

File ID 106861  
Filename Chapter 10 Hyperglycemia accelerates arterial thrombus formation and attenuates the antithrombotic response to endotoxin in mice

---

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation  
Title The role of blood coagulation in cancer, inflammation and embryonic development  
Author L.W. Bruggemann  
Faculty Faculty of Medicine  
Year 2008  
Pages 198  
ISBN 9789064642593

FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/273480>

---

*Copyright*

*It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use.*

---

## **Hyperglycemia accelerates arterial thrombus formation and attenuates the antithrombotic response to endotoxin in mice**

Hjalmar R. Hansen<sup>1,2,3</sup>, Jef L. Wolfs<sup>4</sup>, Lois W. Brüggemann<sup>1</sup>, Dirkje W. Sommeijer<sup>1,2</sup>, Eduard Bevers<sup>4</sup>, Arnaud D. Hauer<sup>5</sup>, Johan Kuiper<sup>5</sup>, C. Arnold Spek, Henri M.H. Spronk<sup>3</sup>, Pieter H. Reitsma<sup>1</sup> and Hugo ten Cate<sup>1,3</sup>

<sup>1</sup>Laboratory for Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, <sup>2</sup>Department of Internal Medicine, Slotervaart Hospital, Amsterdam, Departments of Internal Medicine<sup>3</sup> and Biochemistry<sup>4</sup>, Cardiovascular Research Institute Maastricht, Academic Hospital and Maastricht University, Maastricht and <sup>5</sup>Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden, The Netherlands

## Abstract

Recent human studies reveal that hyperglycemia induces procoagulant and antifibrinolytic effects in blood that may contribute to a greater risk of arterial thrombosis, but the direct relationship between high blood glucose levels and thrombosis has not yet been investigated. We performed a number of experiments to clarify whether hyperglycemia was causally related to arterial thrombosis and whether the combined stimulus of hyperglycemia and inflammation would enhance the thrombotic effect.

In a model of ferric-chloride-induced carotid artery thrombosis, hyperglycemia did not influence the time to occlusion in mice pretreated with streptozotocin, but the rate of thrombus formation was accelerated. This effect was associated with increased thrombin generation and could not be explained by changes in vessel wall tissue factor activity. The prothrombotic effect of hyperglycemia was assessed in a separate experiment, showing that collagen/thrombin-induced platelet procoagulant activity was increased in hyperglycemic mice.

The effect of inflammation was studied by injecting a low dose of endotoxin that caused a systemic inflammatory state after 24 hours (increased plasma levels of tumor necrosis factor  $\alpha$ , interleukin-6 and monocyte chemoattractant protein 1 in diabetic and non-diabetic mice) associated with a mild delay in thrombus formation. This reduced rate of thrombus formation was attenuated by hyperglycemia.

Together, these data establish a discrete, but clear contribution of hyperglycemia in experimental arterial thrombosis.

## **Introduction**

Diabetes mellitus is a major cause of cardiovascular disease in large parts of the world [1] and atherothrombotic complications are more prevalent in patients with diabetes than in non-diabetic individuals [2, 3]. In spite of the major differences in pathophysiology of cardiovascular disease between the main types of diabetes, type 1 and 2, there is at least one common characteristic - hyperglycemia. Hyperglycemia is a hallmark of diabetes, present for variable periods of time in patients with newly detected diabetes and is thought to be involved in many processes that contribute to vascular complications. Although large clinical trials support strict regulation of blood glucose as a method to limit macrovascular disease, the specific contribution of hyperglycemia to large vessel disease has not yet been entirely established (discussed in [4, 5]). In fact, in the vast majority of patients with type 2 diabetes, other risk factors involved in atherothrombosis such as high cholesterol and hypertension may have obscured the influence of hyperglycemia as a specific contributing factor [4].

With regard to its effect on blood coagulation and thrombosis, hyperglycemia is known to be associated with enhanced platelet reactivity and increased production of thromboxane A<sub>2</sub>, a major platelet agonist substance [6, 7]. On the basis of a number of different studies it has been concluded that blood platelets in patients with diabetes are generally hyperreactive to concentrations of agonists that are below threshold levels in non-diabetic persons. Whether this is true for type 1 and type 2 diabetic patients alike is unknown, but a strong correlation between thromboxane B<sub>2</sub> levels and HbA<sub>1c</sub> suggests that at least blood glucose is a common factor in this hyperreactivity [7].

With regard to plasmatic coagulation activity recent studies in healthy persons convincingly showed that hyperglycemia induces a procoagulant response and a reduced fibrinolytic activity, the latter due to increased plasminogen activator inhibitor-1 (PAI-1) concentrations [8, 9]. These experiments also clearly indicated that hyperglycemia rather than insulin levels, is important in the procoagulant reaction. Although these human studies provide important insight into the mechanisms that underlie thrombosis, however, the generation of thrombin and attenuation of fibrinolysis in itself is not yet sufficiently indicative of a greater risk of thrombosis. In fact, no experiments have directly addressed the question whether hyperglycemia contributes to arterial thrombosis and which mechanisms may be involved.

Atherothrombosis develops in the course of atherosclerosis, which is an inflammatory disease [10]. If hyperglycemia would accelerate arterial thrombosis then the contribution of inflammation, which is known to cause procoagulant effects *in vivo*, could theoretically further enhance the prothrombotic effect of high glucose levels.

We undertook the present study to address one principal question: is hyperglycemia a trigger of arterial thrombosis and if so, is this related to vessel wall or blood-borne factors? To answer this question we utilized a model of ferric-chloride-induced carotid artery thrombosis in mice. The second question, addressed in the same experimental model, was based on the knowledge that thrombin generation driven by pro-inflammatory mediators is a recognized

model for prothrombotic mechanisms in inflammation. Since atherosclerosis, the basis of human atherothrombotic complications, is an inflammatory disease, the combination of hyperglycemia and inflammation was expected to enhance the prothrombotic tendency in this model.

## Materials and methods

### *Study design*

The data described in this paper are from three subsequent experimental studies. In the first experiment we explored the thrombogenic effect of hyperglycemia in C57Bl/6 mice. For this purpose we compared two experimental groups: one consisting of normal wildtype C57Bl/6 mice (n=11), the other of mice of the same genotype that were made diabetic by streptozotocin administration (n=10) (see below).

