Coagulation and inflammation in diabetes mellitus
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Diabetes mellitus does not increase thrombogenicity of the arterial vessel wall in atherosclerotic LDLr-/- mice

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Abstract

**Background:** Diabetes mellitus is associated with an increased risk for acute arterial thrombosis. We evaluated the effect of hyperglycemia on expression of proinflammatory and prothrombotic genes, as well as on the prothrombotic potential of the vessel wall in LDLr-/- mice.

**Materials and methods:** LDLr-/- mice were divided in three groups: mice on control chow, mice on Western-type diet and mice on Western-type diet in combination with streptozotocin induced diabetes. The extent of atherosclerosis was assessed at the aortic sinus. Vascular gene expression was measured by rt-PCR. Ferric chloride induced thrombus formation was evaluated in the carotid artery.

**Results:** Diabetic mice possessed significantly higher glucose and cholesterol levels than control mice. Mean atherosclerotic lesion size increased non-significantly to 140% in diabetic mice compared to control mice (p=0.066). Lesion size was correlated to both glucose and cholesterol levels. Tissue factor and p-selectin mRNA levels were increased in mice on Western diet compared to control fed mice. No further augmentation was observed in diabetic mice on Western diet. Surprisingly, no difference was observed in thrombus formation between mice with and without Western-type diet and with or without diabetes.

**Conclusions:** Although induction of diabetes, leading to hyperglycemia and hypercholesterolemia, was associated with larger plaque size, no changes in vascular gene expression and no effect on thrombus formation were observed. This suggests that the increased risk for atherothrombotic complications in diabetes mellitus is probably more related to accelerated atherosclerosis than to diabetes specific prothrombotic changes in the vessel wall.
Introduction

Diabetes mellitus is associated with accelerated atherosclerosis, which ultimately leads to an increased risk for complications such as myocardial infarction and stroke. Myocardial infarction is in general the result of acute arterial thrombus formation on a ruptured or eroded atherosclerotic plaque (1). The development of these complications is largely determined by properties of the preexisting vascular lesion (2-4). Accordingly, there is evidence that diabetes-related changes in the arterial vessel wall may account for the increased risk for acute arterial thrombotic complications (5-7).

Several studies reported increased signs of inflammation such as higher expression of tumor necrosis factor-\(\alpha\) (8), endothelial adhesion molecules (9) and increased NF-\(\kappa\)B activation in diabetic atherosclerotic plaques compared to normal plaques (10). Increases in PAI-1 expression have been observed in atherectomies and arteries from diabetic patients, which may have led to increased thrombogenicity (11; 12). Studies in animals with streptozotocin (STZ) or alloxan induced diabetes report higher levels of the pro-coagulant protein tissue factor (TF), and an upregulation of pro-inflammatory cytokines and adhesion molecules in the vessel wall (13) (14; 15).

Endothelial dysfunction and endothelial activation are also considered to increase the risk for acute thrombotic complications in patients with diabetes (16; 17). Endothelial dysfunction is typically associated with decreased nitric oxide (NO) production and increased platelet and leukocyte adhesion (5). Activated endothelial cells may produce cytokines and express adhesion molecules (18) as well as TF (19) that may shift the normal anticoagulant phenotype of the endothelial cell to a prothrombotic phenotype (6; 20).

Despite these observations the contribution of hyperglycemia, the main characteristic of diabetes mellitus type 1 and 2, to larger vessel disease and atherothrombotic complications remains disputed (21; 22). Our primary objective was to establish whether hyperglycemia changes the anticoagulant properties of the normal arterial vessel wall into a more procoagulant phenotype, increasing the risk of atherothrombosis. Accordingly, the purpose of the present study was two-fold. Firstly, we assessed the relationship between hyperglycemia and procoagulant and pro-inflammatory gene expression in the vessel wall. Secondly, we established the effect of hyperglycemia on the thrombogenic potential of the
vessel wall. These objectives were addressed in a model of streptozotocin (STZ) induced diabetes in LDLr/- mice.

