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ENDOTHELIAL SECRETOGOGUES AND DEFORMABILITY OF ERYTHROCYTES

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Many diseases of the heart and circulatory system have been linked with both dysfunction of vascular endothelium and insufficient deformability of erythrocytes. Using shear stress laser diffractometry we investigated whether deformability of erythrocytes would be regulated endogenously by generation of two endothelial secretogogues: prostacyclin and nitric oxide. Experiments were performed in rats *ex vivo* and with whole blood or isolated erythrocytes *in vitro*. Iloprost - a stable analogue of prostacyclin (10 μg/kg i.v.) and SIN-1 (NO-donor) at a dose of 2 mg/kg/min i.v induced a significant improvement of deformability of erythrocytes *ex vivo*. Improvements of deformability by these two compounds were also evident *in vitro* when they were applied at a range of concentrations of 1 μM and 3 μM, respectively. Cyclooxygenase (indomethacin 20 mg.kg i.v.) and nitric oxide synthase (L-NAME 10 mg/kg i.v.) inhibitors while worsening deformability *ex vivo*, they did not affect (3 mM and 10 μM, respectively) rheological functions of erythocytes *in vitro*. Aggravating effects of these inhibitors on erythrocyte deformability *ex vivo* were reversed by prostacyclin and nitric oxide supplemented exogenously. Aspirin at a low (1 mg/kg i.v.) and high dose (50 mg/kg i.v.), contrary to indomethacin and L-NAME, aggravated erythrocyte deformability either *ex vivo* or *in vitro*. It is concluded that autocrine function of vascular endothelium plays an important role in regulation of rheology of red blood cells in flowing blood. The mechanism of this phenomenon is unclear but some possible explanations are discussed. In addition, in our experiments aspirin revealed unique erythrocyte damaging properties, possibly independent of inhibition of cyclooxygenase, but related to a direct protein acetylation.

Key words: *deformability, red blood cells, erythrocytes, endothelium, nitric oxide, NO, prostacyclin, PGI_2, aspirin, indomethacin*

INTRODUCTION

It has been widely acknowledged that deformability and aggregability of erythrocytes are very important determinants of microvascular perfusion and that they are of a considerable clinical significance in many circulatory disorders including atherosclerosis, essential hypertension (1, 2) and diabetes mellitus (3). Such approach to red blood cell (RBC) biology raises a crucial question whether rheological properties of RBC are affected by secretory function of endothelium known to play a key role in maintaining of vascular homeostasis. This function of endothelium is achieved through the release of a variety of autocrine and paracrine substances (4), with endothelium–dependent prostacyclin (PGI_2) and nitric oxide (NO), the best characterised and probably the most important among them (5, 6).

Essentially, PGI_2 and NO have platelet suppressant, fibrinolytic, thrombolytic, vasodilator and cytoprotective properties mediated by cyclic nucleotides, i.e. c-AMP and c-GMP, respectively. We have recently described that PGI_2 and NO, when released from leukocytes affect rheological functions of RBC (7). In addition, iloprost, stable prostacyclin analogue, as well as SIN-1, an active metabolite of molsidomine that spontaneously releases NO, were very potent modulators of RBC deformability in rats when given exogenously (7). On the other hand, erythrocytes were claimed to influence the secretory function of vascular endothelium and other blood cells such as platelets and/or polymorphonuclear leukocytes (8, 9).

In the present study we aimed to investigate the hypothesis that RBC deformability is regulated endogenously by balanced generation of two endothelial secretogogues: PGI_2 and NO. Accordingly we assessed the deformability of erythrocytes in rats under the influence of non-selective inhibitors of cyclooxygenase (indomethacin, aspirin) or NO-synthase (N^G-nitro-L-arginine methyl ester; L-NAME). Experiments were performed *ex vivo* and with whole blood or isolated erythrocytes *in vitro* to judge whether rheological properties of RBS could be also affected by non-endothelial sources of these two enzymes.