In the second experiment we were interested to determine the potentiating effect, if any, of inflammation inflicted by endotoxin administration, on thrombogenicity in diabetic mice, after having determined in experiment one that hyperglycemia accelerated thrombus formation. Hence, three comparative groups were used: control normal mice (n=10), mice with lipopolysaccharide (LPS) only (n=10) and mice with LPS plus hyperglycemia (n=8).

In the third experiment we were interested in exploring the role of platelets in thrombin generation as a postulated mechanism underlying accelerated thrombus formation by hyperglycemia, and we compared platelets obtained from diabetic versus non-diabetic mice.

### *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 Committees of the Academic Medical Center, University of Amsterdam, as well as that of the Maastricht University, both in the Netherlands.

Hyperglycemia was induced by a single intraperitoneal infusion of streptozotocin (STZ) (200 mg/kg body weight) in 50 mM citrate buffer (pH 4) at the age of 8 weeks [11]. If one STZ injection did not induce hyperglycemia (glucose > 10 mmol/l) after 4 days, the injection was repeated with one single intraperitoneal injection of 150 mg STZ/kg body weight. Control mice were injected with citrate buffer alone.

### *Arterial thrombosis*

Ten weeks after streptozotocin 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) (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 supplied via a tube placed at the nose of the mouse. Acute arterial thrombosis was induced by application of

ferric chloride (25% FeCl<sub>3</sub>) to the left carotid artery, as described [12], resulting in the formation of platelet-rich and fibrin-rich thrombi [13]. 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 after initiation of arterial injury with ferric chloride required for blood flow to decline to < 0.2 ml/min (Figure 4A). Closing time was defined as the time from the start of the flow reduction i.e. thrombus formation to the final occlusion (Figure 4A).

#### *Inflammatory stimulation*

Endotoxin (5 µg *Serratia marcescens* LPS) was injected ten weeks after STZ or control injection in the foot. This dose was based on the priming injection used in the Schwartzman model for disseminated intravascular coagulation (DIC) [14] and does in itself not elicit thrombosis or DIC.

#### *Plasma analysis*

Blood glucose levels were measured with a Glucometer® Elite (Bayer Diagnostics, Mijdrecht, The Netherlands) in a drop of blood from the tail vein. At the end of the experiment 3.2% (w/v) sodium citrate in a total volume of body weight (g) per 13 x 100 µl was intravenously administered in the vena cava 20-30 seconds prior to blood drawing from the same vein into a syringe. Blood samples were centrifuged for 15 minutes at 3000 rpm at room temperature and subsequently plasma was centrifuged for 5 minutes at 13 000 rpm to remove remaining cells and platelets, and immediately frozen at -80°C. Plasma levels of thrombin-antithrombin (TAT) complexes were measured by a specific murine sandwich TAT enzyme-linked immunosorbent assay (ELISA) as described previously [15]. Plasma levels of interleukin-6 (IL-6), macrophage chemotactic protein (MCP)-1 and tumor necrosis factor α (TNF-α) 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, aortas were collected and stored at -80°C whereas the left carotid arteries were formalin-fixed, embedded in paraffin, and sectioned. For immunohistochemical analysis, paraffin sections of 4 µm were deparaffinized and rehydrated. Sections were incubated with 1.5% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS) for 20 minutes and then blocked with TENG-T (10 mM Tris, 5 mM EDTA, 150 mM 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 tissue factor (TF) and fibrin overnight at 4°C. Rabbit anti-mouse TF antibodies were developed in our laboratory by immunization of rabbits with a mixture of 500 µl Freund's Complete Adjuvant (Difco) and murine TF peptide P5 [16]. Final concentration of anti-TF antibody was 1.3 µg/ml. Rabbit anti-rat fibrin antibody was kindly provided by dr. J. Emeis (TNO, The Netherlands) and used in a 1:1000 dilution [17]. 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, St Louis, Missouri, USA) after incubation for 5 minutes. Specificity controls included normal rabbit immunoglobulin in place of specific primary antibodies. For evaluation of TF and fibrin immunostaining, a semi quantitative score approach was chosen using a magnification of 20 times. For TF quantification the following criteria were used: 0: no staining in thrombus and vessel wall; 1: focal staining in thrombus; 2: diffuse staining in thrombus; 3: positive staining in thrombus and focal staining in vessel wall. For fibrin immunostaining the following criteria were used: 0: no staining in thrombus; 1: focal positive staining in thrombus. The degree of staining was evaluated blinded for treatment strategies.

#### *Tissue factor activity*

Tissue factor activity was determined using an in-house assay. In brief, arterial tissue parts were homogenized in TBS, 50 mM n-octyl  $\beta$ -D-glucopyranoside, pH 8.4 and immediately frozen in liquid nitrogen and subsequently thawed at 37°C. Supernatants were collected through centrifugation at 14 000 rpm, 4°C for 15 min, and stored at -80°C. For TF activity, samples were diluted in 25 mM HEPES, 175 mM NaCl, pH 7.7 and incubated with reaction buffer at 37°C for 20 min. Final concentrations were: 0.72 nM recombinant factor VIIa (Novo Nordisk, Bagsværd, Denmark), 60 nM bovine factor X (Sigma-Aldrich, St. Louis, MO), 3 mM  $\text{Ca}^{2+}$ , 7.2  $\mu\text{M}$  20:80 phosphatidylserine : phosphatidylcholine. Factor Xa activity was kinetically measured using the chromogenic substrate S-2765 (final concentration of 0.7 mg/ml diluted in 50 mM Tris-HCl, 175 mM NaCl, 30 mM sodium ethylenediamine tetraacetic acid ( $\text{Na}_2\text{EDTA}$ ), pH 7.4; Chromogenix, Milan, Italy) for 15 minutes, each 15 seconds at 405 nm at 37°C.

