Impaired action of nitric oxide on blood platelets in premature newborns

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Abstract

Introduction: The influence of nitric oxide (NO) on blood platelets in premature newborns remains largely unexplained.
Material and methods: The expression of the activated GPIIb-IIIa complex and P-selectin were monitored by flow cytometry in whole blood platelets originating from 21 premature, 19 full-term neonates and 27 adults. Platelets were incubated with either freshly generated NO, L-arginine or NO donors (SNAP, GSNO) prior to their activation with collagen or ADP.
Results: Platelets from preterm neonates showed extremely reduced reactivity compared to normal full-term neonates and adults, when activated by ADP, collagen or thrombin ($P<0.001$ or less). NO reduced reactivity of neonatal platelets activated with collagen to a lower extent compared to adult donors (up to 28-39% in full-term and 6-15% in preterm newborns compared to 25-49% in adults); the tendency was not so regular for ADP. The observed reduction in platelet function remained in proportion to newborns' gestational age. Moreover, platelets from preterm neonates agonized with ADP showed reduced vulnerability to the action of L-NAME, an inhibitor of NO synthase, and L-cystamine, an inhibitor of guanylate cyclase. The immature responses in platelets from neonates, and especially preterm infants, are specific for various platelet activators, NO sources or modulators of the NO-dependent pathway.
Conclusions: In spite of immaturity and reduced reactivity of platelets from newborns, their NO-dependent inhibition may be efficient to some extent even in most immature babies.

Key words: nitric oxide (NO), platelet reactivity, neonatal platelets, premature neonates, NO generation.

Introduction

Nitric oxide (NO) is used in the therapy of the most serious conditions of respiratory insufficiency, and since 1992 it has been used more commonly also in the therapy of neonates. It improves oxygenation and acts as a selective pulmonary vasodilator, which means that NO has no influence on systemic blood pressure, like other pulmonary vasodilators used before [1-4]. In adults NO has been shown to suppress platelet adhesion and aggregation, and thus it is believed to contribute to the increased risk of haemorrhagic complications [5, 6]. There are only single

Table I. Demographic and clinical characteristics of newborns

Parameter	Preterm newborns	Full-term newborns
Patients:		
• total	21	19
• females	3	7
• males	18	12
Day of life	3.4±1.7	3.8±2.1
Weeks of gestation*	29.7±2.4 (24-34)	38.1±1.0 (37-40)
Birth weight [g]**	1217±462	3135±618
APGAR score in 5 min [points]*	5.2±1.8	8.7±0.7
Type of delivery:		
• caesarean section	13	8
• spontaneous vaginal	8	11

Data presented as mean ± SD or as median, lower quartile-upper quartile, min-max range given in parentheses
**P<0.0001 by lower side Mann-Whitney U test, **P<0.0001 by one-sided Student t test*

studies on the influence of NO on neonatal platelets, and those concerning premature newborns are even more occasional. The influence of NO on platelets in premature newborns still remains unexplained [7]. Neonates are characterized by an immature haemostatic system with hyporeactive platelets [8-10], and such immaturity has been reported to be directly proportional to the gestation age [11, 12]. Due to a fragile balance of haemostasis in premature neonates compared to adults, the peculiar constellation of compounding effects of lowered platelet reactivity and inhibitory NO effects have been considered a potential contributing factor of the increased risk of haemorrhage to the central nervous system in preterm newborns [6, 7, 13, 14]. Even though the preliminary outcomes of the clinical use of NO in the field of neonatology may seem encouraging, the safety and long-term benefits of such therapy require further studies [15].

In this study, we investigated the *ex vivo* effect of nitric oxide on blood platelets in preterm and full-term neonates, monitored as the changes in the expression of two recognized markers of platelet activation: the activated GPIIb-IIIa complex (PAC-1, the monoclonal antibody binding to activated GPIIb-IIIa complex) and P-selectin (CD62P, the antigen of P-selectin). We sought to explore whether nitric oxide is able to attenuate reactivity of neonatal platelets, as it does in adults, how susceptible such inhibition might be to modulators of the NO-dependent pathway, and whether the extent of such inhibition depends on the maturity of neonates.

Material and methods

Patients

The experimental protocol and study design complied with the ethical standards recommended by the Helsinki Declaration and were approved by the Committee on the Ethics of Research in Human Experimentation in the Polish Mothers' Health Centre in Lodz. Informed consent for participation in the study was obtained from all adult individuals and parents of newborns participating in this research programme.

