Effects of sodium nitroprusside on hemodialysis-induced platelet activation

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Background. Hemodialysis (HD) is associated with increased platelet activation as reflected by enhanced P-selectin expression on platelets and by increased formation of heterotypic platelet-leukocyte aggregates. Both may play a pathophysiologic role in HD-associated platelet dysfunction or the propagation of atherosclerosis. As nitric oxide (NO) is a potent inhibitor of platelet activation, we were interested in whether HD-induced platelet activation could be blunted by a NO donor.

Methods. After a pilot study in 12 patients to gain an estimate for the sample size, the main trial was conducted as a randomized, double-blind, placebo-controlled, two-way, crossover study. Twelve patients received an infusion of sodium nitroprusside (1 μg/kg/min for over 15 min) or placebo into the inlet port of the HD device.

Results. Platelet activation increased within five minutes after start of HD (P < 0.05). Infusion of sodium nitroprusside neither decreased platelet activation (P-selectin+ platelets) nor affected the number of platelet-leukocyte aggregates (CD41+ neutrophils) as measured by flow cytometry.

Conclusion. Although NO may have inhibitory effects on platelet activation in vivo, our results confirm recent findings showing that NO donors were ineffective in preventing platelet activation by extracorporeal circulation during cardiopulmonary bypass or platelethpheresis. Thus, NO donors do not appear to be ideal candidate drugs to inhibit HD-associated platelet activation.

The adhesion molecule P-selectin is stored in the Weibel-Palade bodies of endothelial cells and in the α granules of platelets [1]. P-selectin is translocated within minutes from its intracellular storage sites to the surface of activated platelets [2, 3] and of activated endothelial cells [4, 5]. Expression of P-selectin on platelets is followed by rapid release of a soluble (c)P-selectin into the supernatant ex vivo [2, 3] and in vivo [6].

As P-selectin appears to play a role in early atherosclerosis [7, 8], mechanisms that down-regulate P-selectin expression and its release have become a focus of scientific interest.

A clinical situation that is associated with increased expression of P-selectin on platelets is hemodialysis (HD) [9–11]. The number of platelets expressing P-selectin increases as early as five minutes after the start of HD [9, 12].

Others showed that heterotypic platelet-leukocyte aggregates form during HD [13]. However, no increase was seen in the systemic circulation, possibly indicating sequestration of these aggregates in the human body. These aggregates have recently been found in a number of cardiovascular diseases as well as in sepsis [14]. More directly related to HD is the finding that hydrogen peroxide formation increased in the first 20 minutes of dialysis in neutrophils aggregated to platelets, but not in neutrophils not aggregated to platelets. Similar effects were observed in vitro when adenosine diphosphate-activated platelets were incubated with neutrophils but were largely inhibited by addition of anti-P-selectin antibody. These results strongly suggest that platelet-neutrophil aggregates during HD may have relevant pathophysiological effects. Finally, the HD-induced leukocyte margination has recently been demonstrated to correlate with increased expression of the oligosaccharide sialyl-Lewis × (CD15s) on leukocytes. The increased expression of CD15s on leukocytes was accompanied by increased binding of its counter receptor, P-selectin, to monocytes and polymorphonuclear cells [12]. This cell-bound cP-selectin may derive from the release of a soluble P-selectin from platelets or alternatively from the

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binding of cP-selectin pre-existent in the blood. It is therefore conceivable that contact of blood with artificial surfaces does not only cause hematological phenomena such as an HD-associated decrease in circulating platelets [13, 15] and leukocytes, by a P-selectin/CD15s interaction [12], but that it may also play an important role in dialysis-associated platelet dysfunction [16, 17], pulmonary dysfunction [18, 19], and propagation of atherosclerosis [20].

