Impaired Flow-Mediated Vasodilation in vivo and Reduced Shear-Induced Platelet Reactivity in vitro in Response to Nitric Oxide in Prothrombotic, Stroke-Prone Spontaneously Hypertensive Rats

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\textbf{Key Words}
SHRSP \cdot Flow-mediated vasodilation \cdot Nitric oxide \cdot Platelet \cdot Shear \cdot Hypertension

\textbf{Abstract}
Previous investigations using an He-Ne laser-induced thrombosis method have shown that stroke-prone spontaneously hypertensive rats (SHRSP) have an enhanced thrombotic tendency in vivo compared to normotensive, Wistar Kyoto rats (WKY). In addition, studies in the presence of acetylcholine have suggested the presence of endothelial dysfunction in SHRSP. In contrast, shear-induced platelet reactivity in vitro appeared to be depressed in SHRSP. The aim of the present study was to investigate endothelial function in SHRSP using a new physiological in vivo model, and to determine the response of platelets to nitric oxide (NO) in non-anticoagulated blood using a shear-induced platelet function in vitro method (haemostatometry). Endothelial function was estimated by measuring flow-mediated vasodilation (FMV) of the femoral artery. Vessels were exposed and blood flow was arrested using a silicone-coated arterial clamp. Vasodilation was measured by computer-assisted image analysis 3 min after release of stasis. Arterial vasodilation was observed in the femoral artery of WKY, but not in SHRSP. Vasodilation was seen in both WKY and SHRSP; however, in response to the NO donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC 5). In contrast, 100 \textmu M NOC 5 did not affect platelet reactivity in SHRSP. The NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, sodium salt (carboxy-PTIO) and the NO synthase inhibitor, \textit{N}G\textsuperscript{-}nitro-\textit{L}-arginine methyl ester, hydrochloride (L-NAME), did not affect shear-induced platelet reactivity. NOC 5 at 10 \textmu M (final concentration) inhibited shear-induced platelet reactivity in WKY. These results confirm the presence of endothelial dysfunction in SHRSP and indicate that platelets are non-responsive to NO in this hypertensive model. The data suggest that defective endothelial reactions or disturbed thrombogenic mechanisms outweigh the platelet hyporeactivity and contribute to the prothrombotic status in SHRSP.

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Introduction

Arterial platelet-rich thrombogenesis plays an important role in life-style-related athero-thrombotic diseases such as stroke and myocardial infarction. The precise relationships between prothrombotic mechanisms and the development of cardiovascular disorders remain to be fully evaluated, however, and a number of useful animal models have been described for this purpose. In this respect, we have utilised a sensitive and reproducible He-Ne laser-induced thrombosis technique to assess thrombotic status in vivo [1–3] and a well-defined test of primary haemostasis (haemostatometry) to analyse shear-induced platelet function in native, non-anticoagulated blood [4–8].

Using these techniques, we determined that one breed of congenitally diabetic rat (Goto-Kakizaki) appeared to have an enhanced thrombotic tendency in vivo and platelet hyper-aggregability in vitro compared to the control Wistar rat [9]. In contrast, another breed of rat with congenital diabetes (Otsuka Long-Evans Tokushima Fatty rat) appeared to have a reduced thrombotic tendency and platelet hypo-aggregability [10]. In these experiments, therefore, in vivo and in vitro reactions were consistent and suggested that platelet function contributed directly to thrombogenic mechanisms. In stroke-prone spontaneously hypertensive rats (SHRSP), however, an enhanced thrombotic tendency in vivo was observed in the presence of acetylcholine suggested that endothelial dysfunction prevailed over the platelet hypo-aggregability [10]. In these experiments, therefore, in vivo and in vitro reactions were consistent and suggested that platelet function contributed directly to thrombogenic mechanisms. In stroke-prone spontaneously hypertensive rats (SHRSP), however, an enhanced thrombotic tendency in vivo was observed in the presence of acetylcholine suggested that endothelial dysfunction prevailed over the platelet hypo-aggregability [10].