Blood was obtained from 21 preterm neonates (group 1) and 19 full-term neonates (group 2). Infants with any clinical or laboratory signs of infection, recognized haemostatic disturbances, or congenital anomalies were excluded from our study. Table I presents demographic and clinical characteristics of neonates enrolled in this study. The reference group consisted of 27 healthy adult donors (10 men, 17 women, aged 28.5±3.8 year), who had not regularly used aspirin or any other drug known to affect platelet function for at least 10 days prior to the beginning of our study.

Blood collection, preparation and flow cytometry assay

Peripheral vein blood was collected from newborns (using the Microlance 3, 22G 1 1/4 0.7 × 30 needle) into a polyethylene tube (Becton Dickinson, USA) containing sodium citrate (0.105 mol/l) as an anticoagulant (final citrate:blood volume ratio was 1:10 v/v). The first two drops of blood were discarded before the collection of 1 ml of blood used for further analyses. All samples were processed immediately after the withdrawal of blood. Platelets were activated with collagen (type I, fibrils) from equine tendons, adenosine 5'-diphosphate (ADP) (Chrono-Log Corp., Havertown, PA, USA) or bovine thrombin (Dade-Behring, Marburg, Germany). With both preparation and staining we referred to the modified Becton Dickinson Protocol for Flow Cytometric Analysis for Platelets [8]. Briefly, in order to analyse the surface membrane expression of the activated GPIIb-IIIa complex and P-selectin, aliquots of whole blood were incubated with NOS substrates/donors (L-arginine, GSNO, SNAP, Oxis International, Inc., Portland, USA) and optionally with the modulator of the NO-dependent pathway, dbcGMP (N^2, 2'-O-dibutyrylguanosine 3':5'cyclic monophosphate); the inhibitor of nitric oxide synthase, L-NAME; and the inhibitor of guanylate cyclase, L-cystamine or dGTP (2'-deoxyguanosine-5'-phosphtate) (Sigma Chemicals Co., USA) [16, 17], then activated with agonists, stained and fixed prior to their analysis on a flow cytometer, as briefly presented in Figure 1.

Flow cytometric assay was performed within 24 hours on fixed platelets with a scatter and FL3