Thus, it seems important to investigate therapeutic avenues that down-regulate dialysis-induced platelet activation: Nitric oxide (NO) inhibits platelet aggregation, activation, secretion and adhesion [21–23]. Recently, it has convincingly been shown in various models that NO effectively [21–23] and reversibly [24] suppresses platelet activation and P-selectin expression, whereas inhibition of NO synthesis [25] has the opposite effect in that it enhances P-selectin expression on platelets. Hence, a NO donor may be an ideal model drug to down-regulate HD-associated platelet activation.

Based on these findings [24–27], we hypothesized that increasing the NO supply to platelets may inhibit their activation induced by contact with artificial surfaces such as dialysis membranes, as evidenced by diminished P-selectin expression and reduced formation of platelet-neutrophil aggregates. Hence, we studied whether NO generated from sodium nitroprusside (SNP) may blunt HD-associated platelet activation. If NO turned out to be effective in this respect, it could become a useful adjuvant in preventing HD-associated complications such as dialysis-associated platelet dysfunction, pulmonary dysfunction, and atherosclerosis.

**METHODS**

**Study design**

The study protocol was approved by the Ethics Committee of the Vienna University Hospital. Written consent was obtained from all patients prior to their inclusion in the study. The aim of the feasibility study was to determine an adequate sample size for the main outcome variable (P-selectin platelets) in the subsequent interventional trial and was therefore conducted as an open prospective trial. The main trial was a randomized, double-blind, placebo-controlled, two-way, cross-over study with a washout period of one week.

**The main study**

The aims of the interventional trial were to compare the effect of standard HD with the effect of dialysis under the influence of increased NO generation (by local SNP infusion into the inlet port of the dialyzer) on changes in P-selectin expression on platelets and platelet-leukocyte aggregate formation and on the dialysis-induced changes in leukocyte and platelet counts.

**Rationale for the use of SNP**

Sodium nitroprusside, a potent vasodilator, reversibly inhibits platelet function and P-selectin expression on platelets [24]. SNP is nonenzymatically degraded and thereby directly releases NO. In turn, NO enhances the activity of guanylate cyclase by formation of S-nitrosothiols and thus the intracellular concentration of cyclic guanosyl monophosphate. Because of the rapid reversible platelet inhibition (duration of pharmacodynamic action on platelet aggregation of less than 8 min), the short half-life, the direct NO release, and the dialysance of SNP, it was assumed to be the ideal candidate for this experimental protocol.

**Rationale for the dosage**

The dose of 1 μg/kg/min SNP is within the lower dose range for treatment of cardiovascular indications (0.5 to 8 μg/kg/min up to 3 days). Harris et al have observed a 50% decrease in epinephrine-induced platelet aggregation ex vivo, under the maximally tolerated systemic SNP dose of approximately 10 μg/kg/min [24]. We did not infuse SNP systemically but through the inlet port of the dialyzer. Considering a flow rate of approximately 300 ml/min in the inlet-line, SNP probably reached approximately 15-fold to 20-fold higher concentrations in the dialyzer than it would have reached when infused into the systemic circulation. Thus, this dose was likely appropriate to inhibit platelet function in the dialyzer circuit (because it was equivalent to a dose of 15 to 20 μg/kg/min administered systemically).

**Patients**

Twelve patients were included in the feasibility study. Based on the results from the feasibility study, we chose to study the same number of patients in the main trial, because it was calculated that 12 patients would be adequate to detect a 50% lower P-selectin expression under SNP as compared with placebo.

Eligible for inclusion in this study were patients routinely undergoing HD because of end-stage renal disease, aged between 19 and 80 years.

Exclusion criteria were hypotension (sitting diastolic tension of less than 60 mm Hg/systolic blood pressure of less than 100 mm Hg), history of migraine in the last month or history of symptomatic cerebrovascular disease.

**Experimental procedures**

In the feasibility study, no drug was administered. Twelve patients with end-stage renal disease undergoing maintenance HD were enrolled. A total of five blood samples (7 ml each) was drawn into citrated Vacutainer® tubes. The total blood loss on the study day was 35 ml. Blood samples were obtained at the following time points: (I) from the inlet-line at the start of dialysis at
zero minutes, \((2 + 3)\) from the inlet line and the outlet line at five minutes, and \((4 + 5)\) from the inlet line and the outlet line at 15 minutes.

