Antiplatelet effects of prostacyclin and nitric oxide in patients with type I diabetes and ischemic or edematous retinopathy

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Antiplatelet effects of prostacyclin and nitric oxide in patients with type I diabetes and ischemic or edematous retinopathy

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The aim of this study was to investigate prostacyclin and nitric oxide (NO) and their platelet second messengers cAMP and cGMP, in patients with type I diabetes with or without retinopathy. We compared 20 healthy volunteers and 97 patients with type I diabetes: 24 with no signs of diabetic retinopathy (DR), 43 with ischemic-proliferative DR, and 30 with edematous DR. The following parameters were recorded: platelet aggregometry, nitrites/nitrates, 6-keto-prostaglandin-F$_1$-a, and intraplatelet cAMP and cGMP. Platelet aggregation was greater in patients with edematous DR. The platelets in patients with diabetes were more resistant to inhibition by prostaglandin E$_2$ or sodium nitroprusside. Nitrite concentration in patients with ischemic-proliferative DR was 80% lower than the value in healthy controls, but there was no significant difference between the control group and patients with edematous DR. In the latter group, stimulation of neutrophils with L-arginine increased nitrite + nitrate production by 44 ± 3.6%, whereas in patients with ischemic-proliferative DR, the increase was 9.8 ± 0.8%. We conclude that NO production is higher in patients with type I diabetes and edematous retinopathy than in those with ischemic-proliferative retinopathy. This finding, together with the possibly greater production of free radicals, may explain the greater impairment of platelet function in the former patients.

Introduction

After 20–25 years of diabetes, more than 80% of the patients have some type of retinal lesion, and these patients are 20-fold as likely to become blind as are members of the general population.1–3 A prolonged increase in levels of blood glucose is recognised as one of the main causes of the appearance and evolution of diabetic microangiopathy, of which retinopathy is one type.4 Sustained hyperglycemia leads to a series of interrelated alterations that can cause evident endothelial dysfunction, which in turn can cause vascular lesions in the retina of patients with diabetes and retinopathy.5

One of the main consequences of endothelial dysfunction is a deficit in nitric oxide (NO) and prostacyclin, substances with vasodilating and platelet-inhibiting functions.6,7 In this connection, platelet hyperactivity has been widely documented in patients with diabetes mellitus, and this hyperactivity is greater in patients with microangiopathy.8–13

The aim of the present study was to measure prostacyclin and NO production, and the production of their platelet second messengers cAMP and cGMP, in patients with type I diabetes mellitus with different types of retinopathy.

Materials and methods

Patient selection

The participants in this study were 20 healthy volunteers and 97 patients with diabetes diagnosed as type I (IDDM). We excluded patients with other types of...
vasculopathy (macro- or microangiopathy). Except for insulin, none of the subjects took any medication at the time of the study or during the 15 days before inclusion. All participants were given the same recommendations regarding dietary measures, exercise and life style. All participants gave their informed consent in writing after receiving information about the aims of the study, and the procedure was approved by the Ethics Committee of the Virgen de la Victoria University Hospital in Málaga, Spain.

All patients and healthy volunteers underwent complete ophthalmological examination, which included evaluation of the visual field, intraocular pressure, lens and fluorescein angiography (FAG). On the basis of the FAG results, the participants were divided into four groups. Group 1 \((n = 20)\) comprised persons without diabetes or signs of retinopathy. Group 2 \((n = 24)\) consisted of patients with IDDM with no signs of retinopathy. The patients in Group 3 \((n = 43)\) had IDDM and predominantly ischemic-proliferative retinopathy, defined as the presence of new vessel formation (intraretinal, preretinal, papillary or vitreous), with leaks in the new vessels in the early phase of FAG. Group 4 \((n = 30)\) contained patients with IDDM and predominantly edematous retinopathy, defined as the presence of abundant, confluent infiltrates that formed plaques, and diffuse retinal edema (more than six leaks). Table 1 summarizes the main characteristics of the participants in each group.

A blood sample was obtained from each participant between 08:00 and 09:00 h, before the subject had had anything to eat or drink, and before the patients had injected their daily insulin dose.

Glycosylated hemoglobin (HbA\(_1c\)), duration of diabetes, daily insulin dose, and other findings of interest were recorded from the patient’s clinical record. For each patient HbA\(_1c\) was measured 2 or 3 times per year, and the value used in this study was the mean of the three most recent measurements.