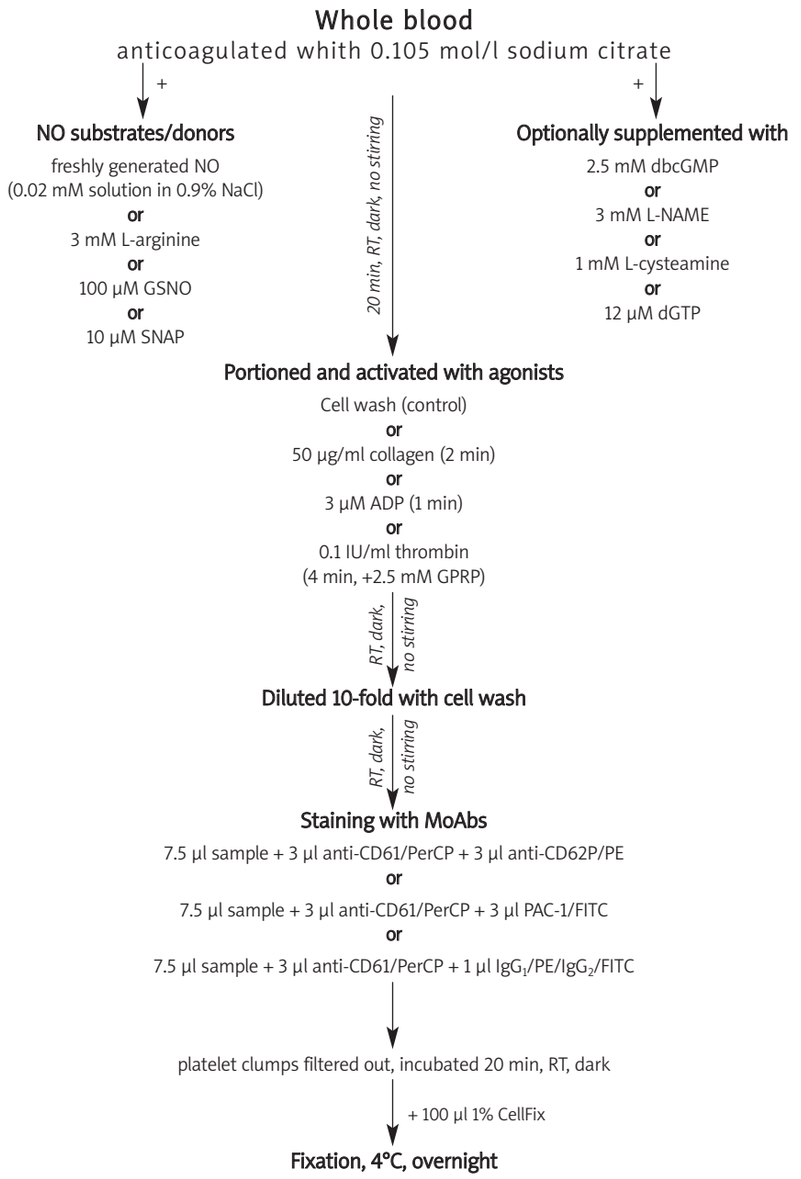

Figure 1. Flow chart presenting the methods used in platelet incubation and staining
RT – room temperature, MoAbs – monoclonal antibodies, GPRP – tetrapeptide Gly-Pro-Arg-Pro, the inhibitor of fibrin polymerization

fluorescence gates set on the platelet fraction. Laser excitation was at 488 nm and green (for FITC, FL1 channel), orange (for PE, FL2-channel), and red (for PerCP, FL3-channel) fluorescence of up to 10 000 platelets was read with a FACSCalibur instrument (Becton Dickinson). All data were acquired by using Lysis II (Becton Dickinson) software and analyzed with WINMDI 2.8 software. The percentage of platelets positive with the specific fluorescence was obtained after the subtraction of non-specific mouse IgG_1 binding and referred to as the expression of a given platelet surface membrane antigen. The extent of forward light scatter was employed to evaluate the fractions of normoplatelets, platelet microparticles and platelet aggregates (range of channels 0-30, 31-180 and 181-256 on the log 4 decade 256-channel scale, respectively) [8, 18-20].

All flow cytometry measurements were fluorescence-compensated on a daily basis for each set of measured samples using calibration beads (Becton Dickinson), to ensure that there was no considerable green, orange, and red fluorescence overlap. Flow cytometric measurements were performed at the Flow Cytometry Unit, Department of Clinical Immuno-

logy of the Institute of the Polish Mother's Health Centre.

Statistical analysis

Data are presented as mean ± SD for normally distributed variables (Shapiro-Wilk's W test) or as median and interquartile range [*Me*; IR: lower quartile (LQ) to upper quartile (UQ)] for parameters showing departure from normality. For time-related parameters we estimated harmonic means instead of common arithmetic means. The comparisons between adults and neonates in the extent of platelet function inhibition by NO or guanylate cyclase modulators were made using the data of the relative changes in antigen expression with respect to platelets incubated with a given agonist alone. Equality of variances (homoscedasticity) was verified with Levene's test. Data which showed a right-skewed distribution and met the remaining criteria of normal distribution were transformed logarithmically and analysed with the relevant parametric tests: unpaired Student *t* test, one-way ANOVA with repeated measures and the randomised complete block design ANOVA for pair-matched data. Multivariate ANOVA (MANOVA) and R Rao statistics were employed to assess the overall ('pooled') trend of inter-group differences in NO efficacy to attenuate platelet reactivity. For the comparison of groups in non-normally distributed data, we used the Mann-Whitney *U* test and the Kruskal-Wallis test. Parametric and non-parametric versions of Tukey's test were used for the multiple comparisons. Spearman's rank correlation was used to evaluate associations between selected parameters. Continuous variables for the analysis with 2 × 2 table χ^2 test were dichotomised based on the cut-off points determined by the use of ROC analysis. Interclass correlation for nominal data was estimated by Cramer's contingency test and expressed as the φ correlation coefficient [21].

Results

Activation of circulating blood platelets and platelet reactivity in response to collagen, thrombin or ADP

The circulating whole blood platelets in preterm and term newborns did not differ significantly in regard to both the markers of platelet activation: the expression of the activated GPIIb-IIIa complex (2.3±0.6 vs. 1.8±0.6%) and surface membrane P-selectin (2.6±1.1 vs. 2.4±0.8%). Likewise, no differences in the activation of whole blood platelets were found between neonates and healthy adult donors (1.8±0.9% for P-selectin and 2.8±1.1% for the activated GPIIb-IIIa complex). Upon the *ex vivo* stimulation of whole blood platelets with either collagen, thrombin or ADP, we noticed 2-3-fold reduced responses in neonates compared to healthy adult donors. Moreover, the agonist-induced increases in the fractions of PAC-1-positive and CD62P-positive platelets remained significantly higher in the group of full-term newborns in comparison to preterm newborns (Figures 2A, 2B). These findings point to greatly reduced platelet reactivity in all of the neonates and particularly profound platelet refractoriness in response to the used agonists in preterm neonates.

