Coagulation and inflammation in diabetes mellitus

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Modified vascular response to endotoxin in streptozotocin-induced diabetic mice

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Abstract

**Background:** Oxidative stress induced vascular dysfunction is thought to contribute to the increased risk for acute arterial complications in patients with diabetes mellitus (DM). In this study we evaluated the effect of streptozotocin (STZ) induced diabetes on vascular gene expression and thrombus formation in mice with endotoxin induced oxidative stress.

**Materials and methods:** C57BL/6 mice were divided in three groups: control mice, mice treated with endotoxin (lipopolysaccharide; LPS) (5 μg Serratia Marcesens) and mice with LPS and STZ induced diabetes. Twenty four hours after LPS injection cytokines were measured in plasma. Ferric chloride-induced thrombus formation was evaluated in the carotid artery by measuring thrombus formation times and thrombus composition. Vascular gene expression was measured by rt-PCR.

**Results:** Endotoxin injection resulted in significantly increased plasma levels of TNFα, IL-6 and MCP-1 in both diabetic and non-diabetic mice. In non-diabetic LPS treated mice a trend towards prolonged time to occlusion (LPS: 11.3 ± 4.6 min vs. control: 8.8 ± 1.8 min) (P=0.1) and an increase in time from the start of thrombus formation until occlusion (closing time) (LPS: 4.9 ± 2.9 min vs. control: 2.5 ± 1.2 min) (P=0.02) was observed. In diabetic mice treated with LPS these occlusion times were not different from non-LPS treated mice. Aortic VCAM-1 mRNA level was upregulated after LPS in non-diabetic mice, but not in diabetic mice. In contrast, eNOS expression increased > 5 times in diabetic LPS treated mice but not in non-diabetic LPS treated mice.

**Conclusions:** Oxidative stress due to low dose endotoxin injection induces an antithrombotic response in control mice, which was absent in diabetic mice, as well as a different pattern of vascular gene expression compared to diabetic mice. We propose that an altered vascular thrombotic response to oxidative stress might play role in the increased risk for acute arterial thrombosis in patients with DM.
Introduction

Severe cardiovascular syndromes like myocardial infarction or stroke result from acute arterial thrombosis on a ruptured or eroded plaque (1). The development of such complications is considered to be primarily determined by preexistent vascular changes (2-4). Endothelial activation and vascular dysfunction leading to a prothrombotic phenotype of the inner vessel wall may be important determinants (5; 6). One of the causes of vascular dysfunction is oxidative stress, characterized by the generation of free radicals, like superoxide (7). Oxidative stress results in vascular dysfunction by activating signaling pathways in endothelial and other vascular cells leading to the enhanced transcription and translation of several pro-inflammatory and procoagulant genes such as vascular endothelial growth factor (8), tissue factor (TF) (9), E-selectin (10), intercellular adhesion molecule-1 (ICAM-1) (11), endothelial nitric oxide synthase (eNOS) (12), interleukin-8 (13), monocyte chemoattractant peptide-1 (MCP-1) (14) leading to reduced nitric oxide (NO) production and increased platelet and leukocyte adhesion (15), ultimately provoking thrombus formation.

A pathophysiological hallmark of cardiovascular disease is its multifactorial nature involving gene-gene and gene-environment interactions provoked by various risk factors like inflammation, diabetes mellitus and hypercholesterolemia. A shared characteristic of these risk factors is their ability to induce oxidative stress and endothelial dysfunction (16-18). Theoretically, interactions between different risk factors may have synergistic or additive effects on the development of cardiovascular complications via similar molecular pathways.