#### *Quantitative reverse transcriptase polymerase chain reaction*

Expression of genes involved in inflammation and coagulation, interleukin-1 $\beta$  (IL-1 $\beta$ ), TF, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), monocyte chemoattractant peptide-1 (MCP-1), and endothelial selectin (E-selectin) was analyzed on the mRNA extracted from homogenates from the descending aorta, by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) on an ABI PRISM 7700 combination of hardware and software (Applied Biosystems, Foster City, California, USA) using SYBR Green technology as earlier described [18]. 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 raise this difference to the square. The 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.

### *Platelet purification and procoagulant activity*

Blood was collected as described above. To each volume of citrated murine blood, five volumes of HEPES buffer containing 10% (w/v) bovine serum albumine (BSA) (10 mM HEPES, 137 mM NaCl, 2.7 mM KCL, 2 mM MgCl<sub>2</sub>, 5 mM glucose, pH 7.4) were added. The platelet-rich plasma was collected after centrifugation for 6 minutes at 200 g and subsequently the platelet concentration was determined using a Coulter counter and adjusted at 1·10<sup>6</sup>/ml using HEPES buffer containing 0.05% (w/v) BSA.

Platelets were activated with collagen (5 µg/ml) and thrombin (4 nM) in the presence of CaCl<sub>2</sub> (3 mM) for 10 min at 37°C under continuous stirring. Platelet procoagulant activity was measured by addition of factor Xa, factor Va and prothrombin to the activated platelet suspension. More specifically, the following conditions were used (final concentrations): 0.7·10<sup>6</sup>/ml platelet concentration, 1 nM factor Xa, 2 nM factor Va, 3 mM CaCl<sub>2</sub>. Thrombin formation was started by addition of prothrombin (555 nM) and arrested after 4 minutes by addition of 5 mM ethylenediamine tetraacetic acid. Thrombin was measured using the chromogenic substrate S2238.

BSA (essentially fatty acid free), was obtained from Sigma (St Louis, MO). Coagulation factors factor Xa, factor Va, prothrombin and thrombin were purified from bovine blood as described before [19]. Thrombin-specific chromogenic substrate S2238, was obtained from Chromogenix (Möln dal, Sweden). Horse tendon collagen was from Horm Nycomed (Münich, Germany). All other reagents were of the highest grade commercially available.

### *Statistics*

Data are represented as the mean with standard error of mean for normally distributed variables, and as median with 25-75% quartiles for non-normally distributed variables. Differences between groups were assessed using non-paired Student's *t*-test for data with a normal distribution or Mann-Whitney test for data with non-normal distribution. P values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS version 12.01 for Microsoft Windows (SPSS Inc., Chicago, Illinois, USA).

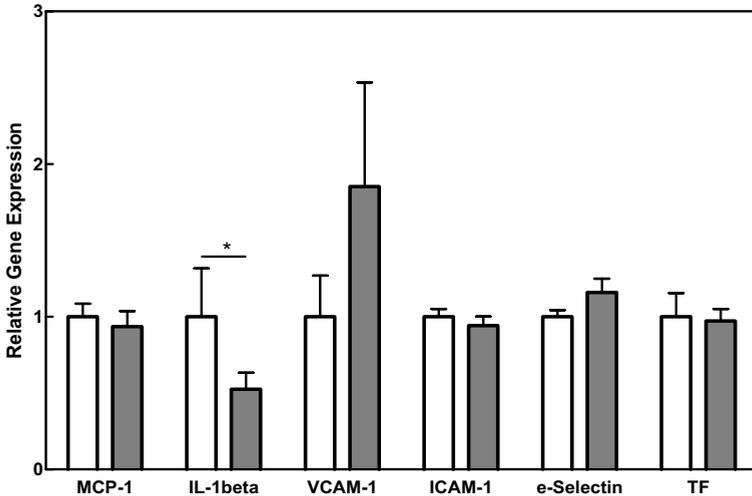
## **Results**

### *Hyperglycemia and systemic inflammation*

In the first experiment, diabetic mice were compared with non-diabetic C57Bl/6 mice with regard to inflammatory gene expression and thrombosis. In this experiment blood glucose levels were 22.2 ± 5.8 mmol/l in the diabetic versus 7.3 ± 2.2 mmol/l in the control mice, respectively. No plasma was obtained for systemic inflammatory cytokines, assuming that hyperglycemia as such would not induce systemic inflammation. The results of aorta gene expression levels are indicated in Figure 1, showing that diabetes as such did not induce inflammatory gene expression in the vessel wall. In fact, only a significant suppression of Il-1β was observed.

