex; ETP, endogenous thrombin potential; HA, hyaluronic acid.

* P < 0.05 compared to baseline; ^ P < 0.05 compared to t = 1
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**Coagulation**

We determined the effects of increasing glucose levels on coagulation by measuring prothrombin fragment 1+2 (F1+2) and von Willebrand factor (vWF). F1+2 are released when thrombin is formed from prothrombin and therefore provide an in vivo measure of thrombin formation. VWF plays a major role in haemostasis by ensuring the arrest of blood platelets at sites of injury, and by binding of coagulation factor VIII, but it is also an established marker of endothelial dysfunction.

The effects of the stepwise increase in blood glucose on these markers of coagulation are depicted in Figure 4 and Table 2. No significant effects on F1+2 levels or vWF were detected after octreotide infusion. Median F1+2 levels showed a significant increase from 170 (124-474) pmol/l to 508 (275-1714) pmol/l when the glucose level was raised to 6 mmol/l, further increased to 731 (446-1095) pmol/l at 8 mmol/l and remained at a stable high level at 10 mmol/l with no significant differences between the glucose plateaus. The following day F1+2 levels had returned to baseline. After raising the glucose level to 6 mmol/l vWF levels dropped to 59% (39-86, \(P = 0.02\)). An increase of glucose to 8 mmol/l led to a further decrease of vWF levels to 58% (39-107, \(P = 0.05\)), but raising the glucose level to 10 mmol/l did not cause further significant changes. After 24 hrs, vWF levels had returned to baseline values.

Finally, we determined the endogenous thrombin potential (ETP), which represents the
balance between pro- and anti-coagulant processes in plasma and provides an ex vivo measure for overall coagulability. No significant effects of the octreotide run-in period or any of the blood glucose levels on peak thrombin values could be detected (Table 2). ETP showed a significant decrease from 1271 (1167-1415) nM.min to 1237 (1074-1429) nM.min after the 1 hr octreotide period ($P = 0.01$) and a subsequent small increase to 1296 (1143-1486) nM.min at the 10 mmol/l glucose plateau ($P = 0.01$). The following day, ETP remained higher than baseline at 1328 (1170-1481) nM.min ($P = 0.05$).

Figure 3 Endothelial glycocalyx
Shedding of endothelial glycocalyx components was assessed by plasma hyaluronan levels (left panel) and activity of the regulatory enzyme hyaluronidase (right panel). Data are depicted as medians with interquartile ranges. Dotted lines represent the time points where glucose infusion was increased to go to the next glucose level. $^*P <0.05$ compared to $t = 1$, after 1 hr of octreotide infusion.

Figure 4 Coagulation
Coagulation was assessed by prothrombin fragment 1+2 (F1+2; left panel) and von Willebrand factor (vWF; right panel) plasma levels. Data are depicted as medians with interquartile ranges. Dotted lines represent the time points where glucose infusion was increased to go to the next glucose level. $^*P <0.05$ compared to $t = 1$, after 1 hr of octreotide infusion.
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Figure 5 Fibrinolysis
Fibrinolysis was assessed by measuring plasmin-alpha2-antiplasmin (PAP) complexes and d-dimer (Figure 5 and Table 2). PAP complexes serve as an indicator of recent in vivo fibrinolytic activity, since alpha2 antiplasmin is the most important circulating inhibitor of plasmin, the main enzyme in the fibrinolytic system. D-dimer is a fibrin degradation product, which is dependent on the amount of fibrin that is generated (coagulation) as well as the ability of the fibrinolytic system to degrade the generated fibrin (fibrinolysis).

Neither PAP complexes nor d-dimer had changed significantly after the 1-hr octreotide infusion period (t = 1; Figure 5). Median PAP levels were not significantly different at a blood glucose level of 6 mmol/l when compared to t = 1, but did show a significant increase at a blood glucose of 8 mmol/l (335 [244-934] μg/l to 603 [398-1060] μg/l, P = 0.01). PAP levels remained at a stable high level when blood glucose was further increased to 10 mmol/l and returned to baseline the following day. Median d-dimer levels showed an increasing trend from 0.00 (0.00-0.05) mg/l to 0.08 (0.00-0.23) mg/l when the glucose level was raised to 6 mmol/l (P = 0.07). At 8 mmol/l d-dimer levels had risen to 0.28 (0.00-0.51) mg/l (P = 0.04, compared to t = 1) and increased further to 0.36 (0.004-0.51) mg/l when blood glucose was raised to 10 mmol/l (P = 0.02, compared to t = 1). After 24 hrs, d-dimer levels remained higher than at t = 0, at 0.19 (0.00-0.39) mg/l (P = 0.04).

