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THE EFFECT OF PROSTACYCLIN AND NITRIC OXIDE ON DEFORMABILITY OF RED BLOOD CELLS IN SEPTIC SHOCK IN RATS

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Six hours after administration of E. Coli endotoxin (LPS) into rats (10 mg kg^{-1}, i.p.) a significant ($P<0.001$) decline in the red blood cell deformability index (RBC D_i) was observed. The control Di value of untreated animals it was 300 ± 39 RBC $\times 10^6$/min (mean $\pm$ S.D.; n = 12) while in LPS treated animals was 140 ± 50 RBC $\times 10^6$/min; n = 12. Pretreatment of the animals with the stable analogue of prostacyclin, iloprost (30 μg/kg, i.p.) or with the inhibitor of thromboxane A_2-synthase, camonagrel (10 mg/kg, i.p.), but not with nitric oxide donor, such as GEA 5285 (10 mg/kg, i.p.), significantly increased deformability of red blood cells in the group of non-septicaemic animals, and antagonized the LPS-induced decline in red blood cell deformability of septicaemic rats. Administration of N^G-nitro-L-arginine (L-NNA, 30 mg/kg, i.p.), as that of aspirin (50 mg/kg, i.p.), did not affect red blood cell deformability in non-septicaemic rats, however, in contrast with aspirin, it significantly improved deformability of red blood cells in LPS-treated animals. It is concluded that prostacyclin, camonagrel and L-NNA can act as protective agents against LPS-induced loss of red blood cell deformability. The mechanisms of this protection are complex and, possibly, related to the specific effects of these agents on biochemical function of leukocytes present in RBC suspension. While the effect of exogenous prostacyclin (iloprost) may be explained on the basis of its direct cytoprotective potency on leukocytes, the effect of camonagrel is indirect and can be attributed both to the release of endogenous prostacyclin and to the inhibition of thromboxane A2-synthase. The protection induced by NO-synthase inhibitor seems to depend upon inhibition of an increase of the generation of nitric oxide which follows administration of LPS.

Key words: *Red blood cell deformability, septic shock, prostacyclin (PGI_2), iloprost, Nitric oxide (NO), Nitric oxide-synthase inhibition, lipopolysaccharide (LPS).*

INTRODUCTION

Administration of lipopolysaccharide (LPS) *in vivo* is associated with over-production of nitric oxide within the vasculature (1, 2), various organs and blood cells (3, 4). Sepsis and endotoxaemia are known to be associated with

alterations in the red cell membrane that result in diminished deformability which may be responsible for the microcirculatory abnormalities and increased peripheral shunting accompanying sepsis. These alterations are claimed to be not a direct effect of endotoxin, but plausibly require the presence and participation of the white blood cells and/or their mediators (5). Our previous studies (6) showed that in non-septicaemic red blood cells *in vitro*, the deformability was modulated mainly by prostacyclin and by polymorphonuclear leukocytes *via* the release of nitric oxide. In this modulatory mechanism the concentration of nitric oxide was of critical importance. NO seemed to preserve or enhance red blood cell deformability within a certain range of concentrations, but these effects were reversed or eliminated at both too low and too high concentrations. Thus study evaluates the effect of endogenous or exogenous prostacyclin (iloprost) and nitric oxide on deformability of red blood cells in septicaemic rats *ex vivo*.

MATERIALS AND METHODS

Experimental protocol

Male Wistar rats (220—270 g) were injected with N^G-nitro-L-arginine (L-NNA; 30 mg kg^{-1}, i.p.), aspirin (ASA, 50 mg kg^{-1} i.p.), camonagrel (10 mg kg^{-1}, i.p.), NO-donor — GEA 5285 (30 mg kg^{-1}, i.p.), iloprost (10—30 μg kg^{-1}, i.p.) or vehicle, 24 h and 15 min before administration of E. Coli LPS (10 mg kg^{-1}, i.p.) or vehicle (saline). Six h later, animals were anaesthetized (pentobarbital, 40 mg kg^{-1} i.p.) and blood samples taken from the carotid artery into trisodium citrate (3.15% w/v) at a ratio of 9:1. Animals were then killed by exsanguination.