We found a significant correspondence between platelet reactivity and gestational age of neonates: reduced platelet reactivity occurred significantly more often in neonates with lower gestational age (Cramer correlation coefficients: φ=0.561, P<0.001 in collagen-activated platelets, φ=0.365, P<0.05 for PAC-1 and φ=0.463, P<0.005 for P-selectin in ADP-activated cells).

Effect of exogenous gaseous nitric oxide, NOS substrates and NO donors on platelet reactivity in response to ADP or collagen

To verify whether nitric oxide is able to hamper the activation of neonatal platelets agonized with either

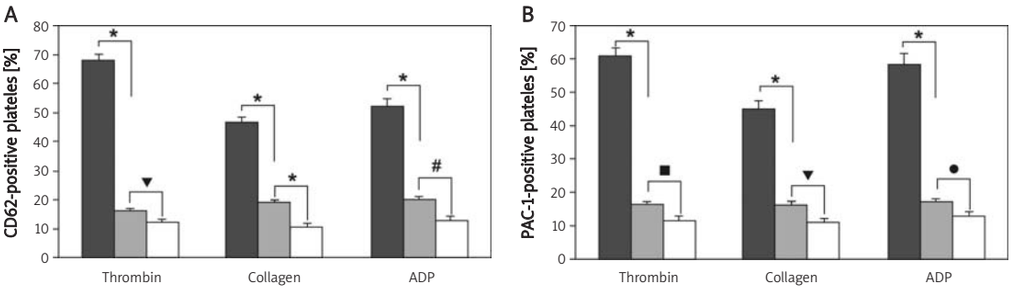

Figure 2. A – expression of P-selectin and **B** – the activated complex of fibrinogen receptor on the surface of whole blood activated platelets from full-term and preterm newborns and adult donors.
Data shown as mean ± SE for measurements performed in duplicate; n=27 for adult donors, n=21 for preterm and n=19 for full-term newborns. Data represent the expression of P-selectin (A) (monitored as the fraction of CD62-positive platelets) and the activated complex of fibrinogen receptor (B) (monitored as the fraction of PAC-1-positive platelets) on the surface of whole blood platelets from adult donors (dark grey bars), full-term newborns (light grey bars) and preterm newborns (white bars) activated with thrombin, collagen or ADP. Significance of differences between groups was estimated by analysis of variance and Tukey test for multiple comparisons: *P<0.0001, #P<0.005, ▾ P<0.02, ■ P<0.03, • P<0.05

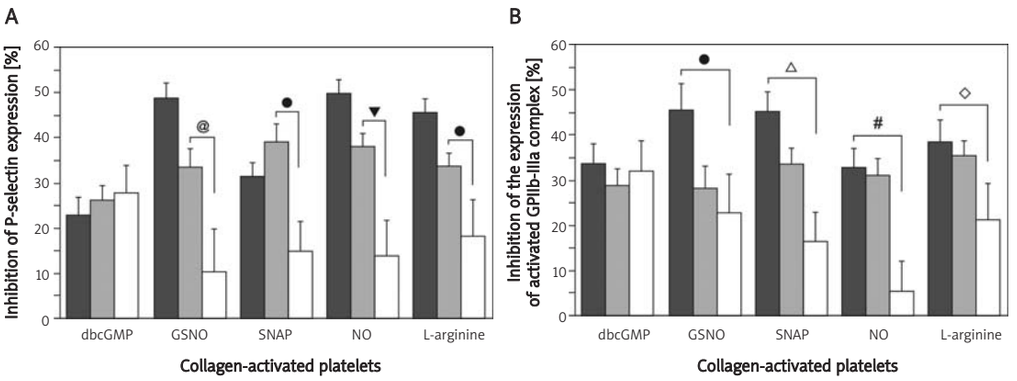

Figure 3. A – effects of NO on the expressions of P-selectin, **B** – the activated complex of fibrinogen receptor in whole blood collagen-activated platelets from full-term and preterm newborns and adult donors.
Data shown as mean ± SE for measurements performed in duplicate; n=27 for adult donors, n=21 for preterm and n=19 for full-term newborns. Data represent the NO-mediated reductions in the expressions of P-selectin (A) (monitored as the fraction of CD62-positive platelets) and the activated complex of fibrinogen receptor (B) (monitored as the fraction of PAC-1-positive platelets) on the surface of whole blood platelets from adult donors (dark grey bars), full-term newborns (light grey bars) and preterm newborns (white bars) incubated with dbcGMP, freshly generated NO, L-arginine, GSNO or SNAP, prior to their activation with collagen. Significance of differences between groups was estimated by analysis of variance and Tukey test for multiple comparisons: @P<0.01, ▼ P<0.02, • P<0.05, △P<0.003, ◊ P<0.004, #P<0.005

collagen or ADP, we employed freshly generated gaseous NO or model systems with L-arginine or NO donors (GSNO or SNAP).

