Increased platelet activation in young Zucker rats with impaired glucose tolerance is improved by acarbose

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Summary
Patients with diabetes display increased platelet activation. Recent data show a markedly increased risk for cardiovascular events already in pre-diabetic individuals with impaired glucose tolerance (IGT). We investigated whether IGT is associated with platelet activation. Blood samples were collected from young lean (control) and obese Zucker rats, an established model of IGT, after single oral application of sucrose (4g·kg⁻¹). Platelet-bound fibrinogen and platelet surface-expression of P-selectin were assessed as indices of platelet activation using flow cytometry. In lean Zucker rats, acute sucrose application induced fibrinogen-binding and P-selectin surface-expression, which was prevented by co-administration of acarbose (10mg·kg⁻¹). In obese Zucker rats, platelet activation was already maximally increased under baseline conditions with no significant increase after sucrose application. Chronic treatment with acarbose (15mg·kg⁻¹·day⁻¹) significantly reduced platelet activation in these animals. Acute ingestion of sucrose induces platelet activation which is prevented by acarbose. IGT is associated with marked platelet activation that can be reduced by chronic administration of acarbose. The positive modulation of platelet activation by acarbose may contribute to the reduction of cardiovascular events in patients with IGT.

Keywords
Platelet activation markers, diabetes / metabolic disorders, animal models

Platelets and Blood Cells

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Introduction
Patients with diabetes mellitus have an increased risk of thrombosis and accelerated atherogenesis, which has been attributed to increased platelet reactivity (1). Hyperactive platelets essentially contribute to microembolisation of capillaries, local progression of vascular lesions and triggering of acute arterial thrombosis (2), ultimately resulting in the development of acute or chronic myocardial ischaemia. Platelets from diabetic patients display enhanced surface-expression of P-selectin, and increased membrane-binding of fibrinogen on activated glycoprotein IIb/IIIa. Furthermore, in patients with diabetes, circulating monocyte-platelet aggregates are increased (3) and adhesion and aggregation of platelets on extracellular matrix is enhanced (4). Agonist-induced release of platelet-derived growth factor is increased in platelets from diabetic patients (5) as well as plasma levels of serotonin, β-thromboglobulin, and soluble von-Willebrand-factor (vWF) (6, 7).
While those changes have been found in several diabetic patient populations and in animal models of overt diabetes mellitus (8), their role in pre-diabetic states has not been investigated. Recent observations suggest increased platelet susceptibility in healthy close relatives of diabetic patients. Even in metabolically still normal individuals tested positive for islet cell antibodies, markers of platelet activation (P-selectin, glycoprotein 53, thrombospondin) were reported to be significantly increased (9).

Insulin resistance and high fasting insulin concentrations are considered as independent risk factors for the development of ischemic heart disease (10,11). Impaired glucose tolerance is a risk factor for cardiovascular disease (12) and leads to increased mortality (13). The ability of acarbose to prevent the progression from IGT to type 2 diabetes mellitus was investigated in the STOP-NIDDM trial. Treatment with the α-glucosidase inhibitor acarbose resulted in less progression from IGT to diabetes (14). Recently, a secondary endpoint analysis of the STOP-NIDDM trial showed a marked reduction of myocardial infarction and hypertension by acarbose in patients with IGT (15).

Therefore, we investigated whether platelets are activated in an animal model of IGT, and whether parameters of platelet activation are affected by acute oral sucrose application. In addition, the acute and chronic effect of acarbose on platelet activation was measured.

Materials and methods

Animal experiments were performed in accordance with the “Principles of laboratory animal care” (NIH publication no. 85-23, revised 1985) and the current version of the German Law on the Protection of Animals.

Animals and protocols

Ten-week-old lean and obese Zucker rats were obtained from Bayer AG, Leverkusen, Germany. The Zucker rat is an inbred rat model that closely mimics human adult onset diabetes and its related complications through genetic mutation of the leptin receptor. The old obese Zucker rat is an accurate model for type 2 diabetes based on impaired glucose tolerance caused by the inherited obesity gene mutation (fa/fa) which leads to insulin resistance. Animals of the age group of about 10 weeks as used in the present study are an established model of impaired glucose tolerance and the lean animals (Fa/fa) are the respective healthy controls. Animals were kept on a normal diet and were subjected to a single acute sucrose load (4 g kg⁻¹ body weight by gavage, 6-10 animals for each group). For assessment of acute effects of acarbose (provided by Bayer), 10 mg·kg⁻¹ were given simultaneously with sucrose. For chronic treatment, acarbose (15 mg·kg⁻¹·day⁻¹) was given for 7 days prior to the experiments. Higher doses of acarbose for rats compared with humans were chosen due to higher carbohydrate metabolism in rodents and increased intestinal enzyme activity as previously described (16, 17).