No cumulative effect of any of the coagulation and fibrinolysis parameters was detected at the three examined blood glucose levels, except for F1+2 at a blood glucose level of 6 mmol/l (p=0.02, Friedman test for repeated measures).
**Endothelial glycocalyx**

The effect of elevation of blood glucose levels on the endothelial glycocalyx was assessed by plasma measurement of its main component hyaluronic acid (HA) and its regulatory enzyme hyaluronidase to detect shedding from the glycocalyx. Plasma HA and hyaluronidase levels were unaffected by 1 hr octreotide infusion ($t = 1$). When raising the blood glucose level to 6 mmol/l, median HA levels remained unaffected but a raise to 8 and 10 mmol/l showed a significant, but small increase compared to $t = 1$ (from 49.5 [47.9-50.2] ng/ml to 49.9 [47.0-51.5] ng/ml, $P = 0.038$, and to 50.3 [47.2-51.2] ng/ml, $P = 0.008$). This increase persisted after 24 hrs. Plasma hyaluronidase activity showed a gradual decrease during the clamp, with significantly lower activity at a blood glucose level of 10 mmol/l (36.0 [33.4-41.4] U/ml compared to 51.6 [43.6-55.3] U/ml at $t = 1$, $P = 0.005$). After 24 hrs, these levels had returned to baseline values (Figure 3 and Table 2).

**Discussion**

In this study we show that oxidative stress, represented by MDA levels, showed a stepwise increase during the clamp, mimicking the glucose curve. The coagulation system was activated even at near normal glucose levels of 6 mmol/l, resulting in a significant increase in prothrombin fragments 1+2 (F1+2) indicating thrombin formation. This was followed by activation of the fibrinolytic system, as measured by PAP complexes and d-dimer, at a glucose level of 8 mmol/l. Relevant endothelial glycocalyx changes were not detected using biochemistry techniques, except for a decrease in hyaluronidase activity when the glucose concentration was raised to 10 mmol/l.

To our knowledge this is the first study examining the effects of isolated mild hyperglycaemia, with a maximum of 10 mmol/l, on vascular homeostasis. Previous studies on oxidative stress show glucose dependent formation of reactive oxygen species (ROS) with blood glucose levels above 10 mmol/l \(^{2,10}\), which is comparable with our findings and in vitro studies \(^{18}\). Our data suggest that hyperglycaemia dependent ROS formation is dose-dependent rather than an on-off phenomenon. This is depicted in Figure 2, showing no cumulative effect within the different glucose plateaus but only an increase in oxidative stress when blood glucose is increased to the next level. Data reported by Ceriello et al. \(^{10}\) support this finding showing higher plasma nitrotyrosine levels at a plasma glucose of 15 mmol/l compared to 11 mmol/l as well as no further increase in plasma nitrotyrosine levels when stabilizing plasma glucose. Also, in type 2 diabetes patients an impressive correlation between MDA and blood glucose (ranging from 6 to 14 mmol/l) was found after a mixed-meal test suggesting an insulin-independent effect \(^{19}\).

Our results are in line with previous observations, which have shown that hyperglycaemia...
activates the coagulation as well as the fibrinolytic system. Unlike oxidative stress, our results indicate that the glucose induced-activation of the coagulation system is an on-off phenomenon showing a more than threefold increase in thrombin generation, measured by F1+2 levels, triggered by a blood glucose level of only 6 mmol/l. This hypothesis is supported by the observation that maximum levels are reached quickly and show no increase, perhaps even a decrease, at the highest glucose level. Moreover, the maximum levels of F1+2 and d-dimer are comparable with the levels reached during a hyperglycaemic clamp at a blood glucose of 15 mmol/l previously performed by our group. The timing of the increase in fibrinolytic activity, closely following the coagulant activity, suggests that the increased fibrinolytic activity is secondary to the coagulation activation. Conversely, diabetes mellitus is associated with impairment in fibrinolysis, which we did not detect in our study. However, Stegenga et al. showed that fibrinolysis was mainly affected by hyperinsulinemia as opposed to hyperglycaemia, and insulin was suppressed throughout our experiments.