Erythrocyte deformability

Immediately after withdrawal, the blood was centrifuged at 1400 g (4000 r.p.m.) for 10 min, and the supernatant and the buffy coat were removed by aspiration. The number of erythrocytes was determined and then they were resuspended in their native platelet-poor plasma to give a hematocrit value of 0.39—0.4 in each experiment. Microscopic examination revealed that such suspension consisted mainly of red blood cells and a small number of neutrophils (1.2×10^6 cells/ml). Only single platelets were seen.

Erythrocyte deformability was measured by the erythrocyte flow rate method (7), as previously described (6). Briefly, under standard conditions erythrocytes were passed through a membrane filter using a negative pressure of 20 cm water. The deformability of red blood cells was determined by the speed of flow, the pore diameter of 5 μm being less than that of red blood cells and thus limiting flow according to the flexibility of the red blood cell membranes. The time was recorded for the passage of 0.5 ml of suspension of red blood cells. The results were expressed as a number of a red blood cells filtered per min (RBC number/min), which we defined the "deformability index" (D_j). The assay system contained two identical filters, allowing to assess control and treated samples simultaneously.

Materials and reagents

The membrane filters had pores of a diameter of 5 μm, with 4×10^5 pores per cm^2 and a thickness of 10 μm (Nucleopore filters — GSI Darmastadt, Germany). Chemicals used were

iloprost (gift from Schering AG, Germany), N^G-nitro-L-arginine (L-NNA, Sigma), camonagrel (Ferrer int.), donor of nitric oxide — GEA 5285 (GEA Ltd, Denmark), E. Coli endotoxin (LPS serotype 0127:B8, Sigma).

Statistical analysis

In each experiment the permutation test and checking the experiment related error probability for first-order error was calculated. The results were expressed as means ± SD of n experiments, and analyzed by using a two-way analysis of variance followed by a least significance procedure to determine the nature of the response. A P value of less than 0.05 was considered statistically significant.

RESULTS

The red blood cell suspension all the time contained polymorphonuclear neutrophils, but never by more than 1.2×10^6 cells/ml. Administration of LPS produced a significant ($P < 0.001$) decline in the red blood cell deformability index (D_j) (*Fig. 1*). The control Di value of untreated animals was 300 ± 39 RBC $\times 10^6$/min (mean ± S.D.; n = 12) and in LPS treated animals it was 140 ± 50 RBC $\times 10^6$/min; n = 12 (*Fig. 1*). Pretreatment with a stable analogue