MATERIALS AND METHODS

Ex vivo experiments

Male Wistar rats were anaesthetised with thiopental (50 mg/kg i.p., Biochemic GmbH) and heparinized (800 U/kg i.v., Polfa). Blood samples (0.5 ml) were taken from the right carotid artery before (control sample) and after administration of following compounds into the left femoral vein: 1) SIN-1 (Sigma) infused at a dose of 2 mg/kg/min for 10 min and samples taken after 5 and 10 min (in some experiments 10 min infusion of SIN-1 was preceded with bolus injection of L-NAME (Sigma) at a dose of 10 mg/kg), 2) iloprost (Schering) in bolus injection at a dose of 10 μg/kg and sample taken after 15 min, 3) N^Gnitro-L-arginine methyl ester (L-NAME)

in bolus injection at a dose of 10 mg/kg and samples taken after 15, 30 and 45 min, 4) aspirin (1 or 50 mg/kg, Sigma) or indomethacin (20 mg/kg, Sigma) in bolus injection and samples taken after 15, 30 and 45 min.

In vitro experiments

Male Wistar rats were anaesthetized with thiopental (50 mg/kg i.p.). Blood (8 ml) was collected from the right carotid artery into heparin (25 U/ml) solution. Erythrocytes were isolated using the method described by Jubelin and Giennan (10). Red blood cells were separated from white blood cells on a Ficoll packed column, then washed three times in PBS solution (Imperia Lab. UK), enriched in albumin (Sigma) and glucose (POCH S.A.) Cells were suspended in solution of PBS with 3% dextran for 30 min and again rinsed to remove dextran. Finally, cells were resuspended in PBS solution. Haematocrit was adjusted to the value of whole blood haematocrit. Microscopic examination of prepared suspension revealed neither white blood cells nor platelets. Samples of whole blood or suspension of erythrocytes in PBS were incubated with SIN-1 (3 μM), iloprost (1 μM), aspirin (0.05 mM or 3.0 mM), indomethacin (3.0 mM), L-NAME (10 μM) or placebo (saline) at 22°C for 15 min.

Red blood cell deformability

Erythrocyte deformability was measured by a laser shear stress diffractometer (Rheodyn SSD). This instrument measures ellipsoidal elongation of red blood cells in response to defined, physiologically relevant shear stress conditions, forced by rotation rate. For all experiments deformability of RBC was assessed at the shear stress of 60 Pa. Samples of blood (30 μl) were suspended in 2 ml of dextran solution (MW 60 000, osmolarity 300 mOsm, pH=7.4, viscosity 24 mPa). The instrument projects actual level of deformability of red blood cells as a deformability index (DI%) calculated automatically according to equation: DI=100(L-W)/(L+W) where L and W are the means of length and width of elongated red cells, respectively. In our data every change in erythrocyte deformability resulting from the activity of investigated compound was expressed as ΔDI(%) – the result of a subtraction between reading of control DI and reading of DI in the presence of the compound or placebo. Thereby the values of ΔDI above zero (plus sign) or less than zero (minus sign) indicate improvement or worsening of RBC deformability by particular compound, respectively.

Statistical analysis

Results were expressed as arithmetical means ± SD of n numbers of experiments and analyzed by Student`s T test for paired means to determine the significance of the response; “p” values of less than 0.05 were considered as statistically significant.

The Institutional Review Committee approved the protocol for animal experiments.

RESULTS

NO and PGI_2 administered exogenously

In experiments *ex vivo* 10 min intravenous infusion of SIN-1 (NO-donor) at a dose of 2 mg/kg/min induced significant improvement of deformability of erythrocytes. The change of deformability index (Δ DI) as compared with control

reading before drug infusion was 1.41% ± 0.23, p<0.001 (*Fig. 1a*). Also iloprost – stable analogue of PGI_2, efficiently increased deformability index of RBC 15 min after its bolus intravenous injection at a dose of 10 μg/kg (ΔDI=2.05% ± 3.0, p<0.05) (*Fig. 2a*).

Improvements of RBC deformability by these two compounds were also evident *in vitro* when they were applied and incubated for 15 min in whole blood or in suspension of isolated erythrocytes. For SIN-1 at a concentration of 3 μM in whole blood and in isolated erythrocytes ΔDI was 2.41% ± 1.4, p<0.05 and 4.08% ± 2.6, p<0.01, respectively (*Fig. 1b*). For iloprost at a concentration of 1 μM the corresponding data were 2.09% ± 1.06, p<0.01 and 2.95% ± 1.93, p<0.01 (*Fig. 2b*).