The efficacy of NO-dependent attenuation in platelet response to agonists depended upon the agonist used to determine the in vitro platelet reactivity, as well as the monitored marker of platelet reactivity (P-selectin or the activated GPIIb-IIIa complex). In general, regardless of the source of nitric oxide, the NO-mediated reductions in platelet reactivity upon activation with collagen were significantly lower in preterm neonates compared to adults, and this was due to significantly reduced NO capability to attenuate platelet activation in the group of preterm newborns (Figures 3A, 3B). Although the platelet response to NO observed in full-term neonates tended to be diminished compared to adult individuals, these discrepancies were not statistically significant. Overall, we found that NO attenuated platelet reactivity to the highest extent in adult individuals, was less effective in full-term neonates, and its efficacy was significantly reduced in preterm newborns (MANOVA P<0.0001). This effect was statistically more distinct with regard to platelet release of P-selectin (Figure 3A) than activation of the GPIIb-IIIa complex (Figure 3B) (MANOVA R Rao 4.62, $P=6 \times 10^{-5}$ vs. 3.73, $P=6 \times 10^{-4}$).

The extent of NO-mediated reduction in platelet response to agonists in premature neonates was dependent on the duration of pregnancy for L-arginine and NO donors (Cramer φ in the range of 0.348-0.779, P<0.05 or less). In platelets agonized by collagen, platelet reactivity was significantly hampered in the presence of dbcGMP, the activator of adenylyl cyclase; these effects occurred to approximately the same extent in adult individuals and neonates (Figure 3).

When ADP was used as a platelet agonist, the observed efficacy of nitric oxide to attenuate the agonist-induced platelet activation varied according to the monitored marker of platelet activation. The effect of NO on the inhibition of intraplatelet granule release of P-selectin was not significantly different between healthy adult donors and neonates (Figure 4A), whereas the inhibitory effects of NO donors on the activation of GPIIb-IIIa complex were more profound in neonates compared to adults, although no differences were found between full-term and preterm neonates (Figure 4B). In general, we found that NO reduced the expression of the activated GPIIb-IIIa complex in the ADP-agonized platelets to a higher extent in neonates than in adult individuals (MANOVA P<0.005).

The alterations in the NO-mediated inhibition of platelet response to agonists in premature and full-term neonates were not dependent on the duration of pregnancy (Cramer φ in the range of 0.142-0.204, P>0.05). The activator of adenylyl cyclase, dbcGMP, greatly suppressed the expressions of P-selectin and the activated GPIIb-IIIa complex in platelets agonized by ADP, and this inhibitory effect remained much higher in preterm neonates compared to full-term neonates and adult individuals (Figures 4A, 4B).

Effects of NOS and guanylate cyclase inhibitors on NO-mediated inhibition of platelet reactivity in response to ADP or collagen

The effects of the two guanylate cyclase inhibitors, dGTP and L-cystamine, as well as the inhibitor of