### Laboratory procedures

All reagents were from Sigma (St. Louis, MO, USA) unless otherwise noted. Blood samples were collected with 3.8% sodium citrate as the anticoagulant at a proportion of 1:10. Each sample was divided into aliquots for analyses of whole blood, platelet-rich plasma (PRP), mononuclear leukocytes (MNL) and neutrophils. Platelet-rich plasma was obtained by centrifuging whole blood at 180 \(\times g\) for 10 min at 20°C. Mononuclear leukocytes were obtained on a Ficoll density gradient (Hystopaque 1077), and resuspended (final count, 3.1 \(\pm 4 \times 10^9\) leukocytes/l) in Ca\(^{2+}\) physiological saline solution that contained 140 mM NaCl, 4.6 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM glucose and 10 mM Hepes, pH 7.4. Neutrophils were also obtained on a Ficoll density gradient (Hystopaque 1119) and resuspended in Ca\(^{2+}\) physiological saline solution (95% neutrophils, 95% viability according to Trypan blue exclusion) to a final count of 3.2 \(\pm 0.3 \times 10^9\) neutrophils/l.

### Platelet aggregation

Maximum intensity of platelet aggregation induced by ADP or collagen in whole blood or PRP was measured with an electrical impedance method\(^{14}\) (Chrono-Log 530S aggregometer, Chrono-Log, Haverton, PA, USA). Samples were incubated at 37°C for 5 min, then different concentrations of collagen or ADP were added (Menarini Diagnóstica, Barcelona, Spain). The recording electrode was immersed in the sample, and maximum intensity of aggregation was recorded as the maximum change in impedance 10 min after the inducing agent was added. In some experiments with PRP, the samples were incubated with different concentrations of prostaglandin E\(_1\) (adenyl-cyclase stimulant) or sodium nitroprusside (SNP, a nitric oxide donor), and aggregation was induced by adding 1 \(\mu\)g/ml collagen.

### Nitrite/nitrate concentration

Nitric oxide is rapidly converted to NO\(_2^-\) and NO\(_3^-\) (NO\(_x\)) in an oxygenated aqueous solution such as human plasma. Samples were filtered through Ultrafree-MC microcentrifuge filters (Millipore, Gif-Sur-Yvette, France) to remove the hemoglobin from cell lysis. A commercial kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure NO\(_x\) concentration. The method is based on the Greiss reaction after conversion.
of nitrates and nitrites via nitrate reductase. The concentration of NOx was determined spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite. Concentrations of NOx were measured in plasma and in the supernatant of neutrophils stimulated for 30 min with 100 µM L-arginine.

**Prostacyclin concentration**

The plasma concentration of prostacyclin was quantified by determining 6-keto-prostaglandin-F₁α in samples of MNL. Calcium ionophore A23187 (1 µM) was added, and the mixture incubated for 5 min at 37°C and then centrifuged at 10000 × g for 3 min. The supernatant was frozen at −80°C until analysis. The concentration of 6-keto-prostaglandin F₁α was measured by enzyme immunoassay (Biotrak RPN 220, Amersham International, Little Chalfont, Buckinghamshire, UK).

The sensitivity of this method is 3.6 pg/ml, the within-assay variability for duplicate determinations was 2.5% and the between-assay variability was 9.9%. The cross reactivity of this method is as follows: 100% 6-keto-PGF₁α, 6.1% 6-thromboxane B₂, 0.01% 6-keto-PGE₂, 0.011% PGD₂, 0.18% PGD₁, 0.01% PGE₂, 0.01% thromboxane B₂, 0.06% PGF₂α, < 0.01% arachidonic acid.

**Platelet cAMP**

Platelets from PRP were washed in a medium composed of 5.6 g/l NaCl, 0.7 g/l NaH₂PO₄, 0.5 g/l KH₂PO₄, 0.9 g/l glucose, 0.05 g/l aspartate, and 50 nM prostaglandin E₁. The sample was centrifuged at 1000 xg for 15 min at 4°C, and the resulting pellet was resuspended in a solution consisting of 7.7 g/l NaCl, 0.6 g/l NaH₂PO₄, 1 g/l NaHCO₃, 0.3 g/l CaCl₂, 0.2 g/l MgCl₂, 1.2 g/l HEPES, 0.9 g/l glucose, 0.05 g/l aspartate, and 0.5 g/l bovine albumin. The number of platelets was adjusted to 2.5 × 10¹¹ cells/l, and the sample was divided into aliquots to which we added 10 µM isobutyl-methyl-xanthine and 1 µM prostaglandin E₁. After incubation for 5 min at 37°C, the process was stopped by adding 1 N HCl. The sample was centrifuged at 10000 xg for 3 min and the resulting supernatant was neutralized with NaOH. The production of cAMP was measured by enzyme immunoassay (Amersham). The sensitivity of this method is 38.4 pg/ml, the within-assay variability for duplicate determinations was 7.9% and the between-assay variability was 11.0%. The cross-reactivity of this method is as follows: 100% cAMP, 0.014% cTMP, 0.0007% cGMP, 0.034% cCMP, 0.0036% cTMP, 0.006% AMP, 0.0002% ADP, 0.00015% ATP, < 0.00015% EDTA, 0.00015% theophylline, 0.0071% isobutyl-methyl-xanthine.