Endogenous generation of NO and PGI_2

In *ex vivo* experiments an inhibitor of NO-synthase – L-NAME (10 mg/kg, i.v.) caused significant decrease of RBC deformability, reaching the maximal effect 45 min after its injection (ΔDI = -2,21% ± 0.55, p<0.01) (*Fig. 3a*). Contrary to the above, L-NAME did not significantly affect RBC deformability *in vitro* (*Fig. 3b*).

Worsening of deformability by NO-synthase inhibition *ex vivo* was spectacularly reversed by infusion of a NO-donor – SIN-1 at a dose of 2 mg/kg/min i.v. (ΔDI = -0.14% ± 0.89, after 5 min infusion). As compared with the change in deformability induced by L-NAME (ΔDI = -1.31% ± 0.83) statistical significance was at the level less than 0.001 (*Fig. 4*). Five minutes after infusion of SIN-1 deformability returned to the initial value.

Inhibition of cyclooxygenase in experiments *ex vivo* by intravenous injection of aspirin significantly worsened RBC deformability in a way similar to observed with L-NAME. The effect of aspirin appeared both at a low (1 mg/kg) and high (50 mg/kg) dose of the drug. ΔDI 45 min after bolus injection was –2.32% ± 1.32, p<0.01 and –1.39% ± 0.72, p<0.01, respectively (*Fig. 5*). Surprisingly, aspirin at a concentration of 0.05 mM and 3.0 mM worsened RBC deformability also in suspension of isolated erythrocytes: ΔDI = -1.37% ± 0.98, p<0.001 and -1.24% ± 1.0, p<0.001, respectively (*Fig. 6*). Other cyclooxygenase inhibitor - indomethacin (20 mg/kg i.v.) aggravated RBC deformability *ex vivo* similarly to aspirin (ΔDI = -1.34% ± 0.9, p<0.05 15 min after injection), but contrary to aspirin it did not affect deformability *in vitro* up to 3 mM concentrations (*Fig. 7*).

DISSCUSSION

So far distempered deformability of red blood cells in hypercholesterolaemia (11), arterial hypertension (1, 2), diabetes mellitus (3), coronary artery disease (12) and ageing (13) has been suggested to result from the injury of vascular endothelium with subsequent deficit of endothelium-

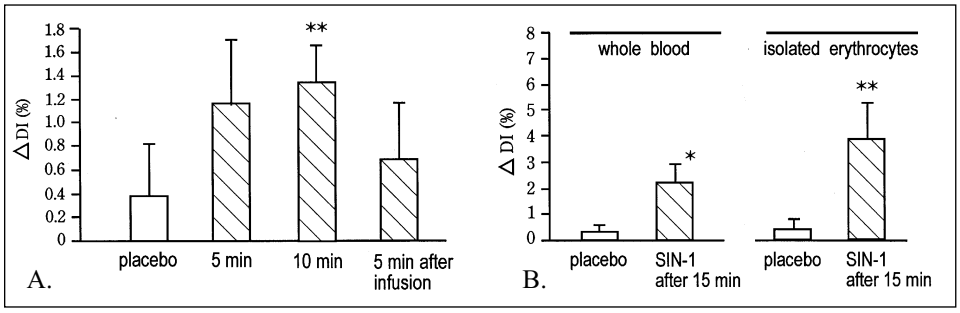

Fig. 1. The effect (ΔDI% mean ± SD) of NO-donor - SIN-1 on RBC deformability in rats *ex vivo* (2 mg/kg/min i.v. infusions - A) and *in vitro* (3 μM - B). ** p<0.01, * p<0.05, n=6

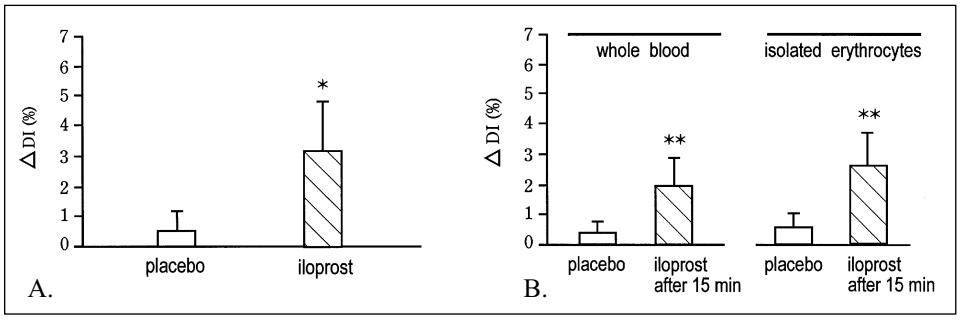

Fig. 2. The effect (ΔDI% mean ± SD) of iloprost on RBC deformability in rats *ex vivo* 15 minutes after its bolus injection at a dose of 10 μg/kg i.v. (A) and *in vitro* (1 μM - B).
** p<0.01, * p<0.05, n=6