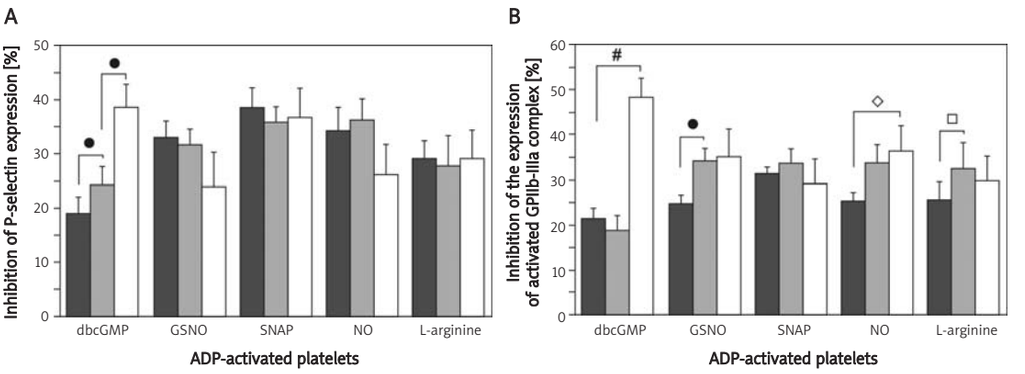

Figure 4. A – effects of NO on the expressions of P-selectin and **B** – the activated complex of fibrinogen receptor in whole blood ADP-activated platelets from full-term and preterm newborns and adult donors.
Data shown as mean ± SE for measurements performed in duplicate; n=27 for adult donors, n=21 for preterm and n=19 for full-term newborns. Data represent the NO-mediated reductions in the expressions of P-selectin (A) (monitored as the fraction of CD62-positive platelets) and the activated complex of fibrinogen receptor (B) (monitored as the fraction of PAC-1-positive platelets) on the surface of whole blood platelets from adult donors (dark grey bars), full-term newborns (light grey bars) and preterm newborns (white bars) incubated with dbcGMP, freshly generated NO, L-arginine, GSNO or SNAP, prior to their activation with ADP. Significance of differences between groups was estimated by analysis of variance and Tukey test for multiple comparisons: □ $P<0.04$, • $P<0.05$, ◊ $P<0.004$, # $P<0.005$

nitric oxide synthase (NOS), L-NAME, were tested in platelets incubated with L-arginine and agonized with either collagen or ADP. The observed efficacy of the inhibitors to reduce the NO-mediated suppression in the agonist-dependent platelet reactivity, as well as the inter-group differences, strongly depended upon the used inhibitor and agonist. In general, the NOS inhibitor, L-NAME, and the guanylate cyclase inhibitor, L-cystamine, were the most effective modulators of platelet NO pathway. For collagen-activated platelets L-cystamine was more effective in preterm neonates compared to adults, and statistically significant changes occurred for the expression of P-selectin. Similarly, the reduction in the expression of P-selectin in the presence of L-NAME was much lower in preterm neonates compared to adults and full term neonates (Figure 5A). The same trend of changes was typical for another marker of platelet activation, the activated GPIIb-IIIa complex; the changes however, did not reach statistical significance (Figure 5B). Overall, these inhibitors of the platelet NO pathway remained less effective in neonates when their platelets were agonized with collagen (MANOVA $P=0.041$) and the tendency was dependent on the duration of pregnancy, especially in regard to the expression of platelet P-selectin (Cramer φ in the range of 0.348-0.513, $P<0.05$ or less).

In platelets activated with ADP the tested NO pathway inhibitors were more efficacious in reducing the NO-mediated inhibition in activated GPIIb-IIIa expression in adult platelets than in neonate platelets; these changes however were statistically significant only between preterm infants and adults (Figure 6B). A similar trend occurred for platelet release reaction (P-selectin expression), but these changes reached statistical significance only in the case of L-cystamine (Figure 6A). Overall, the data in Figure 6 support the finding that NO pathway inhibitors had a more profound effect in adult platelets as compared to neonate platelets stimulated with ADP (MANOVA $P<0.01$). Such a tendency of milder attenuation of the NO inhibitory effects on platelet activation was dependent on the duration of pregnancy, especially in regard to the expression of the platelet-activated GPIIb-IIIa complex (Cramer φ in the range of 0.322-0.406, $P<0.05$ or less).

Discussion

Numerous abnormalities in the functioning of blood platelets in newborns have been reported to date, including transient platelet immaturity and platelet refractoriness to some agonists [8, 10-12, 22, 23]. In line with these reports were the findings that increased thrombopoietin levels and the percentage of reticulated platelets might indicate that thrombocytopoiesis is more active in term and premature newborns [24-26]. The majority of studies concerning full-term neonates pointed to platelet hyporeactivity in the presence of various agonists [8, 10, 12, 27], although evidence concerning preterm newborns is very scarce [22]. The problem whether hyposensitivity of neonatal platelets is a generalized phenomenon observed for various agonists and antagonists and whether platelets from preterm newborns show even a higher extent of immaturity is still a matter of debate [28, 29]. Among others, the higher percentage of platelet-derived microparticles in preterm newborns has been suggested as a compensatory mechanism for the haemostatic system [23]. Accordingly, the effects of NO on neonatal platelets and especially in premature newborns, as of potential use in clinical