**Intraplatelet cGMP**

Samples were prepared as described above for cAMP, except that 100 µM zaprinast were used instead of isobutyl-methyl-xanthine, and 10 µM SNP were used instead of prostaglandin E₁. The sensitivity of this method is 161 pg/ml, the within-assay variability for duplicate determinations was 7.5% and the between-assay variability was 12.2%. The cross-reactivity of this method is as follows: 100% cGMP, < 0.0005% cAMP, < 10⁻⁴% AMP, < 10⁻⁴% ADP, < 10⁻⁴% ATP, < 0.0005% GMP, < 0.00025% GDP, < 0.00025% GTP.

**Statistical analysis**

The results were expressed as the mean ± SEM. Differences between groups were detected with one-way
analysis of variance followed by testing for the least significant difference. Simple linear correlations were also calculated. Differences were considered significant at $P < 0.05$. The SPSS program (version 10.0 for Windows, SPSS, Chicago, IL, USA) was used for all statistical analyses.

**Results**

Platelet aggregation increased in a concentration-dependent way after stimulation with collagen or ADP, in whole blood and PRP (Figures 1 and 2). Aggregation was greater in patients with diabetes than in healthy volunteers; these curves were greater in patients with diabetes and edematous retinopathy than in any other group. Table 2 shows the concentrations of inducers (ADP and collagen) that produced 50% maximal aggregation ($EC_{50}$). In PRP the differences between groups were significant only after induction with ADP for patients with diabetes and edematous retinopathy, and in whole blood the $EC_{50}$ values for ADP and collagen were significantly lower in patients with diabetes than in the other groups. However, in all experiments (Figures 1 and 2), aggregation was greatest in patients with diabetes and edematous retinopathy, the largest difference appearing in whole blood.

Prostaglandin E$_1$ inhibited platelet aggregation induced by collagen in a concentration-dependent way (Figure 3). Inhibition was greater in healthy volunteers than in patients with the disease. SNP also inhibited aggregation induced by collagen in a concentration-dependent way. Resistance to inhibition by SNP was greatest in patients with diabetes.

Plasma concentrations of NOx are shown in Table 3. In patients with diabetes and ischemic-proliferative retinopathy, plasma NOx concentrations were lower than in the other groups.

Nitric oxide production by neutrophils stimulated with L-arginine (Figure 4, left panel) was significantly greater in healthy volunteers than in patients with diabetes and retinopathy. Platelet concentrations of cGMP (Figure 4, right panel) were similar in all four groups, although cGMP production after induction with SNP showed no significant change in platelet samples from patients with diabetes.

Leukocyte production of 6-keto-prostaglandin F$_{1\alpha}$ increased significantly after stimulation with calcium ionophore (Figure 5, left panel), although leukocytes

![Figure 2](image-url) Maximum intensity of platelet aggregation ($I_{max}$) in platelet-rich plasma and whole blood, induced with increasing concentrations of collagen. HV, volunteers without diabetes ($n = 20$); NR, patients with diabetes but without retinopathy ($n = 24$); IPR, patients with diabetes and ischemic-proliferative retinopathy ($n = 43$); ER, patients with diabetes and edematous retinopathy ($n = 30$). *$P < 0.05$ in comparison to all other groups.