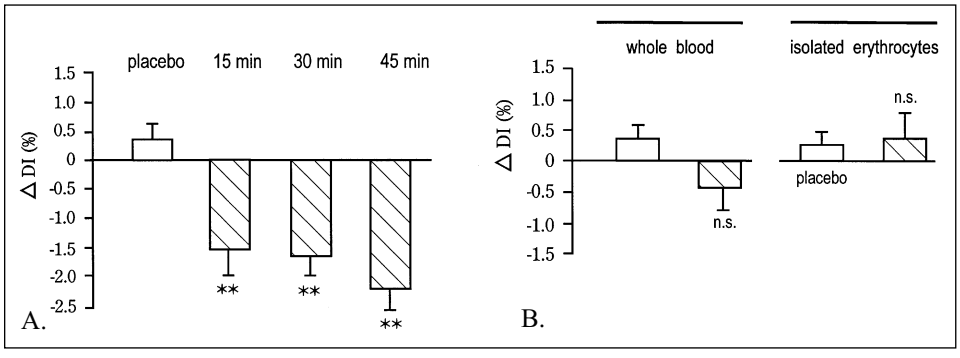

Fig. 3. The effect (ΔDI% mean ± SD) of L-NAME on RBC deformability in rats *ex vivo* (10 mg/kg i.v. - A) and *in vitro* (10 μM - B).
** p<0.01, * p<0.05, n=6

derived relaxing and antiplatelet secretogogues – nitric oxide (NO) and prostacyclin (PGI_2). Here we provide the evidence that RBC deformability may be also aggravated when endogenous generation of these compounds is pharmacologically suppressed by cyclooxygenase (indomethacin or aspirin)

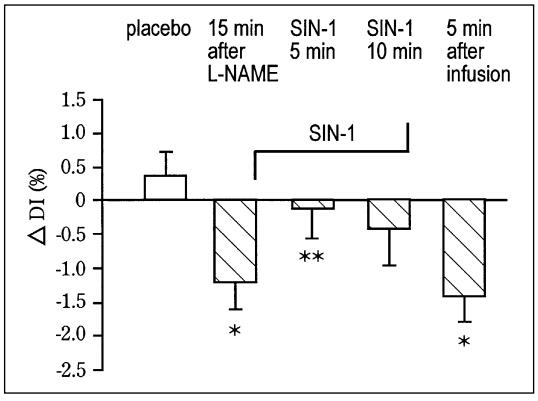

Fig. 4. The effect (ΔDI% mean ± SD) of SIN-1 infusion (2 mg/kg/min i.v.) on L-NAME-induced (10 mg/kg i.v.) worsening of RBC deformability in rats *ex vivo*.
** p<0.01, * p<0.05, n=6

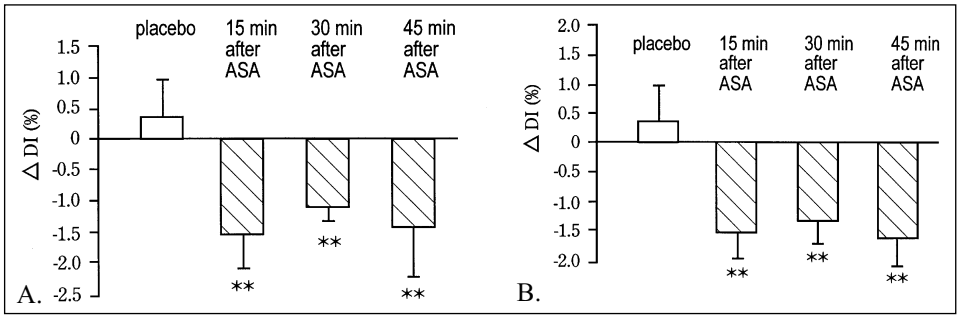

Fig. 5. The effect (ΔDI% mean ± SD) of aspirin on RBC deformability in rats *ex vivo* at a dose of 1 mg/kg i.v. (A) or 50 mg/kg i.v. (B).
** p<0.01, * p<0.05, n=6