Preparation of blood samples

General anaesthesia was induced using diethyl ether. The abdominal cavity was opened under deep anaesthesia, determined by total absence of reaction to pain under spontaneous respiration, and blood was taken by direct puncture of the inferior caval vein into a chilled tube containing 3.8% sodium citrate.

Platelet preparation and flow cytometry

Platelet-bound fibrinogen was determined after dilution with PBS (free of Ca²⁺ and Mg²⁺, enriched with D-Glucose [5.5 mmol/l] and 0.5% BSA) and incubation with a FITC-labelled anti-fibrinogen antibody (WAK-Chemie, Bad Soden, Germany) (18) for 10 min at 37°C. Staining with the directly FITC-labelled anti-fibrinogen antiserum has been extensively controlled in our laboratory in the past using an FITC-labelled isotype control IgY obtained from the manufacturer and, in this study, all samples were analysed at the same voltage setting of the flow cytometer and only included when the background signal had a mean fluorescence of less than 2. For determination of surface-expressed P-selectin, diluted blood was incubated with a polyclonal rabbit anti-P-selectin (CD62P) antibody (Becton Dickinson, Heidelberg, Germany) for 10 min at room temperature followed by incubation with a FITC-labelled goat anti-rabbit IgG-antibody (Jackson ImmunoResearch, West Grove, Pennsylvania). Staining of the samples was also

<table>
<thead>
<tr>
<th>Time from sucrose load (min)</th>
<th>Lean Placebo</th>
<th>Lean Acarbose</th>
<th>Obese Placebo</th>
<th>Obese Acarbose</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>4.0±0.1</td>
<td>3.9±0.1</td>
<td>4.7±0.1**</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>30</td>
<td>6.7±0.3</td>
<td>4.5±0.1**</td>
<td>7.8±0.2</td>
<td>5.6±0.3**</td>
</tr>
<tr>
<td>60</td>
<td>6.1±0.3</td>
<td>5.0±0.1**</td>
<td>8.5±0.4**</td>
<td>6.3±0.4**</td>
</tr>
<tr>
<td>120</td>
<td>6.3±0.4</td>
<td>5.4±0.1*</td>
<td>7.3±0.6</td>
<td>7.4±0.7</td>
</tr>
</tbody>
</table>

* = p<0.05, ** = p<0.01 vs. Placebo; # = p<0.05, ## = p<0.01 vs. Lean; n = 7-8
performed only with the FITC-conjugated secondary antibody in the absence of the primary antibody to arbitrarily adjust the unspecific binding to a mean fluorescence of 10. Platelet glycoprotein 53 (CD63) was determined using a mouse anti-rat CD63 monoclonal antibody and consecutively incubated with a FITC-conjugated anti-mouse IgG antibody (both from Becton Dickinson). Platelets within the leukocyte population were identified by the platelet specific antigen CD42d (glycoprotein V), the expression of which was not significantly modulated in the investigated animals (mean fluorescence for CD42d: lean: 618±34; obese: 634±31; obese+acarbose: 589±32, n.s.), using an anti-rat CD42d monoclonal FITC-conjugated antibody (Becton Dickinson) to assess platelet leukocyte adhesion. Platelets were fixed with methanol-free formaldehyde (1.5%) for 5 min, and subsequently analysed in a Becton Dickinson FACSCalibur at low flow rate. The platelet population was identified on its forward and side scatter distribution, and 20,000 events were analysed for mean fluorescence using CELLQuest software, version 3.1f (19).

Statistics
Values are means ± SEM. Statistical evaluation of platelet parameters was performed by one-way analysis of variance followed by a Tukey post-hoc test, p<0.05 was considered statistically significant.

Results
Blood glucose levels at baseline as well as 30, 60 and 120 minutes following acute sucrose load in the absence or presence of acutely administered acarbose in lean and obese Zucker rats are shown in table 1.

**Platelet activation in lean Zucker rats under resting conditions and following a single acute sucrose application**
Platelet activation was assessed in lean control Zucker rats. Several markers of platelet activation were increased following single acute sucrose application compared with baseline levels. One feature of platelet activation is increased activity of glycoprotein Ib/IIa, also referred to as the fibrinogen-receptor. Platelet-binding of fibrinogen was significantly increased in lean Zucker rats after oral sucrose load (Fig. 1A, open bars). Platelet degranulation was measured by enhanced surface-expression of the adhesion molecule P-selectin (CD62P, Fig. 1B, open bars) and glycoprotein 53 (CD63, Fig. 1C, open bars), which were significantly enhanced 30 and 60 minutes after sucrose application compared with lean Zucker rats only given water instead of sucrose solution (baseline).