**Mice, materials and methods**

**Animals and diets**

All animal experimentation was approved by the regulatory authority of Leiden University and was carried out in compliance with guidelines issued by the Dutch government. Male LDLr/- mice, 9-15 weeks of age, bred in our own colonies, were used in this study. Mice were fed standard chow before being divided in three groups: 10 control LDLr/- mice kept standard chow diet; 8 LDLr/- mice were put on a “Western-type” diet and 9 mice were put on a “Western-type” diet and were injected with streptozotocin (STZ) in order to induce diabetes. The Western-type diet contained 0.25% cholesterol and 15% cocoa butter (Hope Farms, Woerden, The Netherlands). Two weeks after start of the Western-type diet diabetes was induced by a single intraperitoneal infusion of STZ (100 mg/kg bw) in 0.05 M citrate buffer (pH 4) (23). If one STZ injection did not induce hyperglycemia (glucose level of > 10 mmol/L) after 4 days, the injection was repeated. The two other groups (standard chow or Western-type diet) were injected with citrate buffer alone.

**Measurement of glucose, cholesterol and thrombin-anti-thrombin complexes**

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. Cholesterol levels were quantified colorimetrically by enzymatic procedures (Roche, Mannheim, Germany) using Precipath (Roche) as internal standard. Plasma levels of thrombin-antithrombin (TAT) complexes were measured by a specific murine sandwich TAT ELISA, developed in our laboratory (24).

**Carotid injury in mice**

Ten weeks after STZ or citrate buffer injection mice were anesthetized by intraperitoneal injection of 70 μl/g FFM mixture (Fentanyl (0.315 mg/ml)- Fluanisone (10 mg/ml) (Janssen Pharmaceutical, Beerse, Belgium), Midazolam (5 mg/ml)
Ateria ll thrombosi s i n diabete s LDLr-/ - mici e (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 supplemental oxygen (1 L/min) was given through use of a tube placed at the nose of the mouse. Acute arterial thrombosis was induced by application of ferric chloride (40% FeCl₃) to a normal-appearing segment of the common carotid artery, as described (25) resulting in the formation of platelet and fibrin-rich thrombi (26; 27). Before and after application of ferric chloride a Doppler flow probe was placed around the artery to measure blood flow before and for 30 minutes after injury. Time to occlusion (TTO) was defined as the time that elapsed between initiation of arterial injury with ferric chloride and a decline of blood flow to < 0.2 ml/min (see Figure 1).

![Time to occlusion](image)

**Figure 1.** Typical example of flow measurement in carotid artery of the LDLr/- mouse. Basal flow was around 0.7 ml/min. After 5 minutes of ferric chloride treatment it took 10 to 20 minutes before vessel occlusion by thrombus formation occurred.

**Quantification of atherosclerosis**
At the end of the experiments the mice were sacrificed by exsanguination and the hearts were collected and fixed in phosphate-buffered 3.7% formalin, embedded in OCT compound (Tissue Tek; Sakura Finetek, Zoeterwoude, The Netherlands). Subsequently, transverse 10 μm cryosections were prepared and stained with oil red O (BDH,Ltd.,UK). The atherosclerotic lesion area in the sections was quantified using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK). Mean lesion area was calculated (in mm²) from 10 sections, starting at the appearance of the tricuspid valves as described previously (28).
Evaluation of thrombus composition
The left carotid arteries were formalin-fixed, routinely processed for paraffin embedding and 4 μm sections were serially cut and every 60th section was stained with H&E. Thrombus area was measured using Image Pro Plus software (Media Cybernetics, Inc., USA). Mean thrombus area was calculated from measurements at three fixed locations in the carotid artery starting at the carotid bifurcation and at the next 180th and 360th section in the direction of the heart.
For immunohistochemical analysis, sections were incubated with 1.5% H$_2$O$_2$ in PBS for 20 minutes and then blocked with 10% goat serum in PBS for 30 minutes at room temperature. Subsequently, sections were incubated with primary antibodies against fibrin O/N at 4°C. Rabbit anti-rat fibrin antibody was kindly provided by Dr. J. Emeis, TNO, The Netherlands and used in a 1:2000 dilution(29) 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) and 0.0075% H$_2$O$_2$. Specificity controls included the use of normal rabbit immunoglobulin instead of specific primary antibodies. For the evaluation of fibrin immunostaining a semi quantitative score approach was chosen using a magnification of 20 times using the following criteria: 0: no staining in thrombus; 1: focal positive staining in thrombus. The degree of staining was evaluated blinded for treatment strategies.