These sampling times were selected because maximal activation of platelets and leukocytes has previously been observed at those time points \([9, 12, 13, 28]\).

In the interventional study, 12 patients, aged 51 years (range 34 to 67), with a body mass index of 23.7 kg/m\(^2\) (range 20.2 to 28.6 kg/m\(^2\)) were included. Patients were treated for an average of three years (range, 1 to 6 years) with HD. Blood flow in the HD device was 326 ml/min (CI, 260 to 393 ml/min). Blood samples were obtained as described earlier here. Patients were randomized to receive a 15-minute infusion of SNP (Nipruss; Sanol-Schwarz, Monheim BRD, Austria; 1 µg/kg/min dissolved in glucose 5%, infusion rate 2 ml/min) into the inlet port of the HD device, or alternatively glucose (Glucose 5%, infusion rate 2 ml/min) into the inlet port. SNP and placebo infusions were protected from light by using dark orange syringes, which were wrapped between studies \([13]\). However, data are presented as the percentage of P-selectin-positive platelets. The method for determining platelet activation of the HD device, or alternatively glucose (Glucose 5%, Leopold Infusionsflash®; Leopold Pharma, Vienna, Austria). SNP and placebo infusions were protected from light by using dark orange syringes, which were wrapped in aluminum foil, and by black infusion lines. This was done to protect SNP from degradation and to prevent unblinding of the investigator by the yellow color of SNP in solution. This procedure and lot number of SNP were shown to enhance the decrease in blood pressure during extracorporeal circulation on a cell separator \([29]\). After a minimum of eight days, patients crossed over to the alternative treatment protocol. As a general restriction, timing of erythropoietin administration \((N = 3)\) had to be exactly the same before or on both study days. The dialyzer membranes used were NiproFB-210UGA (Tri-acetate; Nisho Corp., Osaka, Japan), F60S and F80S (Polysulfone; Fresenius, Bad Homburg, Germany; \(N = 4\) each) and were not switched between periods. Antiaggregation was achieved by means of a loading dose of heparin and a constant infusion of heparin.

Blood was collected from the arterial inlet and the venous outlet lines of the hemodialyzer through a 21-gauge cannula into citrated Vacutainer tubes. All antibodies were monoclonal (mAb) and were purchased from Immunotech (Coulter, Vienna, Austria). Flow cytometric analysis of platelets was performed as previously described \([3, 30]\). Platelets were not fixed, and blood was therefore processed immediately to avoid storage-associated activation \([31]\). Whole blood was diluted with phosphate-buffered saline to a final concentration of 20 × 10\(^5\) platelets/liter in FALCON 2052 polystyrene 12 × 75 mm tubes (Becton Dickinson, San Jose, CA, USA). Twenty microliters of this dilution were incubated with 10 µl of phosphate-buffered saline without or with histamine 0.9 m as a positive control (yielding an average of 30% P-selectin positive platelets) for 10 minutes at room temperature. Expression of CD62 on platelets was determined in accordance with a recent consensus article \([32]\). A fluorescein–isothiocyanate (FITC)–labeled anti-P-selectin mAb directed against the low-density antigen (CD62P) was used simultaneously with a phycocyanin (PE)-labeled anti-CD41 mAb directed against the high-density antigen (CD41, which was used as a gate control) for double staining: 6 µl of undiluted FITC-labeled anti-P-selectin mAb and 6 µl of PE-labeled anti-CD41 were added to 20 µl of diluted whole blood and were incubated for five minutes at room temperature. The incubation was terminated by addition of 750 µl of ice-cold phosphate-buffered saline, and samples were put on ice; the flow cytometric analysis was started immediately thereafter. An isotype-specific mAb was used to set a threshold for P-selectin–positive platelets. We analyzed 25,000 platelets per sample with LYSIS II software on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Data are presented as the percentage of P-selectin–positive platelets. The method for determining platelet-leukocyte aggregates during HD has been performed as Gawaz et al. originally described to allow better comparison between studies \([13]\). However, data are presented as percentage CD41\(^+\) neutrophils rather than CD41 mean fluorescence intensity of neutrophils. Flow cytometric analysis of neutrophils was performed as previously outlined \([33]\). Soluble P-selectin was measured by enzyme immunoassay (R & D Systems, Oxon, UK) as described previously \([34]\). Plasma levels of F\(_{1+2}\) were determined by enzyme immunoassay (Enzygnost F\(_{1+2}\); Behringwerke, Germany) \([35, 36]\).