**Figure 1**: Resting platelets from healthy lean Zucker rats were investigated under baseline conditions and 30, 60 and 120 minutes after oral sucrose application (4 g·kg⁻¹) with (filled bars) and without (open bars) simultaneous administration of acarbose (10 mg·kg⁻¹). Platelet activation was assessed by membrane-binding of fibrinogen on resting platelets (A) as well as surface-expression of platelet P-selectin (CD62P, B) and platelet glycoprotein 53 (CD63, C). Data are expressed as mean fluorescence ± SEM of 8-10 separate animals, *p<0.05, **=p<0.01 vs. lean basal; #p<0.05 vs. untreated animals at the same time point.
Inhibition of platelet activation following sucrose application in lean Zucker rats by acute co-administration of acarbose

When acarbose (10 mg·kg⁻¹) was given simultaneously with sucrose in lean Zucker rats, fibrinogen-binding on platelets (Fig. 1A, filled bars) as well as surface-expression of P-selectin (Fig. 1B, filled bars) and glycoprotein 53 (Fig. 1C, filled bars) were significantly attenuated. Acute administration of acarbose did not influence platelet activation in rats not given sucrose (data not shown). This could be explained by the mechanism of action of acarbose, an α-glucosidase inhibitor, which is a competitive inhibitor of intestinal enzymatic hydrolysis of oligoglucoids and is not absorbed from the intestine. As in a fasting animal no oligoglucoids are available in the intestine, acarbose administered acutely is expected to remain without effect.

**Platelet activation in obese Zucker rats under resting conditions and following acute sucrose application with and without co-administration of acarbose**

Obese Zucker rats, an established model of IGT, had higher fibrinogen-binding (Fig. 2A) as well as surface-expression of P-selectin (Fig. 2B) and glycoprotein 53 (Fig. 2C) even under resting conditions. Platelet activation was neither enhanced following sucrose application (open bars) nor reduced by acute co-administration of acarbose (Fig. 2, filled bars). Acute administration of acarbose did not influence platelet activation in rats not given sucrose (data not shown).

**Effect of chronic treatment with acarbose on basal platelet activation in obese Zucker rats**

As platelet activation under resting conditions was already markedly increased in obese Zucker rats, and short-term acarbose efficiently prevented the adverse effects of acute sucrose load on platelet activation in lean Zucker rats, we hypothesized that chronic treatment with acarbose might beneficially modulate platelet activation in obese Zucker rats. Therefore, obese Zucker rats (Fig. 3) were treated with acarbose in chow (hatched bars) or standard chow (filled bars) for 7 days and compared with lean Zucker rats on standard chow (open bars).

Fibrinogen-binding was significantly increased in obese vs. lean Zucker rats on placebo and this increment was abrogated by chronic treatment with acarbose (Fig. 3A). Surface-expression of glycoprotein 53 (Fig. 3B) and P-selectin (Fig. 3C) was significantly enhanced in obese Zucker rats on standard chow, and reduced by chronic treatment with acarbose. Especially the modulation of P-selectin might have influenced the changes in circulating platelet-leukocyte aggregates in whole blood from obese Zucker rats (Fig. 3D), which were significantly reduced.

**Discussion**

In this study we show that a single acute sucrose application induces platelet activation in healthy rats within 30–60 minutes. Inhibiting the release of glucose from sucrose by co-administration of the α-glucosidase inhibitor acarbose, and thus delaying glucose reabsorption, prevented the increase in platelet activation.
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In addition, we demonstrate marked platelet activation in animals with IGT, which was reduced by chronic acarbose treatment.