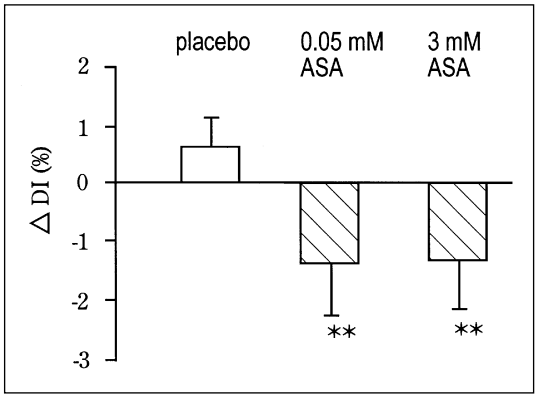

Fig. 6. The effect (ΔDI% mean ± SD) of aspirin (0.05 mM or 3 mM) on deformability of isolated red blood cells of rats.
** p<0.01, n=6

and NO-synthase (L-NAME) inhibitors. Moreover, we demonstrate that prostacyclin and nitric oxide directly improve deformability of red blood cells both *in vivo* and in isolated cells, and that they are capable to reverse damaging effects of their inhibitors. Thereby we propose the hypothesis on the balanced endothelial generation of these two secretogogues being responsible

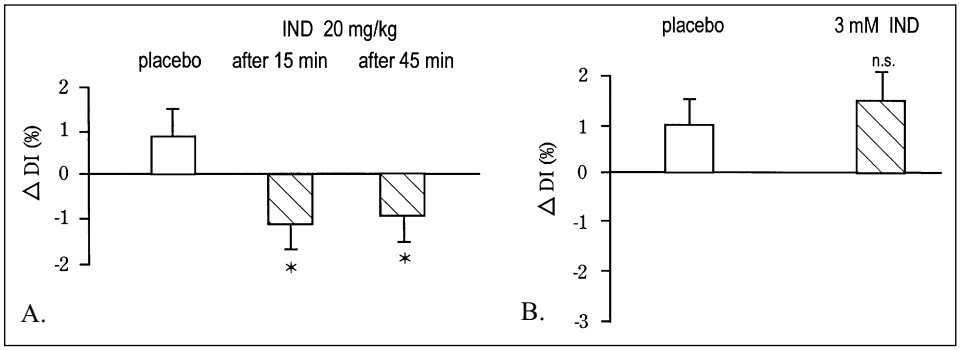

Fig.7. The effect (ΔDI% mean ± SD) of indomethacin on RBC deformability in rats *ex vivo* (20 mg/kg i.v. - A) and in whole blood (3 μM - B).
** $p<0.01$, * $p<0.05$, n=6

for the endogenous regulation of RBC deformability in physiological conditions.

What is the mechanism by which NO and PGI_2 regulate deformability and which endogenous sources of NO and PGI_2 are involved in the regulation of RBC deformability? Up to now numerous clinical studies (10, 14, 15, 16, 17) as well as our own exeprimental results have not enabled us to answer this question thoroughly. In our previous work on RBC deformability rabbit erythrocytes and other blood cells could interact freely and that was why we suggested that the deformability of red blood cells might be modulated by the presence of polymorphonuclear leukocytes (7). However, in experiments *in vitro* reported in the present study and elsewhere (14) NO and PGI_2 did affect erythrocytes even if they were completely isolated from the blood and other blood cells. This is the reason for us to speculate that the regulation of deformability by NO and PGI_2 seems likely to be related to the direct effect of these compounds on erythrocytes and/or on their membranes.