### Biometrics

Because of non-normal distribution, all data were compared with nonparametric statistics. The Friedman analysis of variance and the Wilcoxon signed ranks test were used. Data are expressed as means and the 95% confidence intervals. A two-tailed \(P\) value of less than 0.05 was considered statistically significant.

### RESULTS

#### Effects of hemodialysis on P-selectin expression of platelets

P-selectin expression increased within five minutes after start of HD. In the pilot study, the number of P-selectin\(^+\) platelets increased from 2.1% (CI, 1.0 to 3.1%) by 1.0% (CI, 0.0 to 1.9%) (Fig. 1). This was internally consistent with the findings in the main trial. Due to one outlier who showed an increase in P-selectin\(^+\) platelets from 6% to 27% in the SNP period, there was a larger variability in the SNP period. When this outlier is excluded from analysis, the number of P-selectin–positive platelets increased by 1.3% (CI, –0.1 to 2.7, \(P = 0.02\) vs. baseline) in the placebo period and by 1.1% (–0.1 to 2.3%, \(P = 0.04\) vs. baseline) in the SNP period. Hence, treatment with SNP did not inhibit platelet activation.
Formation of platelet-neutrophil aggregates

Baseline CD41+ neutrophils averaged 4.5% (CI, 3.2 to 5.8%) and 5.4% (CI, 2.8 to 7.9%) in the placebo and the SNP period, respectively. A drop in CD41+ neutrophils occurred within five minutes: −0.4% (CI, −1.3 to 0.5%; P > 0.05) and −1.3% (CI, −2.7 to −0.1%; P = 0.02) in the placebo and the SNP periods, respectively. At 15 minutes, the arteriovenous differences in CD41+ neutrophils averaged 0.2% (CI, −0.5 to 0.8; P > 0.05) and 1.6% (CI, −0.2 to 3.3%; P = 0.03) in the placebo and the SNP periods, respectively.

Changes in soluble P-selectin across the hemodialysis device

Soluble P-selectin levels averaged 59 ng/ml (CI, 49 to 70) and 68 ng/ml (CI, 48 to 87) in the placebo and the SNP periods, respectively. The levels of soluble P-selectin were only slightly higher in the venous outlet: 6% (95% CI, 0 to 12, P = 0.06) and 9% (CI, −3 to 21%, P = 0.024) during the placebo and the SNP periods, respectively. No differences between treatment periods were observed (P > 0.05, data not shown).

Changes in plasma levels of F1+2 across the hemodialyzer

Baseline values of F1+2 averaged 2.3 nmol/liter (CI, 1.6 to 3.1) in the placebo period and 2.3 nmol/liter (CI, 1.3 to 3.2) in the SNP period. There was a moderate arteriovenous increase in F1+2 plasma levels across the dialyzer at 15 minutes: 0.8 nmol/liter (CI, 0.0 to 1.7, P = 0.038) and 0.8 nmol/liter (CI, 0.2 to 1.4, P = 0.004) in the placebo period and the SNP period, respectively.

Changes in blood pressure

Although there was a minor (less than 5%) decrease in diastolic blood pressure at five minutes in both periods, there was no consistent or significant decrease in blood pressure after infusion of SNP (data not shown).