ETP changed only minimally during and after the clamp. This indicates no relevant change in the thrombin generating capacity of the coagulation system itself, but rather suggests that glucose is a trigger for the in vivo activation of coagulation. VWF levels showed a maximal decrease of 5%. This modest change could be due to increased binding to blood platelets, known to be activated by hyperglycaemia, or caused by physical inactivity of the participants. VWF levels have been shown to increase after physical exercise and previous control experiments by our group have shown a similar decreasing effect of a 6-hr saline infusion in healthy males (M. Nieuwdorp, unpublished data).

We did not detect relevant changes in plasma HA levels and a decrease in hyaluronidase activity was found only at a glucose level of 10 mmol/l. Previous investigations show marked increase in HA shedding from 70 ng/ml at baseline to 112 ng/ml with blood glucose levels of 15 mmol/l, suggesting that the trigger for direct endothelial damage as reflected by loss of glycocalyx lies above a blood glucose level of 10 mmol/l. Statistically, there was a change in plasma HA levels at 8 and 10 mmol/l. However, the maximum increase was only 1.6% indicating no significant biological effect. This is supported by the limited effects on vWf levels, which are also considered a marker for endothelial damage. The decrease in hyaluronidase activity at 10 mmol/l does indicate substrate binding to this enzyme. This substrate may consist of other glycosaminoglycans than HA shed from the glycocalyx, such as heparan sulphate or chondroitin sulphate, since these are also bound by hyaluronidase.

The results of our study are in line with epidemiological data, which show that the increase in cardiovascular risk already starts at mildly elevated glucose levels.
Nonetheless, our results indicate that glucose-induced activation of the coagulation system and ROS formation are completely reversible after 24 hrs. Therefore, these changes may not be considered to be pathological in healthy subjects who spent the greater part of the day with glucose levels below 6.1 mmol/l. Conversely, patients with diabetes or pre-diabetes by definition have a fasting glucose level of >5.6 mmol/l if untreated, and are exposed to glucose levels above 6 mmol/l throughout the day. This may interfere with the reversibility of the changes in coagulation and oxidative stress, and translate to pathological effects. Moreover, in diabetes inappropriate activation of the coagulation system may not be counterbalanced because of the fibrinolytic impairment associated with this disease. Our results do not support a role for glucose variability in coagulation activation and ROS formation, since coagulation activation occurred even at a blood glucose of 6 mmol/l and the relationship between blood glucose and oxidative stress was continuous.

Several aspects of our study need comment. First, this study was specifically designed to assess the effects of mild hyperglycaemia on several components of vascular homeostasis and was therefore performed under full suppression of insulin levels. In disease states, such as type 2 diabetes or stress-hyperglycaemia during severe illness, high glucose levels are accompanied by high insulin levels and therefore our results cannot be extrapolated directly to these settings. However, glucose levels are highly predictive of vascular complications although insulin levels are highly variable in these patients.

Second, given the design of our experiment, it can be argued that the effects we detected may not be glucose specific, but rather result from the osmotic effect of raising blood glucose or from prolonged administration of octreotide. However, previous work from our group has shown no effect on coagulation or fibrinolysis in a control experiment during which octreotide was administered in combination with mannitol infusion for six hours, serving as a time and osmolality control. Moreover, in our study no significant effect on any of the parameters after one hour of octreotide infusion was detected. This is supported by literature, showing no significant vaso-active or haemostatic effects of this dose of octreotide.

In conclusion, our results show that glucose-induced changes to vascular homeostasis already start at near normal glucose levels. Furthermore, our study reveals a dose-dependent effect of glucose on MDA formation and an on-off phenomenon for glucose induced coagulation activation, while changes to the endothelial glycocalyx occur at glucose levels of 10 mmol/l or higher. These results give us more insight in the glucose driven mechanisms of vascular complications in humans. To elucidate the difference between acute and chronic mild hyperglycaemia on vascular homeostasis, further studies are needed.
Methods

Patients
The study was approved by the institutional medical ethical committee and conducted according to Declaration of Helsinki principles. Participants signed informed consent prior to inclusion after oral and written explanation of the study.