We hypothesized that in mice treated with endotoxin, well established to induce oxidative stress and endothelial dysfunction, hyperglycemia (as a dominant characteristic of diabetes) would accelerate vascular inflammation and endothelial dysfunction as well as arterial thrombus formation. In this study, we evaluated the effect of endotoxin induced oxidative stress on vascular gene expression in mice with streptozotocin induced diabetes and control mice. Interaction of endotoxin and diabetes mellitus on the prothrombotic potential of the vessel wall was examined by measuring ferric chloride induced thrombus formation in the carotid artery.
Mice, materials and methods

Animals and Treatment

Female C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands). The study was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, the Netherlands. Mice were divided in three groups: 10 control mice, 10 mice received a LPS injection, 8 mice received a LPS injection after induction of diabetes. Diabetes was induced by a single intraperitoneal infusion of STZ (200 mg/kg bw) in 0.05 M citrate buffer (pH 4) (19) at the age of 8 weeks (n= 8 mice). If the STZ injection did not induce hyperglycemia (glucose > 10 mmol/L) after 4 days, the injection was repeated. Non-diabetic mice were injected with citrate buffer alone. LPS (5 μg Serratia Marcesens LPS) was injected ten weeks after STZ or control injection in the foot.

Arterial thrombosis

Twenty four hours after LPS injection mice were anesthetized by intraperitoneal injection of 70 μl/g bw FFM mixture (Fentanyl (0.315 mg/ml) - Fluanisone (10 mg/ml) (Janssen Pharmaceutical, Beerse, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands)). Body temperature was monitored with a rectal probe and maintained at 37°C ± 1°C via a heating pad and a halogen-heating lamp. During anesthesia extra oxygen (1 L/min) was supplemented via a tube placed at the nose of the mouse. Acute arterial thrombosis was induced by application of ferric chloride (25% FeCl₃) to the left carotid artery, as described (20) resulting in the formation of platelet and fibrin-rich thrombi (21). Before and after application of ferric chloride a Doppler flow probe was placed around the artery to measure blood flow. Time to occlusion (TTO) was defined as the time required for blood flow to decline to < 0.2 mL/min after initiation of arterial injury with ferric chloride (Figure 1). Closing time was defined as the time from the start of the flow reduction i.e. thrombus formation to the final occlusion (Figure 1).

Plasma analysis

Blood glucose levels were measured with Glucometer® Elite (Bayer Diagnostics, Mijdrecht, The Netherlands) in a drop of blood from the tail vein. At the end of the experiment blood from the vena cava was sampled into a plastic syringe containing 0.1 volume of 3.2% sodium citrate. Plasma levels of thrombin-
Endotoxin and diabetes in arterial thrombosis model

**Figure 1.** Typical example of flow registration of ferric chloride induced thrombus formation in female C57Bl/6 mouse. "Time to occlusion" is the time from the ferric chloride application to final occlusion. "Closing Time" is the time from the start of the flow reduction to the final occlusion.

Antithrombin (TAT) complexes were measured by a specific murine sandwich TAT ELISA, developed in our laboratory (22; 23). Plasma levels of interleukin (IL)-6, MCP-1 and TNFα were measured by Cytometric Bead Array Analysis (Beckton Dickinson Biosciences, Pharmingen, San Diego, USA). Aspartate aminotransferase (ASAT) and creatinine in plasma and creatinine and microalbumin in urine were measured using standard techniques.

**Tissue harvesting and histological analysis**

At the end of the experiments, the left carotid arteries were formalin-fixed, embedded in paraffin and sectioned. For immunohistochemical analysis paraffin sections of 4 μm were deparaffinized and rehydrated. Thrombus composition was evaluated using haematoxylin-eosin staining. For detection of TF expression sections were incubated with 1.5% H₂O₂ in PBS for 20 minutes and then blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween-20%, pH 8.0) for 30 minutes at room temperature. Thereafter sections were washed and incubated with primary antibodies against TF O/N at 4°C. Rabbit anti-mouse TF antibodies were developed in our laboratory by immunization of rabbits with a mixture of 500 ml Freund's Complete Adjuvant (Difco) and murine TF peptide P5 (23; 24). Final concentration of anti-TF antibody was 1.3 μg/ml. After incubation with the primary antibodies tissue sections were washed and goat anti-rabbit (DAKO A/S, Glostrup, Denmark) was used as the secondary antibody in a 1:250 dilution for 1 hour at room temperature, and then washed with PBS. The sections were incubated with SABC complex (DAKO A/S) for one hour at
room temperature, and washed. Enzyme activity was detected with AEC (Sigma) after incubation for 5 minutes. Specificity controls included the use of normal rabbit immunoglobulin instead of specific primary antibodies. For evaluation of TF immunostaining a semi-quantitative score approach was chosen using 20-fold magnification. For TF quantification in thrombus and vessel wall the following criteria were used: 0: no staining; 1: focal staining; 2: diffuse staining. The degree of staining was evaluated blinded for treatment allocation.