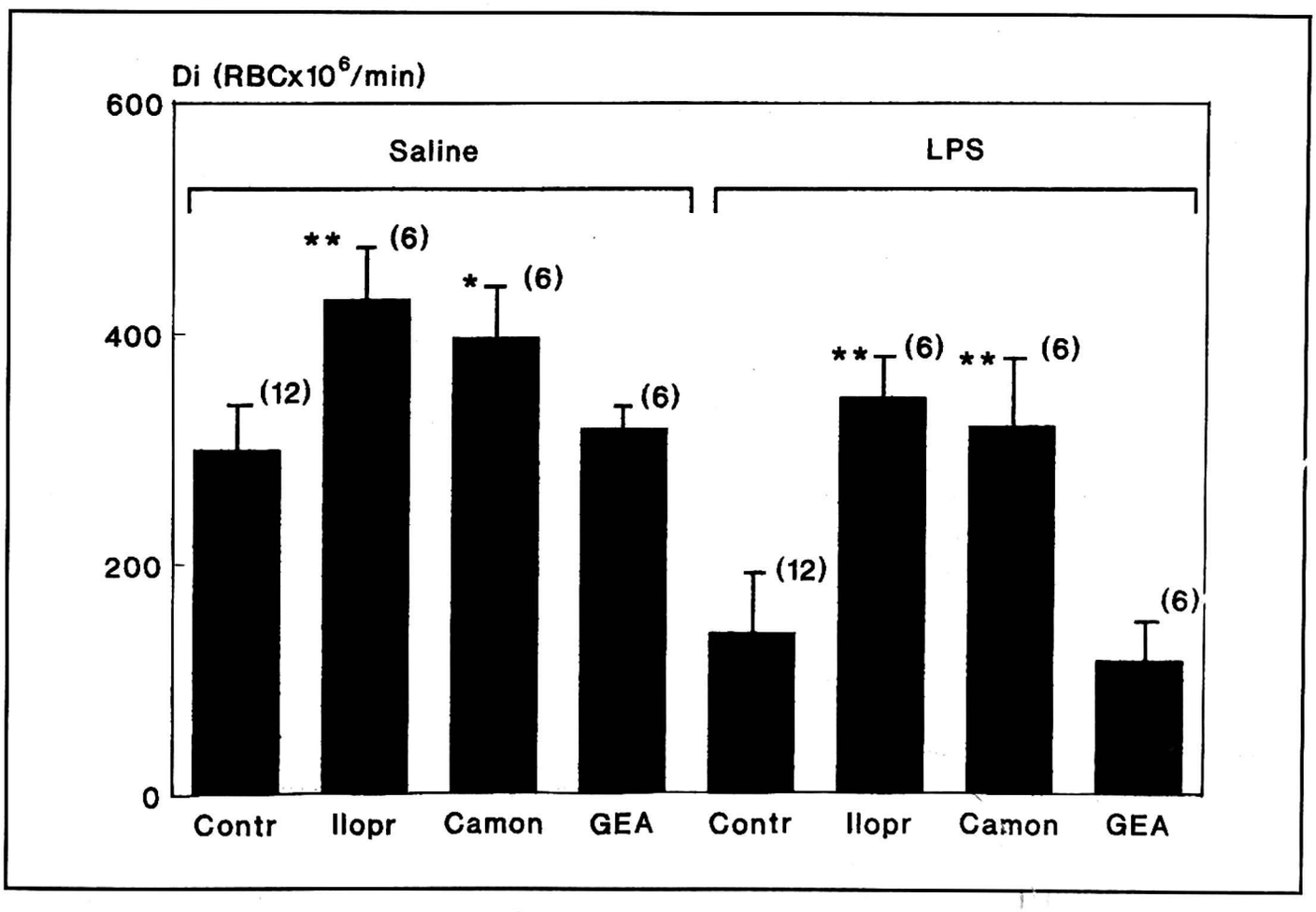

Fig. 1. The effect of iloprost (30 μg kg^{-1} i.p.), camonagrel (10 mg kg^{-1} i.p.) and NO-donor — GEA 5285 (10 mg kg^{-1} i.p.), injected into rats 24 h and 15 min before injection of vehicle (saline) or lipopolysaccharide (LPS, 10 mg kg^{-1} i.p.) on the changes in deformability of red blood cells (RBC). * $p < 0.05$, ** $p < 0.01$ as compared to corresponding controls. Numbers in parentheses indicate number of experiments.

of prostacyclin, iloprost (30 μg kg^{-1}, i.p.) or with an inhibitor of thromboxane A_2-synthase, camonagrel (10 mg kg^{-1}, i.p.), but not with nitric oxide donor, such as GEA 5285 (10 mg kg^{-1}, i.p.), significantly increased deformability of red blood cells in the group of non-septicaemic animals, and antagonized the LPS-induced decline in red blood cell deformability of septicaemic rats. Administration of N^G-nitro-L-arginine (30 mg kg^{-1}, i.p.) did not affect red blood cell deformability in non-septicaemic rats, similarly to that of aspirin (50 mg kg^{-1}, i.p.), however, in contrast with aspirin, it significantly improved deformability of red blood cells in LPS-treated animals (*Fig. 2*).