### Hyperglycemia, systemic inflammation and organ failure after endotoxin injection

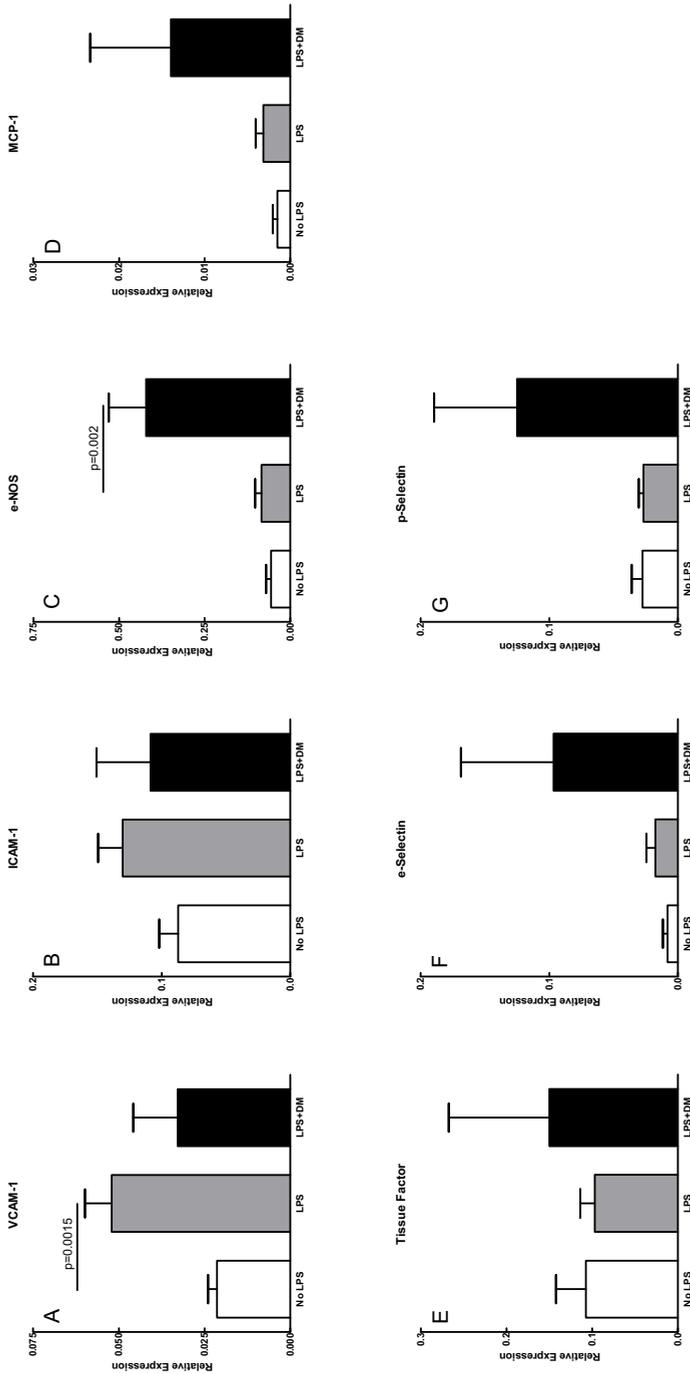
Blood glucose levels were significantly higher in the diabetic mice as compared with all non-diabetic mice (diabetic:  $23.9 \pm 5.8$  vs. non-diabetic:  $7.3 \pm 2.2$  mmol/l;  $p < 0.001$ ) measured before endotoxin injection.



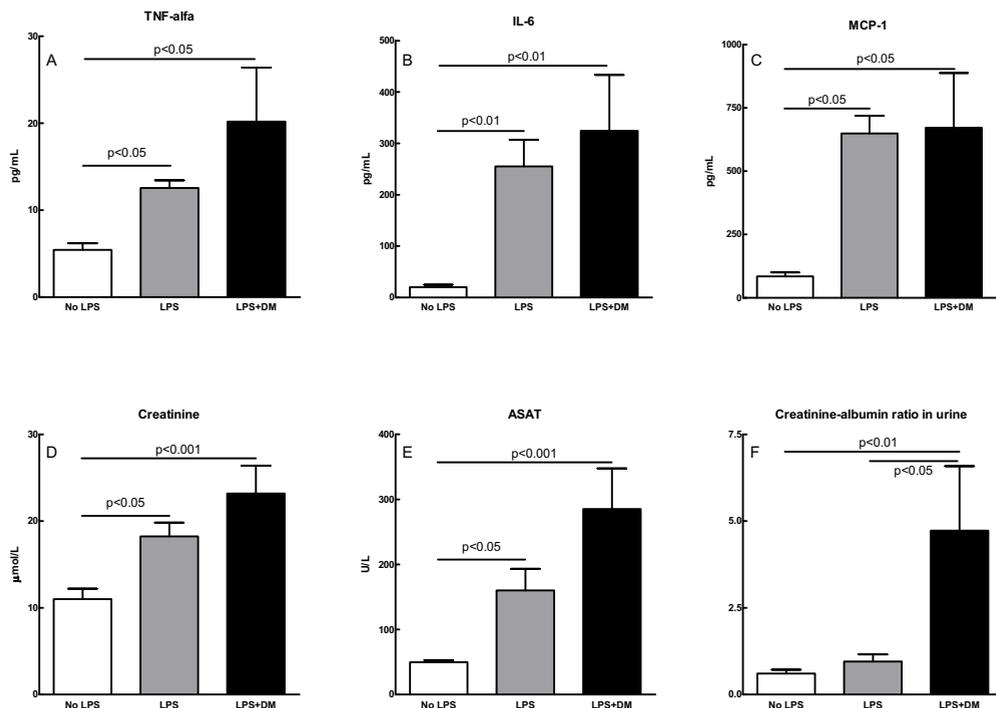
**Figure 1.** Relative mRNA levels in aortas from control mice (white bars,  $n=11$ ) and streptozotocin induced hyperglycemic mice (gray bars,  $n=19$ ). Relative expression levels of MCP-1, IL-1  $\beta$ , VCAM-1, ICAM-1, E-selectin, and TF in aortas from hyperglycemic animals are presented as the ratio of relative expression levels in control aortas. \* Significantly lower IL-1 $\beta$  mRNA in streptozotocin-induced hyperglycemic mice ( $p=0.039$ ) as compared to non-treated control mice.

Figure 2 shows the effect of diabetes against the background of inflammation (after LPS treatment) on mRNA levels in the aorta. Two findings stand out. First, endotoxin injection induced a greater than two fold increase in median VCAM-1 level in the vessel wall of normal mice, but not in diabetic mice. A similar trend of increased expression after endotoxin injection in normal mice, but not in diabetic mice was observed for ICAM-1 expression (Figure 2). Second, in the diabetic mice endothelial nitric oxide synthase (eNOS) expression was more than five-fold increased after endotoxin injection. In non-diabetic mice the increase after endotoxin in eNOS expression was only moderate and not statistically significant. Similar patterns were observed for MCP-1, TF, E-selectin and P-selectin expression, although none of these differences reached statistical significance.

An overview of plasma markers of systemic inflammation and organ failure 24 hours after endotoxin injection is shown in Figure 3. In mice injected with endotoxin, a significant increment in plasma concentrations of TNF- $\alpha$ , IL-6 and MCP-1 was measured, indicating ongoing inflammation. No statistically significant difference was observed in plasma cytokine levels between diabetic mice treated with endotoxin and non-diabetic mice treated with endotoxin. Plasma markers for kidney function (creatinine) and liver damage (ASAT) were also increased 24 hours after endotoxin injection in both diabetic and non-diabetic mice



**Figure 2.** Relative mRNA levels of (A) VCAM-1, (B) ICAM-1, (C) eNOS, (D) MCP-1, (E) TF, (F) E-selectin and (G) P-selectin in aorta from 10 non-endotoxin-treated mice (no LPS), 10 endotoxin-treated mice (LPS) and 8 endotoxin treated mice with diabetes (LPS + DM).