Endothelial dysfunction and NO metabolism are key processes in atherosclerosis and thrombosis [13]. Flow-mediated vasodilation (FMV) is thought to reflect endothelial NO release, and techniques have been described using FMV to quantify endothelial function in humans [14–16]. In animals, however, tests of vasodilation have classically utilised ex vivo arterial segments [17, 18] and vasoactive responses have been studied in situ in only a small number of experiments [19, 20]. The aim of the present study was to establish a physiologically relevant technique based on FMV to evaluate endothelial function in SHRSP in vivo, and to relate the findings to the effects of NO on shear-induced platelet function in this model.

Materials and Methods

Reagents

The NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, sodium salt (carboxy-PTIO), and the NO synthase (NOS) inhibitor, N-nitro-L-arginine methyl ester, hydrochloride (L-NAME), were dissolved and diluted in saline. The NO donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC 5), was dissolved and diluted in 0.1 M NaOH. The reagents were purchased from Dojindo Laboratories (Kumamoto, Japan). Solutions were stored at –80°C and used within 2 weeks of preparation.

Animals

Male SHRSP and normotensive WKY rats were kindly donated by Kinki University School of Medicine (Osaka, Japan) at 8–20 weeks of age. Animals were fed a commercial solid diet (SP diet, Sunabashi farm, Japan) and given tap water ad libitum. They were housed in compliance with the ‘Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences’ published by the Physiological Society of Japan. All experiments were performed at 28–31 weeks of age (body weight in WKY and SHRSP rats, 451.5 ± 6.1 vs. 322.5 ± 8.8 g, p < 0.001; systolic blood pressure, 166.8 ± 7.0 vs. 240.4 ± 7.4 mm Hg, p < 0.001, respectively).

Flow-Mediated Vasodilation

Animals were anesthetized with sodium pentobarbital (60 mg/kg, i.m.). They were artificially ventilated and placed on a heated pad to maintain body temperature. The left femoral artery was carefully exposed and removed from the surrounding tissue. The vessel was compressed at a proximal part of the exposed artery using an arterial clamp coated with silicon tubing. Complete occlusion was established gently and maintained for 3 min. The clamp was removed to restore blood flow and changes in vessel diameter were monitored before and after treatment with the NO donor.

Changes in vessel diameter were continuously recorded on a videotape recorder. Subsequently, images at 30-second intervals were transferred to a personal computer and vessel outer diameters were analysed with Image-Pro Plus software (version 4.0, Media Cybernetics, Inc., Md., USA). Baseline values were obtained 30 s before vessel occlusion or adding NOC 5. Vessel diameters were monitored before and after treatment with the NO donor.

In vitro Shear-Induced Platelet Reactivity Test

(Haemostatometry)

A three-channel purpose-built haemostatometer was constructed in the Physiology Laboratory of the Faculty of Nutrition at Kobe Gakuin University. Details of the measurement of shear-induced platelet reactivity and dynamic coagulation (haemostatometry) have...
been described elsewhere [6–8]. In brief, blood was withdrawn using a 20-gauge needle, without anticoagulant, from the abdominal aorta of SHRSP or WKY rats anaesthetised with nembutal (60 mg/kg; i.m.). Blood was collected in 1.5-ml aliquots into syringes containing 15 or 150 μl of reagent or vehicle. After gentle inversion three times, measurements commenced without delay. Perfusion of the blood samples through standardised polyethylene tubing (outer diameter: 1.00 ± 0.02 mm; inner diameter: 0.50 ± 0.01 mm) was achieved by pumping paraffin liquid into the blood sample at a constant rate of 0.057 ml/min. After 150 s, pressure was stabilised at 60 mm Hg and holes were punched in the tube using a needle (diameter: 0.18 mm). Blood loss into surrounding saline led to the formation of platelet-rich haemostatic plugs, blood coagulation and subsequent arrest of blood flow. Pressure changes were monitored and the parameters H1 and H2 (mm Hg s), CT1 and CT2 (min) were defined as indices of platelet reactivity (adhesion/aggregation) and dynamic coagulation, respectively.