Quantitative rt-PCR
Expression of genes involved in inflammation and coagulation including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial nitric oxide synthase (eNOS), monocyte chemoattractant protein-1 (MCP-1), TF, endothelial selectin (E-selectin) and platelet selectin (P-selectin) were analyzed on mRNA level by quantitative real time PCR as described (25). Total RNA was extracted from homogenates from the descending aorta using TriZol reagent (Life Technologies) according to the manufacturer’s instructions. Purified RNA was reverse transcribed (RevertAid M-Mul V Reverse Transcriptase) according to protocols supplied by the manufacturer. Quantitative gene expression was performed on an ABI PRISM 7700 combination of hardware and software (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers were designed using Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems) and validated for identical efficiencies (slope = -3.3 for a plot of Ct versus log ng cDNA). SYBR Green master mix (19 ml) was added to 5 ml cDNA (corresponding to 50 ng of total RNA input) and 300 nM of forward and reverse primers in water. The samples were heated for 2 min at 50°C and 10 min at 95°C. Subsequently 40 PCR cycles consisting of 15 sec at 95°C and 60 sec at 60°C were applied. At the end of the run samples were heated to 95°C with a ramp time of 20 min to construct dissociation curves to check that single PCR products were obtained. The absence of genomic DNA contamination in the RNA preparations was confirmed by using total RNA samples that had not been subjected to reverse transcription. Acidic ribosomal phosphoprotein PO (36B4) was used as the standard housekeeping gene (25). Ratios of target gene and 36B4 expression levels (relative gene expression numbers) were calculated by subtracting the threshold cycle number (Ct) of the target gene from Ct of 36B4 and raising 2 to the power of this difference. Ct values
are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. Target gene mRNA expressions are thus expressed relative to 36B4 expression.

Statistics
Data are expressed as means ± SEM. Non-normal distributed mRNA data and categorical data are expressed as medians with 25-75 quartiles. For comparison between normal distributed variables of interest ANOVA was used. The association between non-normal distributed variables was assessed using the Kruskal Wallis Test. P values < 0.05 are considered statistically significant.

Results
Determination of levels of glucose, systemic inflammation, and organ failure
Blood glucose levels were significantly higher in the diabetic mice as compared to all non-diabetic mice (diabetic: 23.9 ± 5.8 vs. non-diabetic: 7.3 ± 2.2 mmol/l; P < 0.001) measured before LPS injection.

![Figure 2](image-url)

*Figure 2.* Plasma levels of inflammatory markers were significantly increased in diabetic and non-diabetic mice treated with LPS (LPS + DM and LPS) compared to non-LPS treated mice (no-LPS) (Fig 2ABC). In addition, markers of liver and kidney damage in plasma were higher in all LPS treated mice compared to control mice (Fig 2DE). All LPS induced inflammatory and organ damage markers did not statistically differ between diabetic and non-diabetic LPS treated mice, except for the creatinine-albumin ratio in urine, which was significantly higher in diabetic mice treated with LPS compared to both non-diabetic mice with and without treatment with LPS (Fig 2F).
An overview of markers of systemic inflammation and organ failure 24 hours after LPS injection is shown in Figure 2. In mice injected with LPS a significant upregulation of TNFα, IL-6 and MCP-1 in plasma was measured indicating ongoing inflammation. No statistically significant difference was observed in plasma cytokine levels between diabetic mice treated with LPS and non-diabetic mice treated with LPS. Plasma markers for kidney (creatinine) and liver failure (ASAT) were also increased 24 hours after LPS injection in both diabetic and non-diabetic mice (P<0.05), whereas the albumin-creatinine ratio in urine, a marker for kidney damage, was only significantly increased in diabetic mice treated with LPS.