An acute application of sucrose on top of a normal diet caused rapid platelet activation in healthy rats. Hyperglycemia has repeatedly been discussed as a contributing factor for platelet activation. Especially the extent of postprandial hyperglycemia seems to influence cardiovascular risk more than fasting blood glucose levels (20). Indeed, acute hyperglycemia exponentiated collagen-induced platelet activation in platelet-rich plasma from healthy human donors in vitro (21). Furthermore, increased levels of glucose are independent predictors of platelet-dependent thrombosis in patients with coronary artery disease (8), and prolonged hyperglycemia induces abnormal Ca²⁺ homeostasis similar to changes observed in platelets from diabetic patients (22). Recent data indicate a role for hyperosmolarity in glucose-induced platelet activation (23). Acute hyperglycemia in vivo by infusion of glucose causes platelet activation in diabetic patients and is prevented by simultaneous insulin infusion maintaining an euglycemic state (24). This basically conforms with other studies demonstrating inhibition of platelet interaction with collagen and increase in platelet cGMP by insulin in vivo, which is blunted in obese, insulin-resistant patients (25). In diabetic patients, however, insulin has been described to even enhance platelet-binding of fibrinogen (26). Diabetes leads to enhanced susceptibility of platelets to agonists, fibrinogen-binding and platelet interaction with injured vessels contributing to increased atherosclerosis (27). Patients with exaggerated platelet aggregation have an increased risk for restenosis following coronary angioplasty (28), a common feature of cardiovascular complications in patients with diabetes. Activation of platelets results in an increased activation of glycoprotein IIb/IIIa followed by enhanced binding of soluble fibrinogen and hence platelet-platelet aggregation (29). Indeed, the high risk of diabetic patients undergoing coronary intervention can be reduced to that of non-diabetic patients by infusing the glycoprotein IIb/IIIa inhibitor abciximab (30). The direct influence of postprandial glucose availability in blood on platelet activation is further strengthened by the prevention of platelet activation in lean rats simultaneously treated with the α-glucosidase inhibitor acarbose, which is known to decrease postprandial glucose and

![Figure 3: Platelet activation in obese Zucker rats on standard chow (filled bars) or acarbose for 7 days (15 mg·kg⁻¹·day⁻¹, hatched bars) compared with platelets from lean Zucker rats on standard chow (open bars). Platelet-binding of fibrinogen (A), surface-expression of glycoprotein 53 (CD63, B) and P-selectin (CD62P, C) as well as circulating platelet-leukocyte aggregates (D) were assessed. Data are expressed as mean fluorescence (A-C) or ratio of CD42⁺ leukocytes ± SEM of 12 separate animals, **=p<0.01 vs. lean placebo; #=p<0.05, ##=p<0.01 vs. obese placebo.](http://www.thrombosis-online.com)
insulin levels (31) and leads to decreased progression of IGT to diabetes (14). However, whether platelets are activated during IGT and may contribute to the increased risk for cardiovascular events remained unclear.

In obese Zucker rats, an established model of IGT, we observed marked platelet activation already under resting conditions, which may causally contribute to the increased risk of cardiovascular atherothrombotic events in IGT. In these animals with preexisting platelet activation, acute application of sucrose did not further increase platelet activation, which was already maximal under resting conditions. However, the chronic positive modulation of in vivo platelet activation under resting conditions in IGT observed during acarbose treatment in our present study is likely to contribute to the reduced risk of myocardial infarction in patients with IGT treated with acarbose (15).

The underlying mechanisms of action of acarbose on platelet activation remain uncertain. The main biological effect is reduction of enteric glucose liberation and absorption resulting in less postprandial blood glucose peaks since acarbose is not absorbed from the intestine. In subjects with IGT, acarbose treatment decreases postprandial plasma insulin and improves insulin sensitivity (31). The ability of acarbose, which is widely used in patients, to prevent the progression from IGT to type 2 diabetes mellitus in humans has been clinically investigated in the STOP-NIDDM trial (14). Glucose tolerance was determined on the basis of a yearly oral glucose tolerance test, and during acarbose treatment less progression to overt diabetes and increased reversion from IGT to normal glucose tolerance was observed (14). Recently, a sub-study of the STOP-NIDDM trial reported a marked reduction of cardiovascular events by acarbose in patients with IGT (15). Activated platelets as observed in IGT in the present study show surface-expression of the adhesion molecule P-selectin (32) and subsequent appearance of platelet-leukocyte aggregates (33, 34) predominantly by binding to the leukocyte P-selectin-glycoprotein-ligand 1, which are established predictors for cardiovascular events.

A limitation of this study is that platelet activation was determined by platelet membrane glycoproteins. Therefore, platelets interacting with potentially diseased vessels would not be detected by the methods used in our study. In complex metabolic diseases such as diabetes or pre-diabetes several factors might contribute to enhanced platelet activation. A potential role of glycation of membrane proteins was proposed to be responsible for the hyperreactivity/hypersensitivity of platelets from diabetic patients (1, 35) and altered membrane fluidity were discussed as potential contributing factors (1, 36, 37).

Conclusion

Acute ingestion of sucrose induced platelet activation in healthy rats, which was prevented by co-administration of acarbose. We further demonstrate enhanced platelet activation in an animal model of IGT, which was reduced by chronic administration of the α-glucosidase inhibitor acarbose. The positive modulation of platelet activation by acarbose may contribute to the reduction of cardiovascular events in patients with IGT.

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References