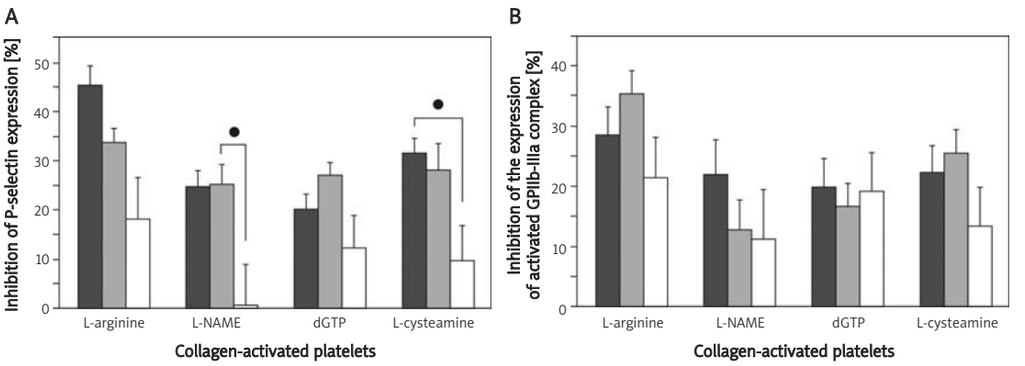

Figure 5. A – effects of nitric oxide synthase inhibitors on the NO-mediated expressions of P-selectin and **B** – the activated complex of fibrinogen receptor in whole blood collagen-activated platelets from full-term and preterm newborns and adult donors.
Data shown as mean ± SE for measurements performed in duplicate; n=27 for adult donors, n=21 for preterm and n=19 for full-term newborns. Data represent the NO-mediated reductions in the expressions of P-selectin (A) (monitored as the fraction of CD62-positive platelets) and the activated complex of fibrinogen receptor (B) (monitored as the fraction of PAC-1-positive platelets) on the surface of whole blood platelets from adult donors (dark grey bars), full-term newborns (light grey bars) and preterm newborns (white bars) incubated with L-arginine, L-NAME, L-cystamine or dGTP, prior to their activation with collagen. Significance of differences between groups was estimated by analysis of variance and Tukey test for multiple comparisons: • P<0.05

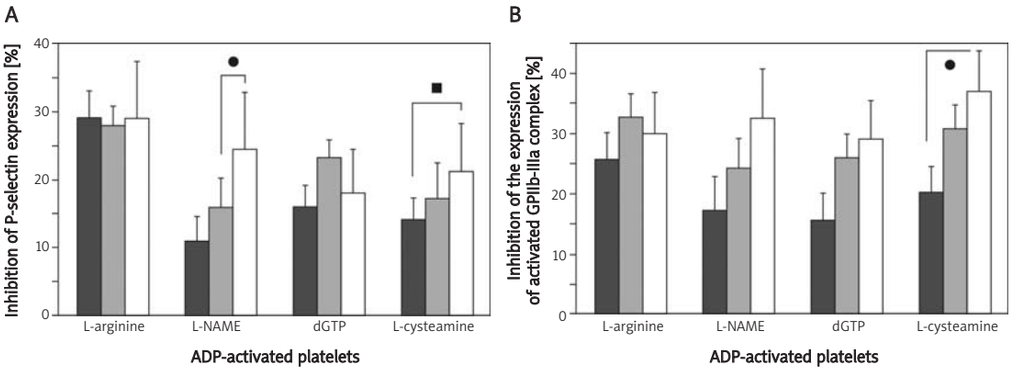

Figure 6. A – effects of nitric oxide synthase inhibitors on the NO-mediated expressions of P-selectin and **B** – the activated complex of fibrinogen receptor in whole blood ADP-activated platelets from full-term and preterm newborns and adult donors.
Data shown as mean ± SE for measurements performed in duplicate; n=27 for adult donors, n=21 for preterm and n=19 for full-term newborns. Data represent the NO-mediated reductions in the expressions of P-selectin (A) (monitored as the fraction of CD62-positive platelets) and the activated complex of fibrinogen receptor (B) (monitored as the fraction of PAC-1-positive platelets) on the surface of whole blood platelets from adult donors (dark grey bars), full-term newborns (light grey bars) and preterm newborns (white bars) incubated with L-arginine, L-NAME, L-cystamine or dGTP, prior to their activation with ADP. Significance of differences between groups was estimated by analysis of variance and Tukey test for multiple comparisons: ■ P<0.03, • P<0.05

practice in the treatment of premature neonates with RDS, may obviously seem worth exploring. In contrast to adult patients [6, 30-32], the influence of nitric oxide on platelets from newborns [14, 33, 34], and considering preterm neonates in particular, has never been extensively examined so far [35]. Due to scarce evidence on the effects of NO on neonatal platelets in general, the question of how nitric oxide influences the platelets of the youngest premature babies still remains unanswered.