**Figure 3.** Plasma levels of inflammatory markers were significantly increased in diabetic and non-diabetic mice treated with endotoxin (LPS + DM and LPS) compared with non-endotoxin-treated mice (no LPS): (a) TNF- $\alpha$ , (b) IL-6, (c) MCP-1. In addition, markers of liver and kidney damage in plasma were higher in all endotoxin-treated mice compared with control mice: (d) creatinine, (e) ASAT. All endotoxin-induced inflammatory and organ failure markers did not statistically differ between diabetic and non-diabetic endotoxin-treated mice, except for the creatinine-albumin ratio in urine (f), which was significantly higher in diabetic mice treated with endotoxin compared with both non-diabetic mice with and without treatment with endotoxin. Data from mice with diabetes only are not available.

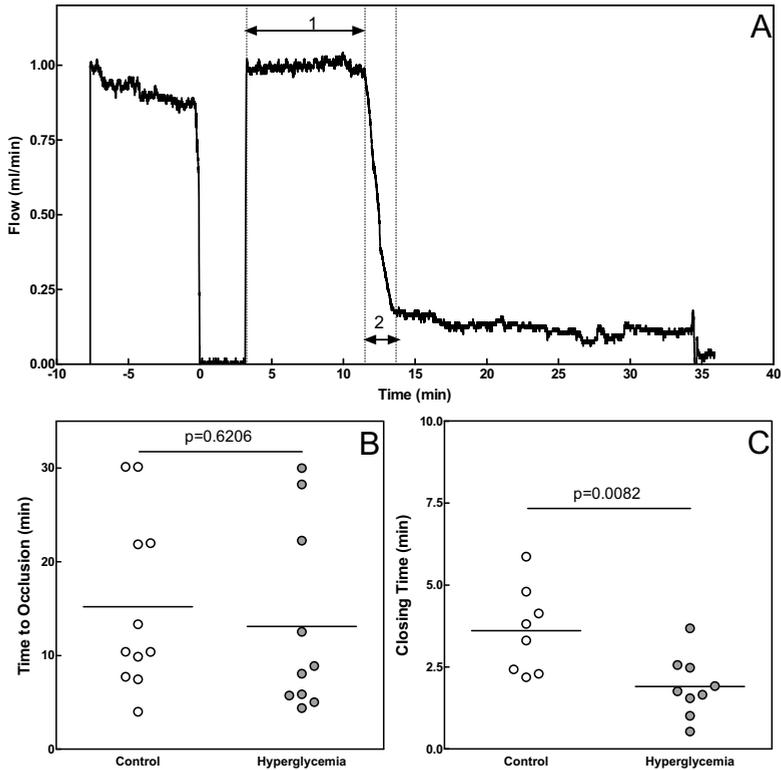
( $P < 0.05$ ), whereas the albumin-creatinine ratio in urine, a marker for kidney damage, was only significantly increased in diabetic mice treated with endotoxin.

### *Hyperglycemia and acute arterial thrombosis*

Application of ferric chloride to the carotid artery resulted in thrombus formation within 30 minutes in most mice. The typical flow curve is schematically indicated in Figure 4a.

In the first experiment we addressed the effect of hyperglycemia on thrombus formation. As illustrated, there was a considerable variation in times to occlusion (Figure 4b), but the closing time showed a statistically significant reduction in the diabetic mice, suggesting accelerated thrombus formation (Figure 4c).

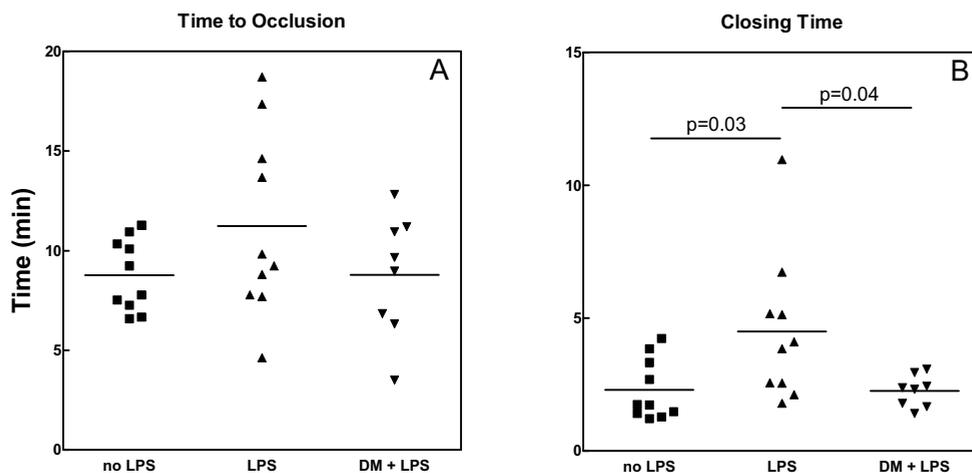
In the second experiment the effect of hyperglycemia superimposed on inflammation was studied. No diabetes only group was included in this particular experiment.



**Figure 4.** Ferric-chloride-induced arterial thrombosis in carotid artery. (A) Typical example of flow pattern during ferric-chloride-induced arterial thrombosis of the carotid artery with measurement of time to occlusion (1) and closing time (2). Effects of streptozotocin-induced hyperglycemia on arterial thrombosis. (B) No effects of hyperglycemia on time to occlusion. Control: ○, Hyperglycemia: ●. (C) More rapid thrombus formation in hyperglycemic mice, measured from the start of thrombus formation to final occlusion (closing time). The closing time was only measured when a thrombus was formed during the experiment (one vessel did not occlude after ferric chloride treatment in 9 diabetic mice and thrombus formation was absent in 3 out of 8 control carotid arteries).

In contrast to our expectation, the endotoxin treated mice showed a trend towards prolonged time to occlusion and a significant prolongation of the closing time (Figure 5). This subtle antithrombotic effect of LPS was attenuated by hyperglycemia, showing a statistically significant reduction in closing time to a level comparable to control levels (no diabetes, no inflammation) (Figure 5).