In most cases biological effects of NO are strictly related to the induction of c-GMP that subsequently, via protein kinase C, phosphorylates cell proteins including proteins of the cell membrane. Could it be also true for erythrocytes? Cyclic GMP-dependent mechanism by which NO may improve RBC deformability has been mainly claimed by advocates of the hypothesis that erythrocytes contain guanylate cyclase and two isoforms of NO-synthase (NOS-2 and NOS-3) thanks to which they are capable to self regulate the deformability by their own generation of NO (10). However, in our hands the NO-synthase inhibitor – L-NAME, while worsening RBC deformability *in vivo*, did not influence rheological properties of erythrocytes either in whole blood or in suspension of isolated cells. This clearly indicates that in physiological conditions RBC deformability is regulated by NO originating rather from vascular endothelium than from blood cells, including erythrocytes. The involvement of c-GMP in mediating the NO-induced changes of RBC

deformability seems to us quite possible. Increase of c-GMP content was found to correlate with changes of RBC deformability after administration of various c-GMP-activators such as sodium nitroprusside, sodium nitrite, atrial natriuretic peptide (ANP) or phosphodiesterase inhibitors (18, 19). On the other hand, among mechanisms by which NO may affect RBC deformability, there are also suggestions indicating that the effects of NO are independent on c-GMP. For instance, there is a possibility that NO directly improves deformability by activation of Ca^{+2}-dependent K^{+} channels known to be induced by nitric oxide in vascular smooth muscle cells and claimed to regulate also rheological properties of RBC (20, 21, 22).

In our experiments, similarly to the effects of nitric oxide, RBC deformability was significantly improved by the stable analogue of PGI_2 – iloprost. So far the mechanism of this phenomenon is unknown and to our knowledge PGI_2 receptors have not been found on erythrocyte membrane. However, since beneficial effects on RBC deformability were observed also for PGE_1 and for β-adrenergic agonists (all of them very potent stimulators of adenylate cyclase), possible role of c-AMP in mediation of biological functions of PGI_2 in erythrocytes appears acceptable (11, 23). In fact, an increased level of c-AMP induced by unspecific inhibition of phosphodiesterase is a commonly accepted mechanism for the activity of pentoxifylline - the only drug with clinically recognised RBC deformability improving properties successfully used for the treatment of peripheral arterial occlusive disease (24).

Interestingly, also NO has been found to inhibit phosphodiesterase E4 (PDE4). So, it could well be that both PGI_2 and NO improve RBC deformability by identical c-AMP-dependent mechanism. At least for the moment we are unable to agree with this opinion and certainly further basic experimental work is required to solve the problem.

As prostacyclin plays a protective role for RBC, the inhibition of its endogenous generation by cyclooxygenase inhibitors was supposed to disturb rheological properties of these cells. Indeed, indomethacin and aspirin when injected into the animal significantly aggravated deformability of erythrocytes. In addition indomethacin did not affect deformability *in vitro,* indicating that the most meaningful portion of prostacyclin involved in the regulation of RBC deformability originated from vascular endothelium. However, aspirin surprisingly decreased deformability *ex vivo* even when used at an antiplatelet dose of 1 mg/kg which was expected to be too low to inhibit cyclooxygenase. Moreover, it had identical effects in isolated erythrocytes. Is there any way to explain such unexpected effects? Along with our observations detrimental effects of aspirin on RBC deformability was also revealed by clinical studies (17, 25) and in a few laboratory experiments (14, 26, 27), but not many comments of this phenomenon were proposed. The most plausible explanation is that aspirin induces acetylation of integral proteins of the red cell membrane that leads to the rigidizing effect on membrane lipid fluidity (26). It should be mentioned as well

that aspirin, like e.g. methyl acetylphosphate, is able to acetylate human haemoglobin which in its acetylated form is known to modify proteins and/or lipids of the red cell membrane changing their rheological properties (28, 29). Thus, in our opinion worsening of deformability by aspirin may result not only from inhibition of cyclooxygenase in vascular endothelium and from depletion of prostacyclin but, above all, it can be due to unspecific effects of aspirin on red cell membrane.

In conclusion, two endothelial secretogogues: prostacyclin and nitric oxide significantly improve deformability of red blood cells and thereby they may play an important role in regulation of rheological functions of red blood cells in flowing blood. The mechanism of this phenomenon is unclear but it seems to be related to the direct effects of these compounds on erythrocytes. Aspirin has a unique property, possibly independent of inhibition of cyclooxygenase, that consists in worsening of the deformability by a direct acetylating effect of red cell membrane. Further exploration of the mechanisms by which NO-donors and prostacyclin analogues improve RBC deformability may contribute to the extension of their therapeutic applications in circulatory disorders.

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