| Table 2. Concentrations of the inducers of platelet aggregation that produced 50% maximum intensity of aggregation ($EC_{50}$)* |
|-----------------|-------|-------|-------|
|                | ND ($n = 20$) | NR ($n = 24$) | IPR ($n = 43$) | ER ($n = 30$) |
| ADP (µM)-PRP   | 2.10 ± 0.29  | 2.43 ± 0.34  | 2.23 ± 0.28  | 1.53 ± 0.13*  |
| ADP (µM)-WB    | 2.13 ± 0.17  | 1.95 ± 0.15  | 0.43 ± 0.06* | 0.32 ± 0.05*  |
| Collagen (µg/ml)-PRP | 0.72 ± 0.08 | 0.75 ± 0.09 | 0.71 ± 0.08 | 0.66 ± 0.08  |
| Collagen (µg/ml)-WB | 0.46 ± 0.05 | 0.41 ± 0.06 | 0.28 ± 0.02* | 0.17 ± 0.02*  |

*HV, healthy volunteers; NR, patients with diabetes without retinopathy; IPR, patients with diabetes and ischemic-proliferative retinopathy; ER, patients with diabetes and edematous retinopathy; PRP, platelet-rich plasma; WB, whole blood.

* $P < 0.05$ with respect to HV and NR.
from healthy volunteers showed the largest increase. Intraplatelet concentrations of cAMP (Figure 5, right panel) were significantly lower in patients with diabetes and retinopathy. In contrast with the results in the control group and patients with diabetes and no retinopathy, stimulation with prostaglandin E\(_1\) led to no significant increase in cAMP levels in patients with diabetes and retinopathy.

### Discussion

We have studied here the effect of two substances produce by the endothelium (prostacyclin and NO) which are clearly affected in patients with diabetes because of the endothelial dysfunction. In addition we analyzed cAMP and cGMP, the intraplatelet second messengers for these two substances.

Our aggregometric findings are similar to those of most authors who have studied this parameter in patients with diabetes, in whom platelet aggregation is more sensitive to stimulation by habitual inducers than in healthy persons.\(^8\)\(^-\)\(^12\) This result is compatible with earlier findings by our group in patients with type I diabetes mellitus,\(^13\) in whom aggregation was also greater than in healthy controls, and among whom the difference was especially striking for patients with edematous retinopathy.

Experiments that investigated endogenous antiaggregant substances (prostacyclin for cAMP, and NO for cGMP) showed that platelets from patients with diabetes were less sensitive to inhibition by these anti-aggregants than those from healthy volunteers. These results are similar to earlier findings that reported a lower inhibition of aggregation when platelets from patients with diabetes were incubated with adenosine or L-arginine.\(^15\)\(^,\)\(^16\)

Plasma concentrations of NO\(_x\) indicated the existence of endothelial dysfunction in patients with diabetes, especially those with ischemic-proliferative retinopathy. These findings, together with the greater platelet activity and lower sensitivity to inhibition by SNP, support the existence of a platelet–vascular wall imbalance that might account for the appearance of ischemia, as prothrombotic factors are stimulated while antithrombotic factors are inhibited.\(^17\) In this connection it has been shown that nitrite levels in patients with diabetes but without vascular complications are similar to those in the healthy population, whereas in patients with vasculopathies, plasma nitrite levels are lowered.\(^18\) Decreased platelet\(^19\)\(^,\)\(^20\) and vascular constitutive nitric oxide synthase (cNOS) activity\(^21\) have been suggested as the causes of reduced NO production in patients with diabetes. Our findings suggest that this reduction is also the result of decreased NO production in neutrophils.

### Table 3.

Plasma nitrite/nitrate levels in healthy volunteers and patients with diabetes and different types of retinopathy

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Nitrites/nitrates (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>20</td>
<td>9.72 ± 0.41</td>
</tr>
<tr>
<td>Patients with diabetes and without retinopathy</td>
<td>24</td>
<td>8.34 ± 0.59</td>
</tr>
<tr>
<td>Patients with diabetes and ischemic-proliferative retinopathy</td>
<td>43</td>
<td>2.14 ± 0.19*</td>
</tr>
<tr>
<td>Patients with diabetes and edematous retinopathy</td>
<td>30</td>
<td>8.14 ± 0.18**</td>
</tr>
</tbody>
</table>

\(^*\)\(^P < 0.05\) with respect to healthy volunteers and patients with diabetes and without retinopathy; \(^**\)\(^P < 0.05\) with respect to patients with diabetes and ischemic-proliferative retinopathy.
Nonetheless, patients with diabetes and edematous retinopathy stood out because of the absence of any reduction in plasma NOx levels. In theory, we would have assumed that because of the increase in NO, platelet inhibition would be greater, but in fact the data showed that inhibition was less intense in this subgroup of patients. To explain this apparent paradox we analyzed NO production by neutrophils, and the activity of cGMP, its second messenger in platelets. In patients with diabetes, nitric oxide synthase activity was lower, as shown by the lower level of NOx in the supernatant after incubation with L-arginine. This result is compatible with earlier reports of a lower activity of cNOS in patients with diabetes.\(^{19,20}\) However, the pattern of NOx production differed in patients with edematous retinopathy: induction with L-arginine led to higher NOx levels than in other subgroups of patients with diabetes.