Quantitative RT-PCR
Expression of genes involved in inflammation and coagulation, TF, interleukin 1-Beta (IL-1β), endothelial nitric oxide synthase (eNOS), plasminogen activator inhibitor type 1 (PAI-1), monocyte chemoattractant peptide-1 (MCP-1), platelet selectin (P-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) was analyzed at the mRNA level by quantitative real time PCR as earlier described (30). Total RNA was extracted from homogenates of 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 the protocols supplied by the manufacturer (Fermentas, St.Leon-Rot, Germany). Quantitative RNA measurements
were performed on an ABI PRISM 7700 system (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 (30). 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 categorical data and mRNA data are expressed as medians with 25-75 quartiles. For comparison between normal distributed variables of interest the one way ANOVA was used. The association between non-normal distributed variables was assessed using the Kruskall Wallis test. Turkey's Multiple Comparison Test was used for post-hoc analysis. P values < 0.05 are considered statistically significant.

Results
Determination of glucose and cholesterol levels
The metabolic parameters of LDLr-/ mice at the time of thrombus induction are displayed in Figure 2. As expected, blood glucose levels were significantly higher in mice injected with STZ-induced diabetes (23.4 ± 2.0 mmol/L) as compared to mice injected with control buffer (standard chow: 7.4 ± 0.5 mmol/L; Western-type diet: 8.1 ± 0.5 mmol/L) (p<0.0001). Cholesterol levels were much higher in mice
on Western-type diet (1086 ± 126 mg/dL) as compared to mice on standard chow diet (271 ± 31 mg/dL) and significantly further increased in mice on Western-type diet plus STZ-induced diabetes (1699 ± 215 mg/dL) (p<0.0001). Regression analysis showed that an increase in glucose level was highly correlated to an increase in cholesterol level ($r^2=0.613$) (p<0.001) in LDLr/- mice (see Figure 3).

Atherosclerosis
Aortic root atherosclerotic lesion area was measured in LDLr/- mice on a Western-type diet and compared with LDLr/- mice on a Western-type diet and STZ induced diabetes. Lesion area more than doubled in mice with diabetes as compared to non-diabetic mice, but the difference was not statistically significant (p=0.066), see Figure 4. To identify possible mechanisms for the increase in lesion area in STZ-mice, regression analysis for relation between lesion area, glucose and cholesterol were assessed. A highly significant correlation was observed between glucose level and lesion area ($r^2=.585$) (p < 0.001). In addition, a highly significant correlation between cholesterol level and lesion area was found ($r^2=.563$) (p<0.001).
Atherosclerotic plaque

\[ p = 0.066 \]

Figure 4. Mean atherosclerotic plaque size in LDLr-/- on Western-type diet and in LDLr-/- mice on Western-type diet and diabetes

Thrombus formation after ferric chloride application

After induction of ferric chloride injury, thrombotic occlusion occurred in 90% of mice fed control diet and in 100% of mice fed Western-type diet and in 100% of mice on Western-type diet in combination with diabetes. The time to occlusion was similar in all groups (Fig. 5). Also the mean size of the thrombus did not differ between the normal chow fed mice (21 ± 6 arbitrary units (a.u.)), Western-type diet fed mice (23 ± 9 a.u.) and Western-type diet fed with STZ induced diabetic mice (19 ± 7 a.u.) (p=0.614). In contrast to our expectations a trend towards smaller thrombus area was observed at the site of the bifurcation in the Western-type diet fed mice with diabetes (11 ± 7 a.u.) compared to Western-type diet fed mice (17 ± 1 a.u.) (p=0.06). Western-type diet and diabetes induction did not increase in vivo thrombin formation, indicated by similar levels of TAT complexes in all three groups after thrombus induction.