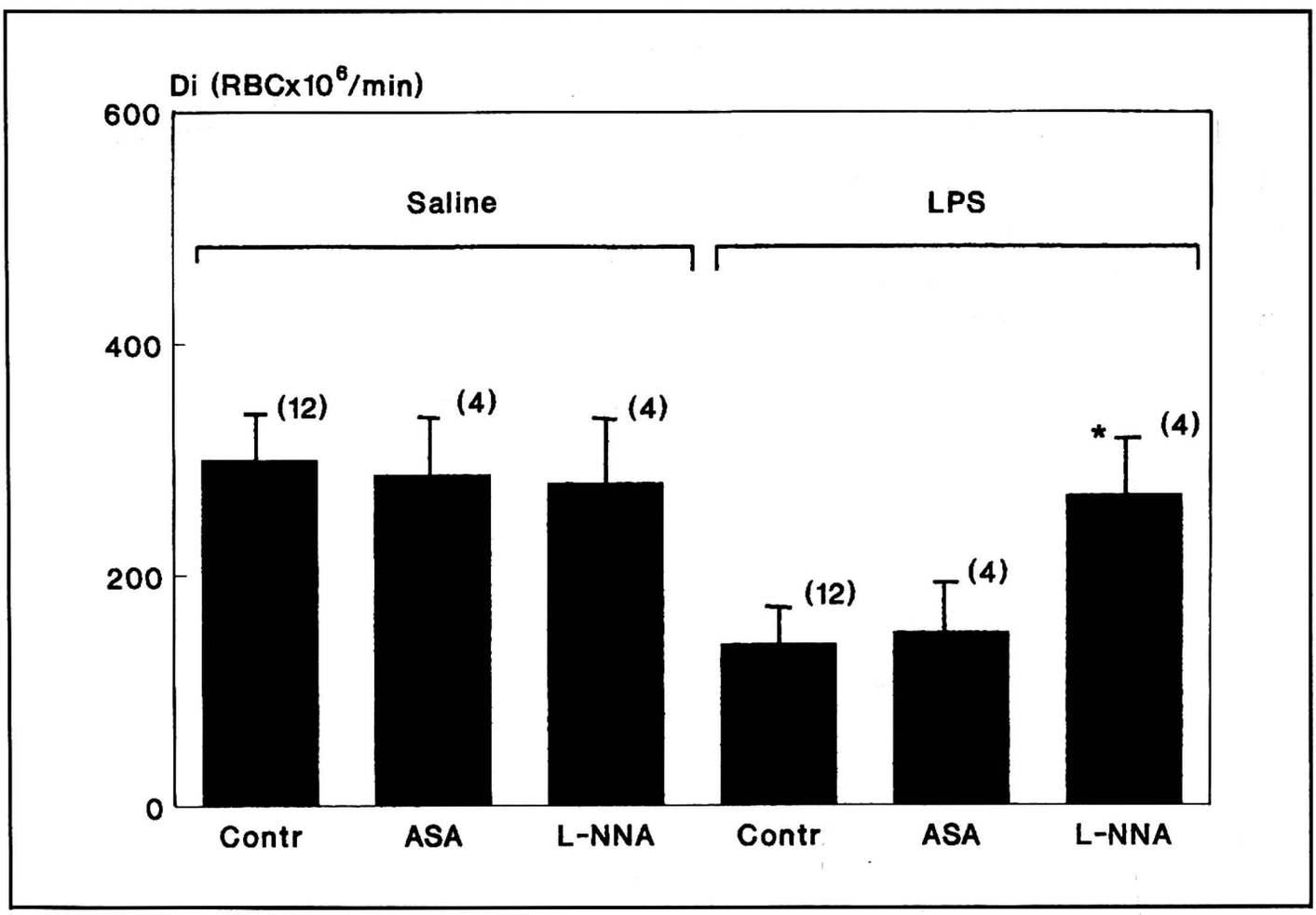

Fig. 2. The effect of aspirin (ASA, 50 mg kg i.p.) and L-NNA (30 mg kg^{-1} i.p.) injected into rats 24 h and 15 min before injection of vehicle (saline) or lipopolysaccharide (LPS, 10 mg kg^{-1} i.p.) on the changes in red blood cell deformability. * $p < 0.05$ as compared to corresponding controls. In parentheses numbers of experiments

DISCUSSION

The fundamental purpose of erythrocyte metabolism is to maintain membrane deformability, thus ensuring the capacity of erythrocyte to traverse the microcirculation (8). Failure of the red cell membrane to maintain this fluidity has been amply demonstrated both in clinical and experimental sepsis (9, 10) indicating that sepsis-associated changes in fluidity of erythrocytes may play a role in the pathophysiology of multiorgan system failure.