After the induction of carotid thrombosis, plasma TAT complexes were considerably higher in diabetic mice (220 (50-2120) ng/ml) than in non-diabetic mice (38 (20-100) ng/ml;  $p=0.032$ ) in the first experiment. In the second experiment, such TAT levels were significantly higher in endotoxin-treated mice with diabetes (189.8 (133.9-482.7) ng/ml) compared to normal control mice (45.4 (10.6-172.5) ng/ml), or normal mice after treatment with LPS (81.4 (79.3-94.8) ng/ml) by analysis of variance (ANOVA);  $p=0.03$  for difference among the groups).



**Figure 5.** Time to occlusion (A) and closing time (B) after ferric chloride application to the carotid vessel wall in control mice (no LPS: ■), endotoxin-treated mice (LPS: ▲) and endotoxin-treated mice with diabetes (DM+LPS: ▼). There were no statistical differences in the Time to Occlusion between the groups. Compared with the control group, Closing Time was significantly longer in the LPS group and was reduced to baseline in mice with diabetes and LPS (DM+LPS)

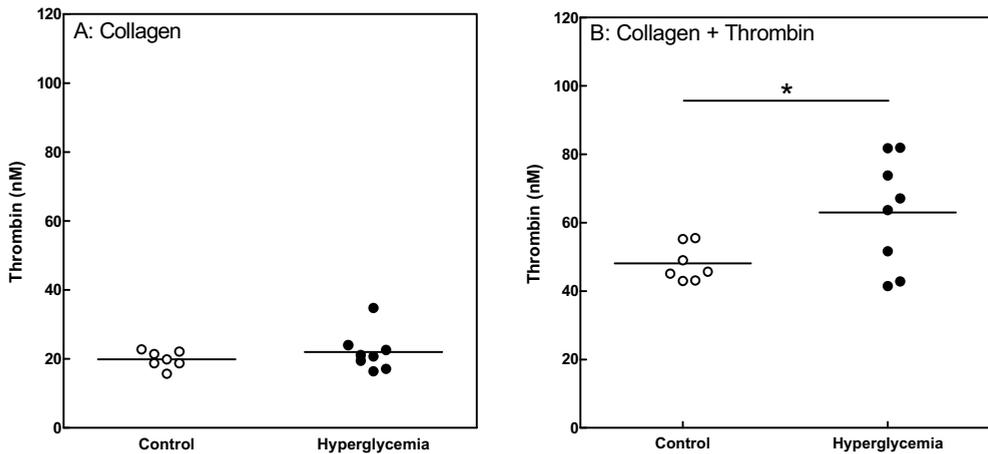
#### *Thrombus composition and immunohistochemistry*

Serial sections of the left carotid artery segment that had been treated with ferric chloride showed total occlusion by a platelet-rich thrombus (data not shown). 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 significant differences among any of the groups in the two experiments were observed with regard to the semi quantitative analysis of immunostaining for tissue factor in the occluded vessel walls, or thrombus composition (data not shown).

#### *Platelet prothrombinase activity and vessel wall tissue factor in relation to hyperglycemia*

Since the mild prothrombotic effect of hyperglycemia observed after ferric chloride induction was not readily explained by local thrombus and vessel wall factors, we carried out a third experiment to address platelet function related to thrombin generation in streptozotocin-treated mice, without additional endotoxin stimulation. The main argument for this separate experiment was that the accelerated clot formation, together with the enhanced thrombin generation (TAT levels) post thrombosis, suggested a circulating prothrombotic factor, with the literature suggesting platelets to be the likely factor involved. In a separate, therefore, C57Bl/6 mice were made hyperglycemic by streptozotocin as indicated above. Mean blood glucose values were  $15.9 \pm 1.3$  mmol/l in the diabetic group and  $8.2 \pm 0.4$  mmol/l in control mice. To ensure that hyperglycemia would not influence vessel wall tissue factor activity we determined this in aorta homogenates. This analysis showed comparable levels in aortas from control and hyperglycemic animals ( $1762$  pM  $\pm 272$  and  $2086$  pM  $\pm 566$ , respectively;  $p > 0.05$ ).

Platelet prothrombinase activity as analyzed by thrombin formation in response to collagen alone was not different between hyperglycemic and control animals (Figure 6A). In an assay in which collagen and thrombin were added in combination to induce platelet prothrombinase activity, thrombin formation was enhanced in hyperglycemic animals ( $62.8 \pm 4.6$  nM/min) as compared with control mice ( $48.1 \pm 2.0$  nM/min,  $p=0.016$ , data given for the 4 minutes time point) (Figure 6B).



**Figure 6.** Effect of hyperglycemia on platelet procoagulant activity induced by collagen (A) and collagen plus thrombin (B) in control (open circles) and hyperglycemic (closed circles) animals. The concentration of thrombin produced in the prothrombinase reaction was determined 4 minutes after the reaction was initiated. Values are the mean and SD of  $n=8$  animals per experiment. \* Significantly increased with respect to the control group.

## Discussion

Although hyperglycemia is a characteristic feature of diabetes type 1 and 2, its influence on atherothrombosis remains poorly characterized. Recent studies in humans, however, indicate that in addition to the known effects of hyperglycemia on platelet reactivity, a procoagulant as well as an anti-fibrinolytic effect is initiated upon experimentally induced hyperglycemia [8, 9]. Direct proof of a hyperglycemic effect on occurrence of arterial thrombosis had however not yet been reported.

Our experiments provide two novel outcomes that link hyperglycemia to thrombosis. First, although hyperglycemia does not have any impact on time to occlusion in the established model of ferric-chloride-induced carotid artery thrombosis, the rate of thrombus formation is accelerated. Second, while in the experimental model of inflammation a (temporary?) delayed thrombus formation occurred, hyperglycemia attenuated this effect, again pointing in a prothrombotic direction.

In the experimental thrombosis model the application of ferric chloride is known to induce endothelial cell damage, leading to exposure of subendothelial platelet binding and activating proteins including collagen [20]. This model has been widely used for testing prothrombotic conditions and investigating mice with a functional deficiency of the anticoagulant cofactor thrombomodulin indeed showed a shortened time to occlusion, confirming the validity of the model in our hands [21].