Figure 5. Thrombotic response after ferric chloride application to the carotid artery in LDLr-/- mice. No difference was observed in mean time to occlusion and median levels of TAT complexes in mice on control diet (no diet), Western-type diet (diet) and on Western-type diet and STZ-induced diabetes (diet + DM).
Thrombus composition and immunohistochemistry

A typical example of an occluded artery is shown in Figure 6. Serial sections from the left carotid artery segment that had been treated with ferric chloride showed in all arteries total occlusion by a platelet rich thrombus. Some accumulation of erythrocytes was seen in adjacent sections. Ferric chloride could be retraced in the vessel wall and on the endothelium in the vessel lumen of the segment that had been treated. There was no evidence for differences in thrombus composition between mice on standard chow and the two groups of mice on Western-type diet. No difference was observed in immunostaining for fibrin between the mice on control diet (1 (0;2)), mice on Western-type diet (2 (1; 2) or mice on Western-type diet and STZ-diabetes (2 (1;2) (p=0.7).

RT-PCR

Gene expression of TF, IL-1β, eNOS, PAI-1, MCP-1, P-selectin, ICAM-1 and VCAM-1 was studied in the aortas from LDLr/- mice, LDLr/- on Western-type diet and LDLr/- on Western-type diet and diabetes (Figure 7). The levels of mRNA encoding TF and P-selectin were significantly increased in mice on Western-type type diet as compared to mice on control diet (both 20 % increase) (p<0.05). Also, a trend towards increased IL-1β (p= 0.06) and MCP-1 (p= 0.07) was observed. The increase in MCP-1 gene expression was statistically significant in mice on Western-type diet and diabetes compared to mice on control chow. In diabetic mice also an increase in TF expression was observed as compared to mice on control chow. However, no significant additional effect of diabetes induction on gene expression was observed when mice on Western-type diet and mice on Western-type diet and diabetes were compared (see Figure 7). No differences were observed in PAI-1 and eNOS gene expression among the three groups.

Figure 6. Representative example of an occluded carotid artery after ferric chloride in LDLr/- mouse.
In order to pinpoint possible mechanisms for the change in gene expression between the mice on standard chow and the mice on Western-type diet with or without diabetes the correlations between gene expression, glucose and cholesterol were assessed. In contrast to our expectations, no significant correlations were observed between glucose levels and any of the mRNA levels that were tested. On the other hand, cholesterol did correlate with TF (r=0.539) (p=0.005), P-selectin (r=0.420) (p=0.037), ICAM-1 (r=0.458) (p=0.024) and MCP-1 (r=0.511) (p=0.009) expression. No significant correlation was observed between PAI-1 and cholesterol concentrations.

**Discussion**

In the present study the effects of STZ induced diabetes on procoagulant and inflammatory gene expression were assessed in relation to the thrombogenic potential of larger vessels in atherosclerosis prone LDLr-/ mice.

The major findings can be summarized as follows. Firstly, the combination of diabetes and Western-type diet had a substantial effect on total cholesterol levels in blood, which was more robust than the effect of Western-type diet alone. Secondly, the size of the atherosclerotic lesions was more pronounced in diabetic LDLr-/ mice than in non-diabetic mice although the difference in lesion size was borderline not significant. Thirdly, a Western-type diet increased mRNA levels of a number of pro-inflammatory genes as well as of the TF gene in the aorta, but this effect was not further enhanced by the combination of Western-type diet.
and diabetes, with the exception of MCP-1 of which the level of expression was significantly higher in diabetic mice. mRNA levels were significantly correlated to cholesterol levels in blood, but not to glucose levels. Fourthly, there were no significant differences in the level of thrombin generation between diabetic and normal LDLr-/- mice and there was no indication of altered thrombus formation in the carotid artery upon stimulation with ferric chloride.