In the present study we investigated the effect of a platelet activation inhibitor, nitric oxide, in mature and immature neonates, and analyzed our findings in the context of reduced neonatal platelet response to common platelet agonists, collagen and ADP. In this study, we have shown that neonatal platelets, compared to healthy adult individuals, remain deeply hyporeactive to thrombin, collagen and ADP, which corresponds to several earlier reports, including our own findings [8, 10, 28, 36]. Furthermore, platelets from preterm neonates showed extreme refractoriness in this respect, as deduced from the attenuated increase in the expression of platelet surface antigens in platelets activated with ADP, thrombin or collagen.

Otherwise, no such distinction was found by Uçar et al. The authors reported transient, 10-14 day hyporeactivity of platelets in both term and preterm groups, but there was no significant discrimination between these groups [22]. Moreover, we evidenced that such depressed platelet reactivity was categorized by the extent of the maturity of babies, and hence probably also the maturity of transduction pathways in neonatal platelets [37, 38]. The effect of NO on platelet reactivity was monitored in full-term and preterm neonates, including the most immature babies (with a body weight below 750 g). We used nitric oxide originating from three different sources – (1) physiological NOS substrate, L-arginine, (2) non-physiological artificial NO donors, such as GSNO and SNAP, and (3) a solution of gaseous NO generated in a model system. Regardless of the NO source, nitric oxide efficiently inhibited the expression of both P-selectin and the activated GPIIb-IIIa complex on the surface of the activated platelets from term and premature neonates, although the extent of NO effect differed depending on the platelet agonist. Whereas no apparent and regular differences occurred between adult and neonatal platelets agonized with ADP, in collagen-activated platelets the inhibitory effect of NO remained essentially dependent on the duration of the pregnancy and was the lowest in very premature newborns. Thus, our present observations are partly inconsistent with the conclusions of Keh et al., who claimed that the relative response of ADP-agonized platelets to NO donors was similar in neonates and adults [34]. Of note, we observed significant inhibitory effects of NO on neonatal platelet reactivity, not only for physiological NOS substrates and non-physiological NO donors, but also when using freshly generated gaseous NO. It is worth emphasizing even more that the final concentration of gaseous NO in blood samples in our model was 20 ppm – which is around the maximal NO concentration achieved during inhaled nitric oxide therapy in neonates under clinical conditions.

In general, the immature responses in platelets from neonates, and especially preterm infants, are specific for various platelet activators, NO sources or modulators of the NO-dependent pathway. L-NAME and L-cystamine were much less efficient inhibitors of the platelet NO pathway in ADP-agonized neonatal platelets compared to adults, whereas the opposite effects were observed for collagen-stimulated platelets. Interestingly, these effects appeared distinct and significant merely for P-selectin, as a marker of platelet activation. It seems that platelet functional immaturity, particularly with respect to the expression of intracellular enzymes of signal transduction, might be a reasonable common denominator linking NO's reduced ability to attenuate agonist-mediated platelet activation with the suppressed efficacy of NOS inhibitors. On the other hand, we observed that dbcGMP, which mimics the effect of NO-dependent inhibition of platelet activation, was more efficient in preterm neonates. This finding might be interpreted, by analogy to other, similar reported mechanisms, in terms of a possible compensation of reduced NO synthesis by increased efficiency of the cGMP-dependent signal transduction pathway [38].

The *in vitro* design may be considered the main limitation of this study. Its encouraging outcomes clearly indicate that it might be worth performing an *in vivo* prospective clinical observation including premature newborns with differentiated gestational age, who receive NO inhalation. Using some alternative methods for the monitoring of platelet reactivity, like the golden standard, platelet aggregometry, would certainly validate the data recorded in the present approach. As far as we considered this report a preliminary one, the group sizes were chosen to be relatively small. Therefore, to reasonably compare NO-affected platelet reactivity in dependence with the extent of 'prematurity', it seems to be worth re-estimating group sizes accordingly in such a clinical study.

To sum up, in spite of their immaturity and reduced reactivity to agonists compared to adults, platelets from newborns can respond to the action of nitric oxide, and the NO-dependent platelet inhibition may be efficient to some extent even in the most immature babies [34, 35].

Acknowledgments

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