In general, thrombus formation depends on interactions of the damaged vessel wall with blood components. The lack of difference in time to occlusion between hyperglycemic and normoglycemic mice in our study suggests that the factor “hyperglycemia” did not have a major impact on most of the components of Virchow’s triad. Indeed, we did not find a difference in TF gene expression (both mRNA and protein activity) and TF activity in the arterial vessel wall (aorta), nor a conspicuous difference in composition of the clot. In addition, we did not observe a pro-inflammatory action of high blood glucose on the vessel wall as indicated by unaltered gene expression levels of a number of pro-inflammatory mRNAs. The arterial vessel wall therefore appears not to be grossly perturbed with regard to pro-inflammatory and procoagulant gene expression levels. To explain the accelerated rate of arterial thrombus formation we assumed that an effect on platelets may be responsible, taking into account the similarly increased thrombin generation in hyperglycemic mice. In order to study the contribution of platelets in this process we set up a separate experiment involving hyperglycemic mice. Platelets were isolated from control and hyperglycemic mice and diluted to an appropriate concentration for the study of thrombin generation after collagen stimulation. These conditions mimic the *in vivo* situation, where platelets stick to collagen on the inner surface of the damaged vessel wall, resulting in activation and aggregation. The *ex vivo* experiment showed increased procoagulant activity after collagen/thrombin induced activation of platelets from hyperglycemia animals. Activation of platelets by collagen alone, however, had no effect on procoagulant activity, most likely due to the synergistic effect of collagen and thrombin [22].

Platelet procoagulant activity is mainly determined by the extent of surface-exposed phosphatidylserine (PS) and the increased procoagulant activity in hyperglycemic mice suggests either an increase in the platelet fraction which exposes PS or an increase in PS exposure within the fraction of platelets exposing PS [22]. The mechanisms involved may consist of different factors that act in concert to lead to a platelet phenotype of enhanced reactivity to usually subthreshold concentrations of stimuli, which is known to occur in platelets from diabetic individuals [6, 7]. These factors may be related to altered biomembrane properties due to glycation of surface phospholipids and proteins. Whether this also includes an alteration in the distribution of PS and phosphatidylcholine (PC) in the outer platelet membrane remains to be established. Although one study reported such a finding induced by high glucose concentrations *in vitro* [23], we have not been able to confirm this in a model of erythrocytes subjected to different glucose concentrations (Wolfs and Bevers, unpublished data).

In patients with diabetes mellitus and atherosclerosis, inflammation is considered to contribute to the pathogenesis of the increased risk for acute arterial complications, like myocardial infarction or stroke [1]. In the present experiment inflammation was imposed by endotoxin injection in mice. Next the combination of hyperglycemia and this inflammatory state on arterial thrombus formation was determined.

In wildtype mice, low dose endotoxin resulted in sustained systemic inflammation and organ damage 24 hours after injection. This condition was not markedly different in hyperglycemic mice, although some levels of vessel wall gene expression differed from non-diabetic animals. One unsuspected finding was that at this stage of inflammation arterial thrombus formation was delayed as compared to non-inflammatory mice. A second unsuspected observation was that hyperglycemia appeared to neutralize this delayed thrombus formation.

These data suggest that systemic inflammation due to low dose endotoxin injection induces a (transient) 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 diabetes. The causes of this antithrombotic state may be due to a temporary hypo-responsiveness of endothelial cells, inflicted by inflammation and oxidative stress [24]. 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 endotoxin administration are seen in *in vivo* studies in relation to myocardial ischemia and reperfusion [25-27] and this effect is referred to as "preconditioning". Interestingly, endotoxin 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 the inflammatory state secondary to hyperglycemia and present before the endotoxin 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 [28, 29]. 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 endotoxin-treated mice was reduced compared with the control endotoxin-treated mice. The mechanisms behind these different reactions to endotoxin, however, remain unexplained.

Several limitations of this study should be taken into account. First, the experiments are all descriptive in nature, not proving beyond doubt the direct association between blood glucose and thrombosis. Another factor related to the experimental model is that the "closing time" is not a common determinant in ferric-chloride-induced thrombosis. Indeed, the commonly used "time to occlusion", subject to marked variation in the present experiments, is not significantly influenced, which appears to rule out a large thrombotic effect. The reproducible finding of differences in the rate of occlusion, however, leads us to think that this is a relevant novel marker of thrombus formation that may be quite suitable as determinant of blood-related thrombogenic (or antithrombotic) influences.

In conclusion, we observed that experimentally induced hyperglycemia caused a mild prothrombotic influence in normal C57/Bl6 mice, while the antithrombotic response induced by a low dose of endotoxin was attenuated by hyperglycemia. Both effects are illustrative of a thrombotic effect of high glucose concentrations in blood and may explain part of the risk of cardiovascular disease in patients with type 1 diabetes. The reduced antithrombotic response may also be pathophysiologically relevant, since this conditioning mechanism may provide a natural defense against thrombosis during infections. In a condition of hyperglycemia (diabetes), however, possibly related to increased thrombin generation and vascular oxidative stress responses, this protective mechanism fails. It is tempting to speculate that this difference in thrombotic responsiveness may put patients with diabetes who are at increased risk of infectious complications also at greater risk of atherothrombotic complications. This observation may also give an additional explanation for the clinical benefit of aggressive glucose regulation in critically ill patients [30].

## **Acknowledgments**

The authors would like to thank Joost Daalhuisen en Ingvild Kopp from the Academic Medical Center for their invaluable assistance.