Role of hyperglycemia in atherosclerosis and vascular homeostasis
In line with other publications we observed that STZ-induced diabetes is associated with increased progression of atherosclerosis in LDLr-/- mice (31-33). Although this increase did not reach statistical significance (p=0.066) in the present study, probably due to the wide variation in plaque size among the diabetic animals, the association between diabetes and atherosclerotic progression was confirmed by the strong relationship ($r^2= .585$) (p < 0.001) between hyperglycemia and the size of the atherosclerotic plaque. However, we cannot conclude that hyperglycemia per se is associated with atherosclerotic progression and vascular changes in this model for the following reasons. First, cholesterol levels were markedly increased in diabetic mice and cholesterol levels were strongly related to glucose concentrations. Second, cholesterol levels correlated to the same extent to plaque area as did glucose levels. Third, the observed induction of pro-inflammatory genes and TF in the aorta was only correlated with cholesterol levels and not with glucose concentrations. Even MCP-1, the gene differentially upregulated in the diabetic mice on a Western-type diet, was not correlated to hyperglycemia, but to cholesterol level. Taken together, these data suggest that the influence of hyperglycemia in this model of diabetes induced progressive atherosclerosis may be predominantly dependent on the secondary hypercholesterolemia rather than hyperglycemia per se. Thus, our observations strongly suggest that hypercholesterolemia is the dominant factor for the increased risk for atherosclerosis in STZ-induced diabetes.

Vascular inflammation and thrombus formation
In the present experiments we observed clear pro-inflammatory effects of Western-type diet and diabetes indicated by increased gene expression of MCP-1, P-selectin and TF in the vessel wall. Such alterations are indicative of an increased prothrombotic potential of the vessel wall that may translate in an increased risk of thrombus
formation (34). However, our experiments did not reveal an increase in thrombin generation nor arterial thrombus formation after ferric chloride exposure. One factor of importance may be that in contrast to TF, MCP-1 and p-selectin gene expression, eNOS and PAI-1 expression were not affected by Western-type diet and diabetes. E-NOS regulates nitric oxide production, which is a key player in the regulation of thrombus formation. There is evidence that changes in nitric oxide (35) influence thrombus formation in this ferric chloride induced thrombosis model. In addition, it has been shown that changes in PAI-1 expression play a dominant role in regulating thrombus formation in ferric chloride induced thrombus formation (36). In fact, eNOS and PAI-1 may be the prominent factors in this process of ferric chloride induced thrombus formation. This might explain that in the present study no change in thrombogenicity is observed by Western-type diet and STZ induced diabetes since they did not affect eNOS and PAI-1 expression in the vessel wall.

Effect of diet and diabetes on in vivo thrombus formation
Epidemiologic studies have clearly shown that both diabetes mellitus and hypercholesterolemia increase the risk for acute arterial thrombotic complications, such as myocardial infarction. In vivo studies on the relation between diet and hyperglycemia and the risk of atherothrombosis have, however, yielded conflicting data (36-43). In the present study we did not observe any effect of Western-type diet or diabetes neither on thrombus size, nor on time to occlusion. A lack of effect of high fat diet on in vivo thrombus formation was also recently reported by Schäfer and co-workers when they compared wild-type and apoE/-/- mice in the same ferric chloride induced thrombosis model (36). In that study an increased time to occlusion was observed in the Apo-E/-/- mice compared to wild-type mice, but not in the wild-type mice on Western-type diet. Other studies also reported comparable times to occlusion in mice with and without high fat diet (37; 41) or only changes in size or composition of the thrombus in hypercholesterolemic (37; 38; 41) or diabetic animals (43). In the present study we did not observe an increase in thrombus size, in contrast, we noted a trend towards smaller thrombus size in the mice fed Western type diet and diabetes at the carotid bifurcation, which is at the distal end of the thrombus. From these and literature data we cannot explain the differences in study data which could be due to many factors including exact type of diet, duration of experiment, animal
species and sex and so on. One specific factor involved in the size and composition of thrombi is the altered platelet function in the diabetic animals compared to the non-diabetic animals (42). Indeed, preliminary data show that platelets from hyperglycemic mice have a greater response to collagen ex vivo as compared to non-diabetic animals (E. Bevers et al, personal communication).

In conclusion, our data favor the concept that large arterial vessel disease and changes in vascular gene expression in diabetes are primarily due to secondary hypercholesterolemia rather than hyperglycemia. The observed vascular alterations are not associated with an increased thrombogenicity in the chosen animal model. Given the experimental limitations, we speculate that pro-inflammatory and prothrombotic changes in the vessel wall are not the main determinants of thrombogenicity in diabetes. Accelerated atherosclerosis, increased plaque vulnerability, and/or features of the metabolic syndrome are probably more significant contributors to the risk of atherothrombosis in diabetes mellitus.

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