However, the specific mechanisms responsible for LPS-induced changes in red blood cell deformability remain a matter of considerable debate. So far, the theory has been accepted that the sepsis-evoked changes in the erythrocyte membrane are secondary to neutrophil-derived, oxidant-induced membrane damage (5, 11). Recently, it has been shown that the effect of LPS on red blood cell deformability depends on the exposure of lipid A of LPS to binding to red blood cell membranes (12). Interestingly, changes of deformability in septicaemia are associated with significant elevations of intacellular calcium concentration and some studies attempted to modify endotoxin-induced alterations in erythrocyte intracellular calcium dynamics with such agents as pentoxifylline and free radical scavengers which are known to modulate the activation of leukocytes and their products (11).

Here, we have analyzed the influence of exogenous (iloprost) and endogenous prostacyclin and nitric oxide on rheological properties of red blood cells in control and septicaemic rats and we were able not only to confirm that the deformability of red blood cells is decreased in septicaemic rats, but for the first time we have found that this decrease may be significantly improved by pretreatment with iloprost — a stable prostacyclin analogue as well as with camonagrel — a selective inhibitor of thromboxane A_2 synthase (13, 14). LPS-induced impairment of red blood cell deformability was also improved by the inhibition of the generation of nitric oxide with N^G-nitro L-arginine. In contrast with N^G-nitro-L-arginine, which improved deformability only in septicaemia, iloprost and camonagrel were potent and rapidly acting in preserving red blood cell deformability both in septicaemic as well as in control animals.

The effect of prostacyclin or its stable analogue — iloprost on red blood cell deformability in physiological conditions has been reported by many investigators (15—17) including ourselves (6). However, despite these studies and numerous studies of clinical conditions, such as Raynaud's disease, systemic sclerosis in which eicosanoid production is reduced (18), the mechanism of the effects of prostacyclin is rather speculative. What we know is only that the drugs affecting the deformability of red blood cells, including prostacyclin, are also potent modulators of biochemical functions of leukocytes (19). In consideration of the fact that LPS decreases deformability *in vitro* only in the presence of leukocytes, it seems very likely that this blood cells are crucial for any drug to improve red blood cell deformability, possibly also in septicaemia. In the experiments reported here, red blood cells and polymorphonuclear leukocytes interacted freely with each other and we believe that the effects of prostacyclin analogue could be due to the direct protection of leukocyte functions by this agent. This last phenomenon has been recently demonstrated by us (19).

Could an inhibition of TXA_2 synthase *in vivo* be an explanation for protective effect of camonagrel on erythrocytes in our experimental model? So

far there are not any results of investigations available on the possible role of TXA_2 in red blood cell deformability, but the following evidence from the present work speaks against this assumption. Aspirin at a dose which completely inhibits the generation of TXA_2 and prostaglandin endoperoxides hardly have any protective action on erythrocytes deformability both in control and in septicaemic animals. Secondly, unlike aspirin, camonagrel does not inhibit the formation of prostaglandin endoperoxides (14), and these act on the same receptors as TXA_2. Speculating, if camonagrel-induced inhibition of TXA_2-synthase was responsible for its protective activity on red blood cell deformability, this effect should be rather weak due to excessive generation of endoperoxides. However, this is not the case in our experiments and we would rather incline towards our original conception (14) that camonagrel *in vivo* shifts the metabolism of prostaglandin endoperoxides from TXA_2 to prostacyclin, inducing the release of this endogenous endothelial hormone. Consequently the release of prostacyclin could be responsible for the mechanism of the activity of camonagrel. However, on the other hand, the inhibition of endogenous prostacyclin by cyclooxygenase inhibitor — aspirin does not affect the deformability of red blood cells both in control and in septicaemia. Thus; our final conclusion is more cautious. We believe that the phenomenon of inhibition of TXA_2 by camonagrel cannot be totally excluded from the mechanism of its protective activity on erythrocyte deformability and, moreover, the endogenous prostacyclin seems to influence the deformability only in very special conditions i.e. during excessive generation of endoperoxides. This is likely inasmuch as endoperoxides are a better substrate for PGI_2-synthase than any other source and as camonagrel always shows dual activity: apart from the activation of the release of endogenous prostacyclin it also inhibits TXA_2 formation (14).