Changes in differential blood counts

All changes were corrected for hemoconcentration that occurred across the hemodialyzer (Fig. 2). Baseline neutrophil counts averaged \(3.8 \times 10^9/\text{liter} (\text{CI}, 2.8 \text{ to } 4.7)\) and \(3.3 \times 10^9/\text{liter} (\text{CI}, 2.5 \text{ to } 4.1)\) in the placebo and SNP periods, respectively. Five minutes after the start of HD, neutrophil counts dropped by \(-1.5 \times 10^9/\text{liter} (\text{CI}, -2.5 \text{ to } -0.5)\) and by \(-1.0 \times 10^9/\text{liter} (\text{CI}, -1.7 \text{ to } -0.3)\) in the placebo and the SNP periods, respectively (P < 0.005). However, no significant changes in platelet counts were observed (P > 0.05).

DISCUSSION

The aim of this investigation was to test whether an increase in NO supply would inhibit the HD-induced increase in platelet activation. Contrary to the hope that motivated the design of this study, we found that SNP infusion did not inhibit platelet activation as measured by P-selectin expression on platelets.

Numerous studies have demonstrated the ability of NO and SNP to inhibit platelet activation [24–27]. Thus, we have no ready explanation for the lack of effect of SNP. However, the question arises as to whether the dose of SNP was adequate. The dose was selected on the basis of results from previous studies with SNP. Infusion of SNP (40 \(\mu\text{g/min}\)) decreased diastolic blood pressure only slightly during the first 15 minutes during plateletpheresis, which is in good agreement with this study [29]. However, marked hypotensive effects were recorded after one hour of SNP infusion [29]. In another study, SNP infusion (1 \(\mu\text{g/kg/min}\)) did not cause any adverse events in healthy subjects not undergoing extra-
corporeal circulation, although it induced significant hemodynamic effects [37]. Doubling of the dose induced side effects such as headache or dizziness even in healthy subjects. Taking possible hypotensive effects of HD into account, we assumed that the lower dose would be better tolerated by our patients. Considering a flow rate of approximately 300 ml/min in the inlet port, SNP levels were probably 20-fold higher in the dialyzer than SNP concentrations expected after systemic infusion. Thus, we calculated that a dose of 1 μg/kg/min administered into the inlet port of the dialyzer would be equipotent to 20 μg/kg/min administered systemically. This was deemed adequate, since a maximally tolerated systemic SNP dose of approximately 10 μg/kg/min decreased the epinephrine-induced platelet aggregation ex vivo by 50%.

The discrepancy may be explained by the fact that not only a single but a multitude of stimulators of platelet activation (such as thrombin or adenosine diphosphate) may become operative in HD-induced platelet activation. This is underscored by the finding that NO is a more potent inhibitor of epinephrine than thrombin-induced platelet activation [38], which increased across the hemodialyzer. It should also be emphasized that the inhibitory effects of NO on platelet activation may be compromised in the presence of hemoglobin (whole blood as opposed to platelet-rich plasma). Finally, Langford et al’s study [39] and our data [29] demonstrate that NO donors are ineffective in inhibiting platelet activation during extracorporeal circulation, such as cardiopulmonary bypass or cell separators.

The second issue that has to be considered is the sample size, which was based on the pilot study in 12 patients undergoing HD, showing approximately a 50% increase in P-selectin expression during HD. Repeat samples from the same subjects showed a coefficient of variation of 10% in the degree of P-selectin expression [3]. Thus, although we had the power to detect a relatively large effect of SNP, minor effects of SNP may have gone undetected because of the day-to-day variability in the response of individual patients to HD.

Regardless of what may be regarded as a clinically relevant suppression of P-selectin expression, Figure 1 shows that there was not even a trend toward decreased P-selectin expression when SNP was administered. Hence, SNP, when administered at tolerable doses, is unlikely to be an effective inhibitor of HD-induced platelet activation. Further, SNP infusion did not prevent HD-induced neutropenia (Fig. 2).

We conclude that nitroprusside is not an ideal candidate drug to inhibit platelet activation during therapeutic HD or during extracorporeal circulation in general.

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