Acute arterial thrombosis
Application of ferric chloride to the carotid artery resulted in thrombus formation within 30 minutes in all mice. In non-diabetic mice treated with LPS a two minute mean increase of time to occlusion (11.3 ± 4.6 min) compared to non-LPS treated mice (8.8 ± 1.8 min) was observed. This difference was however not statistically significant (P=0.1) (Figure 3A). In addition, we observed an increase in the time from the start of thrombus formation until occlusion (closing time) (Figure 3B) in the LPS treated mice (4.9 ± 2.9 min) compared to control mice (2.5 ± 1.2 min) (P=0.02). In the LPS treated mice with diabetes no increase in time to occlusion nor in closing time was found compared to control mice (Figure 3B). In contrast, the closing time was significantly shorter in LPS treated diabetic mice compared to LPS treated non-diabetic mice (P=0.04).

![Figure 3](image-url)

**Figure 3.** Time to occlusion (figure 3A) and closing time (figure 3B) after ferric chloride application to the carotid vessel wall in control mice (no LPS), LPS treated mice (LPS) and LPS treated mice with diabetes (LPS + DM).
After the induction of carotid thrombosis the level of plasma TAT complexes was significantly higher in LPS treated mice with diabetes compared to non-diabetic mice with and without LPS treatment (control mice (81.4 (79.3-94.8 ng/ml), LPS treated mice (45.4 (10.6-172.5) ng/ml) and diabetic LPS treated mice (189.8 (133.9-482.7) ng/ml; P=0.03).

**Thrombus composition and immunohistochemistry**

Serial sections of the left carotid artery segment that had been treated with ferric chloride showed total occlusion by a platelet rich thrombus (Figure 4). Erythrocytes amidst platelets were seen in adjacent sections. Ferric chloride was seen in the vessel wall and on the endothelium in the vessel lumen of the segment that had been treated. No difference could be observed in thrombus composition between mice with and without LPS treatment. No difference between the three groups was observed in immunostaining for TF in the occluded vessel wall (no LPS: 1 (1; 1.5) vs. LPS: 1 (1; 1.5) vs. LPS and diabetes: 1 (1; 1.75) (P = 0.4) and in the thrombus (no LPS: 2 (1; 2) vs. LPS: 2 (2; 2) vs. LPS and diabetes: 2 (1.25; 2) (P = 0.5).

*Figure 4.* Typical example of thrombus in carotid artery after ferric chloride application.

**RT-PCR**

Gene expression of VCAM-1, ICAM-1, eNOS, MCP-1, TF, E-selectin and P-selectin in the aorta was compared between control mice (n=10), LPS treated mice (n=10) and LPS treated mice with diabetes (n=8). For an overview see figure 5. Two main findings were observed. First, in normal mice LPS injection induced a greater than two fold increase in median VCAM-1 level in the vessel wall, but did not increase VCAM-1 expression in diabetic mice (Fig 5A). A similar trend of increased
Figure 5. Relative mRNA levels of VCAM-1, ICAM-1, eNOS, MCP-1, TF, E-selectin and P-selectin in aorta from non-LPS treated mice (no LPS), LPS treated mice (LPS) and LPS treated mice with diabetes (LPS + DM).
expression after LPS injection in normal mice, but not in diabetic mice was observed for ICAM-1 expression (Fig 5B). Second, in the diabetic mice eNOS expression was more than 5 fold higher after LPS injection than in non-LPS treated mice. This increase was only moderate and not statistically significant after LPS in non-diabetic mice (Fig 5C). A similar pattern was observed for MCP-1, TF, E-selectin and P-selectin expression, although none of these differences reached statistical significance (Fig 5DEFG).