Stepwise hyperglycaemic clamp protocol (Figure 6)
After an overnight fast, two catheters for venous access were placed, one in every arm. First, basal measurements of haemostasis and ROS formation were performed. Octreotide was dissolved in saline 0.9% and albumin 20% (proportion 59:1 in a 60 ml syringe) and administered at a final concentration of 30 ng/kg/min octreotide, to suppress endogenous insulin production. To confirm that this infusion did not influence the parameters of interest, the basal measurements were repeated after 1 hr of octreotide infusion. Hereafter, glucose infusion with 20% glucose solution was started to reach the desired glucose concentration based on a steady state principle. The plasma glucose concentration was held constant at the desired plateau for 2 hrs by determination of the plasma glucose concentration every 5 minutes and adjusting the glucose solution accordingly. When a stable glucose concentration was reached, glycocalyx dimension, ROS formation, and haemostasis parameters were measured every 30 minutes; 4 times per plateau, the last measurement being the baseline value of the next step. Glucose infusion was then increased to reach the next level of glycaemia and measurements were repeated. In total, the actual clamp took 7 hrs. Blood samples were centrifuged within 1 hr after collection and stored at -80°C.

![Figure 6 Glucose clamp protocol](image)

Depicted in the lower boxes are the times from baseline where the several actions were performed. An arrow indicates an assessment point for oxidative stress, glycocalyx and coagulation/fibrinolysis parameters.
Oxidative stress
Plasma MDA concentration was determined using high HLPC-MS/MS as described by Pilz with minor modifications. Total (free and bound) malondialdehyde (MDA) in human plasma samples was determined as the 2,4-dinitrophenylhydrazine (DNPH) derivative. After addition of the stable isotopically labeled analogue (\(^{2}H_{2}\)-MDA) as internal standard (IS), alkaline hydrolyzation, deproteinization and derivatization with DNPH, MDA-hydrazone was analyzed by HPLC-MS/MS and positive electrospray ionization. Using an Acquity UPLC system (Waters Corporation, Milford, MA), samples were injected on a LC-18-DB analytical column (250 · 4.6 mm, 5 μm particles, Supelco) hyphenated to a Quattro Premier XE mass spectrometer (Waters Corporation, Milford, MA). Analytes and IS were eluted with acetonitrile/water/acetic acid (50/50/0.2) and detected in multiple reaction monitoring (MRM) mode for the transitions m/z 235 → m/z 159; m/z 237 → m/z 161. Samples were quantified against calibration standards.

Endothelial glycocalyx
Hyaluronic acid was measured by a commercially available ELISA kit (Corgenix, Inc., Broomfield, Colorado, USA). In short, HA reacted with hyaluronic acid binding protein. Thereafter horseradish peroxidase was added to form complexes with bound HA. After addition of a chromogenic substrate the intensity of the colour was measured in optical density units with a spectrophotometer at 450 nm. Hyaluronidase activity was determined by a previously described assay.

Coagulation and fibrinolysis
D-dimer was measured on an automated coagulation analyzer (Behring Coagulation System, BSC) using protocols and reagents from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany). Antigen levels of vWF were assayed by ELISA using antibodies from Dako (Glostrup, Denmark). F1+2 and PAP were determined by ELISA from Siemens Healthcare Diagnostics and DRG (Marburg, Germany), respectively. The ETP was determined using the Calibrated Automated Thrombogram as described by Hemker et al. and the Thrombinscope manual (Maastricht, the Netherlands). Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin, Siemens Healthcare Diagnostics), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored using the Fluoroskan Ascent fluorometer (ThermoLabsystems, Helsinki, Finland), and the ETP and peak thrombin were calculated using the Thrombinscope software.

Data interpretation
The study was conducted to assess the influence of a certain level of plasma glucose on the parameters described above. We excluded samples taken at a certain glucose plateau
when the desired glucose level was exceeded by more than 1 mmol/l since crossing the desired glucose level could interfere with the study results. For example, when a plasma glucose level of 7.1 mmol/l occurred at any point during the 6 mmol/l plateau phase, all subsequent samples taken at the 6 mmol/l plateau were excluded from analysis. Moreover, samples were only included in the analysis when the desired glucose level was truly reached. This was determined by calculation of the mean glucose level of the 30 minutes before sampling which had to be within 0.5 mmol/l of the desired glucose level.

**Statistical analysis**

Baseline characteristics are expressed as mean (SD) and outcome parameters as median (IQR). Differences between plateaus were assessed by a Wilcoxon signed ranks test for paired data. The influence of time on the measurements at each glucose level was assessed using the Friedman test. All analyses were performed using Predictive Analytics Software (PASW) statistics version 18.0 (SPSS Inc., Chicago, IL, USA). A $P$-value $<0.05$ was considered statistically significant.

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References


Mild hyperglycaemia disturbs vascular homeostasis in humans