## References

- 1 Cohen RA. ATVB in focus: Diabetic vascular disease: pathophysiological mechanisms in the diabetic milieu and therapeutic implications. *Arteriosclerosis, thrombosis, and vascular biology*. 2004; 24: 1340-1341.
- 2 Laing SP, Swerdlow AJ, Slater SD, Burden AC, Morris A, Waugh NR, et al. Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. *Diabetologia*. 2003; 46: 760-765.
- 3 Dorman JS, Laporte RE, Kuller LH, Cruickshanks KJ, Orchard TJ, Wagener DK, et al. The Pittsburgh insulin-dependent diabetes mellitus (IDDM) morbidity and mortality study. Mortality results. *Diabetes*. 1984; 33: 271-276.
- 4 Libby P, Plutzky J. Diabetic macrovascular disease: the glucose paradox? *Circulation*. 2002; 106: 2760-2763.
- 5 Nathan DM, Cleary PA, Backlund JY, Genuth SM, Lachin JM, Orchard TJ, et al. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *The New England journal of medicine*. 2005; 353: 2643-2653.
- 6 Watala C, Boncler M, Gresner P. Blood platelet abnormalities and pharmacological modulation of platelet reactivity in patients with diabetes mellitus. *Pharmacol Rep*. 2005; 57 Suppl: 42-58.
- 7 Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost*. 2004; 2: 1282-1291.
- 8 Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. *Diabetes*. 2006; 55: 202-208.
- 9 Stegenga ME, van der Crabben SN, Levi M, de Vos AF, Tanck MW, Sauerwein HP, et al. Hyperglycemia stimulates coagulation, whereas hyperinsulinemia impairs fibrinolysis in healthy humans. *Diabetes*. 2006; 55: 1807-1812.
- 10 Ross R. Atherosclerosis--an inflammatory disease. *The New England journal of medicine*. 1999; 340: 115-126.
- 11 Like AA, Rossini AA. Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science*. 1976; 193: 415-417.
- 12 Farrehi PM, Ozaki CK, Carmeliet P, Fay WP. Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice. *Circulation*. 1998; 97: 1002-1008.
- 13 Smyth SS, Reis ED, Vaananen H, Zhang W, Collier BS. Variable protection of beta 3-integrin--deficient mice from thrombosis initiated by different mechanisms. *Blood*. 2001; 98: 1055-1062.
- 14 Slofstra SH, Cate HT, Spek CA. Low dose endotoxin priming is accountable for coagulation abnormalities and organ damage observed in the Shwartzman reaction. A comparison between a single-dose endotoxemia model and a double-hit endotoxin-induced Shwartzman reaction. *Thrombosis journal [electronic resource]*. 2006; 4: 13.
- 15 Sommeijer DW, van Oerle R, Reitsma PH, Timmerman JJ, Meijers JC, Spronk HM, et al. Analysis of blood coagulation in mice: pre-analytical conditions and evaluation of a home-made assay for thrombin-antithrombin complexes. *Thrombosis journal [electronic resource]*. 2005; 3: 12.
- 16 de Waard V, Hansen HR, Spronk HH, Timmerman JJ, Pannekoek H, Florquin S, et al. Differential expression of tissue factor mRNA and protein expression in murine sepsis. The role of the granulocyte revisited. *Thromb Haemost*. 2006; 95: 348-353.
- 17 Wasser MN, Koppert PW, Arndt JW, Emeis JJ, Feitsma RI, Pauwels EK, et al. An antifibrin monoclonal antibody useful in immunoscintigraphic detection of thrombi. *Blood*. 1989; 74: 708-714.

- 18 Hoekstra M, Kruijt JK, Van Eck M, Van Berkel TJ. Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *The Journal of biological chemistry*. 2003; 278: 25448-25453.
- 19 Rosing J, Tans G, Govers-Riemslog JW, Zwaal RF, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. *The Journal of biological chemistry*. 1980; 255: 274-283.
- 20 Dorffler-Melly J, Schwarte LA, Ince C, Levi M. Mouse models of focal arterial and venous thrombosis. *Basic research in cardiology*. 2000; 95: 503-509.
- 21 Dorffler-Melly J, de Kruijff M, Schwarte LA, Franco RF, Florquin S, Spek CA, et al. Functional thrombomodulin deficiency causes enhanced thrombus growth in a murine model of carotid artery thrombosis. *Basic research in cardiology*. 2003; 98: 347-352.
- 22 Wolfs JL, Comfurius P, Rasmussen JT, Keuren JF, Lindhout T, Zwaal RF, et al. Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol Life Sci*. 2005; 62: 1514-1525.
- 23 Wilson MJ, Richter-Lowney K, Daleke DL. Hyperglycemia induces a loss of phospholipid asymmetry in human erythrocytes. *Biochemistry*. 1993; 32: 11302-11310.
- 24 Parker JL, Adams HR. Selective inhibition of endothelium-dependent vasodilator capacity by *Escherichia coli* endotoxemia. *Circulation research*. 1993; 72: 539-551.
- 25 Brown JM, Grosso MA, Terada LS, Whitman GJ, Banerjee A, White CW, et al. Endotoxin pretreatment increases endogenous myocardial catalase activity and decreases ischemia-reperfusion injury of isolated rat hearts. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86: 2516-2520.
- 26 Zacharowski K, Otto M, Hafner G, Chatterjee PK, Thiemermann C. Endotoxin induces a second window of protection in the rat heart as determined by using p-nitro-blue tetrazolium staining, cardiac troponin T release, and histology. *Arteriosclerosis, thrombosis, and vascular biology*. 1999; 19: 2276-2280.
- 27 Zacharowski K, Frank S, Otto M, Chatterjee PK, Cuzzocrea S, Hafner G, et al. Lipoteichoic acid induces delayed protection in the rat heart: A comparison with endotoxin. *Arteriosclerosis, thrombosis, and vascular biology*. 2000; 20: 1521-1528.
- 28 Bouchard JF, Lamontagne D. Protection afforded by preconditioning to the diabetic heart against ischaemic injury. *Cardiovascular research*. 1998; 37: 82-90.
- 29 Ebel D, Mullenheim J, Frassdorf J, Heinen A, Huhn R, Bohlen T, et al. Effect of acute hyperglycaemia and diabetes mellitus with and without short-term insulin treatment on myocardial ischaemic late preconditioning in the rabbit heart in vivo. *Pflügers Arch*. 2003; 446: 175-182.
- 30 Van den Berghe G, Wilmer A, Hermans G, Meersseman W, Wouters PJ, Milants I, et al. Intensive insulin therapy in the medical ICU. *The New England journal of medicine*. 2006; 354: 449-461.