Discussion
Oxidative stress is considered to be involved in the pathogenesis of the increased risk for acute arterial complications, like myocardial infarction or stroke, in patients with diabetes mellitus. In the present experiment the effect of oxidative stress, inflicted by a subacute inflammatory trigger, on thrombus formation and vessel wall gene expression was measured in mice with STZ induced diabetes and control mice. The major findings are as follows: 1) Low dose LPS resulted in sustained systemic inflammation and organ failure 24 hours after injection in both normal and diabetic mice. 2) In non-diabetic mice VCAM-1 expression in the vessel wall was enhanced 24 hours after LPS injection. In diabetic mice LPS injection did not result in increased VCAM-1 expression, but did induce a more than 5-fold increase in eNOS expression as compared to non-LPS treated mice. 3) In non-diabetic mice LPS treatment induced a delay in thrombus formation and similar thrombin generation as compared to non-LPS treated mice. In contrast, in diabetic mice treated with LPS thrombus formation was comparable to non-LPS treated mice, while thrombin generation was elevated. These data show that systemic inflammation due to low dose LPS injection induces a (temporary) antithrombotic state in normal mice, but not in diabetic mice. This absence of an inflammation induced antithrombotic state might play a role in the increased risk for acute arterial thrombosis in patients with DM.

Effect of LPS on acute arterial thrombosis
LPS administration induces oxidative stress and thereby activation and dysfunction of endothelial and other vascular cells (26) (27). Since inflammation and oxidative processes are key components of atherothrombosis, we used the LPS induced inflammation model to study the effect of endothelial activation on thrombus
formation in diabetic mice. In contrast to our expectations a prolonged duration of thrombus formation was observed 24 hours after low-dose LPS injection in non-diabetic mice. At the same time we observed signs of systemic inflammation and organ failure, indicating that the LPS effect was still present at the time of thrombus induction.

An explanation for the reduced rate of thrombus formation 24 hours after LPS infection might be adaptation of the endothelial cells to the oxidative stress induced by LPS. Previous in vitro observations show that 24 to 48 hours of incubation with pro-inflammatory stimuli such as LPS or IL-1β induces a state of hyporesponsiveness in endothelial cells (28). During this refractory period endothelial TF expression and TF related procoagulant activity is reduced in response to the initial stimulus. Similar thrombo-protective effects of LPS administration are seen in in vivo studies in relation to myocardial ischemia and reperfusion (29-31) and this effect is referred to as “preconditioning”. The latter studies suggest that one of the mechanisms involved in LPS preconditioning is adaptation of coronary endothelial cells to oxidative stress resulting in reduced myocardial infarction. Since this adaptive mechanism of endothelial cells to oxidative stress or low dose LPS has also been demonstrated in various other arterial beds such as pulmonary arteries (32) and in the gastric mucosa (33), it seems likely that also the carotid arteries may become adapted to oxidative stress. Thus, we speculate that the antithrombotic phenotype in the vessel wall of the LPS-treated mice might be explained by adaptation to the oxidative stress induced by LPS injection 24 hours before in the present study. The mechanism behind adaptation to oxidative stress is not known but may involve upregulation of eNOS and thereby increased NO production (34), which is presumably the critical element in TNFα induced antithrombotic activity (35). Such mechanisms might have played a role in the antithrombotic phenotype in the LPS treated mice in the present study, although the upregulation of eNOS in the LPS treated mice was not statistically higher (no LPS: 0.06 (0.03; 0.07) vs. LPS: 0.08 (0.06; 0.14) (P > 0.05)).