Administration of lipopolysaccharide *in vivo* is associated with the induction of nitric oxide synthase in various organs and blood cells (3, 4). Although inhibitors of nitric oxide synthase may be beneficial both in clinical treatment of endotoxaemia (20) as well as in the treatment of LPS-induced disseminated intravascular coagulation (DIC) in experimental animals (21), it is not as yet known whether such treatment could affect septicaemia-induced impairment of red blood cell deformability. In our previous work (6) we showed that *in vitro* nitric oxide produced by leukocytes was an important factor in the regulation of red cell deformability under non-activated conditions and that the amount of NO available to red cells was of critical importance in that mechanism. Below a certain concentration, NO from leukocytes or from NO-donors, like sodium nitroprusside, enhanced deformability; nevertheless, above that concentration a toxic effect, resulting in a significant reduction in red cell deformability was observed. In our present studies nitric oxide donor — GEA 5285 (22) did not significantly influence the

deformability of red blood cells both in control and septicaemic animals. This, however, does not seem to imply that exogenous nitric oxide does not affect deformability of erythrocytes *in vivo*. First of all, in contrast with our *in vitro* work, nitric oxide donor had been administered to the animal as a bolus injection more than 6 hours before the procedure of deformability measurements was started. Simply, during such a period of time the capacity of NO-donor to release nitric oxide was probably by then decreased or even completely exhausted (22). Moreover, it would be very peculiar if additional amount of nitric oxide, delivered by NO-donor, affected deformability of erythrocytes during septicaemia, since the concentration of NO, as a result of leukocytes activation by LPS was possibly at that time above any physiological limits. According to our previous work *in vitro* (6), one could expect in such conditions even a suppression of deformability by NO. In fact, we did observe insignificant but a slight impairment of red blood cell deformability in septicaemic animals pretreated with GEA 5285. On the other hand, in control animals when the concentration of endogenous NO was probably not sufficient to affect deformability, a slight improvement was induced by this compound. As expected, opposite effects were caused by NO-synthase inhibitor. Improvement of red blood cell deformability by N^G-nitro-L-arginine was so much pronounced in septicaemic animals that it could be very tempting to speculate on the protective activity of this agent against LPS-induced loss of red blood cell deformability in septicaemia. The better potency of NO-synthase inhibitor in protection of deformability in sepsis than in control animals simply suggests that the dependence between the percentage reduction of deformability caused by L-NNA and the amount of NO available for red cells is inversely proportional. In other words, the more intense the generation of NO (septicaemia) the less damaging NO-synthase inhibitor can be for red blood cell deformability. Interestingly, a similar dependence on the generation of NO by increased number of leukocytes and the activity of NO-synthase inhibitor was observed in our previous work *in vitro* (6).

In conclusion, we propose that prostacyclin, camonagrel and L-NNA can act as protective agents against LPS-induced loss of red blood cell deformability. The mechanisms of this protection are complex and related to the specific effects of these agents on biochemical function of leukocytes. While the effect of exogenous prostacyclin may be explained on the basis of its direct cytoprotective potency on leukocytes, the effect of camonagrel is indirect and can be attributed both to the inhibitor of thromboxane A_2-synthase and to release of endogenous prostacyclin. The protection induced by NO-synthase inhibitor seems to depend upon an inhibition of increased generation of nitric oxide following LPS administration. However, the use of NO-synthase inhibitors in septicaemia would be hazardous, because it is never known what

concentration of NO is adequate to be inhibited. The role of endogenous nitric oxide and prostacyclin requires further investigations in physiological conditions.

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