Intraplatelet cGMP formation was clearly diminished in patients with any type of retinopathy, a result that is in line with earlier work that reported decreased guanylate-cyclase activity in patients with type II diabetes.\(^{22}\) Hence the defect in the NO–cGMP pathway appears to occur in NO formation. The increased production of NOx in patients with edematous retinopathy might be explainable by the formation of peroxynitrites: in patients with diabetes the formation of free radicals is greater, and the antioxidant defense capacity lower, than in healthy persons.\(^{23,24}\) In addi-

**Figure 4.** Left panel: NO\(_2^-\)/NO\(_3^-\) production in neutrophils under basal conditions (white bars) and after stimulation with 100 \(\mu\)M L-arginine (hatched bars). Right panel: intraplatelet concentrations of cGMP under basal conditions (white bars) and after stimulation with 10 \(\mu\)M sodium nitroprusside (SNP, hatched bars). HV, volunteers without diabetes (\(n=20\)); NR, patients with diabetes but without retinopathy (\(n=24\)); IPR, patients with diabetes and ischemic-proliferative retinopathy (\(n=43\)); ER, patients with diabetes and edematous retinopathy (\(n=30\)). *\(P<0.05\) in comparison to all other groups.

**Figure 5.** Left panel: 6-keto-prostaglandin-1α production in mononuclear leukocytes under basal conditions (white bars) and after stimulation with 1 \(\mu\)M calcium ionophore A23187 (hatched bars). Right panel: intraplatelet concentrations of cAMP under basal conditions (white bars) and after stimulation with 10 \(\mu\)M prostaglandin E\(_1\) (hatched bars). HV, volunteers without diabetes (\(n=20\)); NR, patients with diabetes but without retinopathy (\(n=24\)); IPR, patients with diabetes and ischemic-proliferative retinopathy (\(n=43\)); ER, patients with diabetes and edematous retinopathy (\(n=30\)). *\(P<0.05\) in comparison to all other groups.
tion, superoxide anions react with NO to form peroxynitrite radicals,\textsuperscript{25} which are highly oxidizing and markedly increase platelet aggregation.\textsuperscript{26,27} On the basis of this explanation it might be suspected that in patients with type I diabetes and edematous retinopathy, more free radicals would form and react with NO to give rise to peroxynitrites, which are detected when NO\textsubscript{x} production is measured. This would explain the absence of any apparent decrease in plasma NO\textsubscript{x} in these patients, and the increase in NO\textsubscript{x} production by neutrophils incubated with L-arginine. In this connection, an elevated platelet peroxynitrite production in patients with type II diabetes has been shown to result from the 3-fold increase in induced nitric oxide synthase activity in comparison to healthy persons.\textsuperscript{28} However, to confirm this hypothesis in type I diabetes, further research will need to show that more free radicals and peroxynitrites are formed in these patients than in patients with diabetes and other types of retinopathy.

The prostacyclin–cAMP pathway is also altered in patients with diabetes: the production of the prostanooid is lower, and sensitivity to platelet adenylyl-cyclase is diminished. These alterations are further evidence of the platelet alterations in patients with diabetes. Experimental studies have shown a relationship between the vascular production of prostacyclin and retinal vascularization in rats with streptozotocin-induced diabetes,\textsuperscript{29} and changes in prostacyclin synthesis modify the intensity of retinal ischemia in this model.\textsuperscript{30,31} However, in animals made diabetic, only ischemic retinopathy has been reproduced, but not edematous retinopathy.

In conclusion, there is a clear imbalance between platelet activity and endothelial function patients with type I diabetes mellitus, and this imbalance is especial in patients with edematous retinopathy. In these patients we found evidence of an alteration in the platelet NO–cGMP pathway. The differences we found between patients with different types of retinopathy suggest new approaches to research on the mechanisms that favor or impede the appearance of each type of retinopathy, and raise the possibility that different treatments may be appropriate for each group. However, further studies will be needed to learn more about these mechanisms in patients with different types of retinopathy.

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References