Interestingly, LPS injection in the diabetic mice resulted in a different vascular reaction compared to the non-diabetic mice: no reduced thrombus formation, no increase in VCAM-1, significantly higher expression of eNOS and increased TAT levels were observed. We speculate that oxidative stress secondary to hyperglycemia and present before the LPS injection might have been responsible
for these differences in thrombotic responses. There is evidence that hyperglycemia is associated with a reduced adaptive response to endothelial injury caused by ischemia/reperfusion damage (36; 37). For instance, it was shown that endothelial dysfunction due to ischemia/reperfusion injury can be prevented by preconditioning with only one period of ischemia in control mice, whereas in diabetic hearts three periods of ischemia are necessary to obtain the same endothelial protective effect. It is possible that in the present study a similar diminished capacity to adapt to endothelial injury explains why the thrombotic occlusion time in diabetic LPS treated mice was reduced compared to the control LPS treated mice. The mechanisms behind these different reactions to oxidative stress remain unclear. One explanation may be the observed difference in upregulation of eNOS expression in the vessel wall between the control and diabetic mice. In general, upregulation of eNOS can be explained as a protective reaction of the vessel wall via production of NO. However, there is also evidence that chronic overexpression of eNOS accelerates atherosclerosis in apoE knockout mice and is associated with reduced production of NO (38). The present data show that a more than 5-fold increase in eNOS expression, observed in the diabetic mice injected with LPS, is not associated with a thrombo-protective effect, whereas a small increase (although not statistically significant) observed in normal mice injected with LPS is associated with a prolongation of thrombus formation. Thus, these data are in line with observations that in a state of oxidative stress due to for instance diabetes mellitus increased eNOS expression is not automatically vasoprotective, but may even contribute to endothelial dysfunction. This unfavorable action of eNOS, called uncoupling, leads to a shift towards production of superoxide instead of NO and is probably induced by reduced availability of cofactor tetrahydrobiopterin (39).

In addition to eNOS expression, increased thrombin activation, measured as higher TAT levels may have contributed to the accelerated thrombus formation in diabetic mice treated with LPS as compared to non-diabetic LPS treated mice. It has been clearly established that diabetes mellitus is associated with higher levels of circulating coagulation proteins (40). Whether these markers have a causal relation with the increased risk for arterial thrombosis remains unclear. The present data suggest that higher TAT levels may be responsible for the differences in thrombus formation between LPS treated mice with and without diabetes.
TF gene expression remained unchanged in the aortic vessel wall after LPS treatment. It is, however, assumed that TF upregulation in endothelial cells and monocytes plays a crucial role in thrombotic complications of patients with sepsis and DIC (41). There is indeed a huge body of in vitro data showing that both endothelial cells (42-45) and monocytes (46; 47) express TF after stimulation of LPS and other inflammatory agents. In addition, various in vivo studies show high levels of circulating soluble TF (48) and TF positive monocytes in patients with severe sepsis (49). However, in vivo data on TF expression by vascular cells argue against TF upregulation in the vessel wall during sepsis (50; 51). Drake et al. observed TF expression only in the splenic microvasculature from baboons treated with a lethal dose of Escherichia coli (50). Erlich and co-workers showed LPS induced TF expression in many different cell types, such as glomeruli and epithelial cells, but not in any vascular bed in rabbits (51). In addition we did not observe upregulation of TF gene expression in the vessel wall of mice treated with low-dose LPS. Our finding further suggests that TF expression in the vessel wall is not importantly affected by LPS, at least 24 hours after induction. In contrast to TF expression, VCAM-1 expression did significantly increase after LPS treatment in the present experiment. This is in line with other observations in various in vivo sepsis models indeed showing increased VCAM-1 expression in lungs (52), liver (53) and hearts (54). Increased VCAM-1 expression is supposed to be a marker of disturbed endothelial homeostasis, which favors neutrophil adhesion and thereby increases organ damage during sepsis.

In conclusion, in normal mice a single inflammatory stimulus results in a partial protection against arterial thrombosis after 24 hours. However, in conditions of hyperglycemia, and probably related to oxidative stress responses, the protective influence of inflammation against thrombosis is no longer detectable. This difference in thrombotic responsiveness may in part explain the greater susceptibility of patients with diabetes mellitus to atherothrombotic complications. We speculate that this mechanism may be clinically relevant considering the increased susceptibility to infections of diabetic patients.

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References

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