Measurement of Blood Pressure
Systolic blood pressures were measured by the tail cuff method (UR-1000, Ueda Seisakusyo, Tokyo, Japan).

Statistical Analysis
All data are presented as mean ± SEM. The H1 and H2 parameters obtained by haemostatometry were converted to logarithms before statistical analysis. Results were analysed by the unpaired t test, one-way factorial ANOVA or one-way repeated-measures ANOVA, followed by post-hoc test (Fisher’s PLSD) using the commercially available statistical package Stat View (version 5.0; SAS Institute Inc., Cary, N.C., USA). p < 0.05 was considered to be statistically significant.

Results
Vasodilation Induced by Flow and Exogenous NO – Comparison of SHRSP and WKY
Vessel diameters in WKY after occlusion for 3 min were significantly increased compared to baseline. In contrast, vessel diameters in SHRSP were not changed after temporary vessel occlusion. Thus, FMV was evident in the control rats but not in the hypertensive animals (fig. 1a, p < 0.0001). Exogenous NOC 5-induced vessel dilation in both SHRSP and WKY rats (109.8 ± 1.7 % and 109.6 ± 3.0 %, at 30 s after NOC 5 adding). The changes mediated by NOC 5 were not significantly different between SHRSP and WKY (fig. 1b).

Effects of NOC 5, Carboxy-PTIO and L-NAME on Shear-Induced Platelet Reactivity and Dynamic Coagulation
The effects of the NO scavenger, carboxy-PTIO, and the NOS inhibitor, L-NAME on shear-induced platelet reactivity and dynamic coagulation were assessed by haemostatometry in WKY rat (fig. 2). H1 and H2, and, CT1 and CT2, respectively, showed good correlation. Representative data for H2 and CT2 are illustrated in figure 2. Carboxy-PTIO did not affect shear-induced platelet reactivity but inhibited dynamic coagulation at concentration of 1 mM. L-NAME had no effect on either shear-induced platelet reactivity or dynamic coagulation.

The NO donor, NOC 5, significantly and dose-dependently inhibited platelet reactivity at a concentration of 10 μM (p < 0.01) in WKY rats. It had no effect on dynamic coagulation in WKY. Neither platelet reactivity nor dynamic coagulation was affected by NOC-5 in
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Discussion

The classical Virchow’s triad hypothesis describes interactions between blood cells, endothelial cells and blood flow as central to the pathogenesis of thrombosis. Moreover, recent investigations have clarified the role of blood flow or shear forces in endothelial-related mechanisms and have emphasized the relevance of physiological calcium and thrombin to thrombogenesis [21–24]. It is clear,

Fig. 3. The effects of NOC 5 on shear-induced platelet reactivity (H2) and dynamic coagulation (CT2) measured by haemostatometry. NOC 5 at a concentration of 10 μM significantly inhibited platelet reactivity in WKY but not in SHRSP. Dynamic coagulation was not changed by NOC 5 in either strain. The basal value of H2 was significantly lower in SHRSP than in WKY. Values are means ± SEM. n = 9–11 rats in each group. * p < 0.05; ** p < 0.01; *** p < 0.001.
therefore, that the principles of the tests employed to fully evaluate thrombotic mechanisms in vivo should take account of these structural and functional relationships. In this respect, studies of experimental arterial platelet-rich thrombi in animals provide a good approximation of pathophysiological processes and can be utilised to assess the effects of antithrombotic agents in vivo. Studies of this nature are not applicable to humans, however. Platelets play a central role in arterial thrombogenesis, and platelet function has been examined by various in vitro tests. The technique of haemostatometry has been developed to assess platelets activated by high shear forces in non-anticoagulated blood and has been used to determine thrombotic tendency in man under various conditions [4–6, 8, 25]. Gorog and Kovacs [26] proposed that the H1 parameter is an index of ADP-induced and that H2 reflects thrombin-induced platelet mechanisms. Later experiments have shown, however, that this distinction might not be totally valid. Therefore, both H1 and H2 are used as indices of general platelet reactivity. The CT1 and CT2 parameters identify stages of the blood coagulation process [7]. Antiplatelet agents inhibit only platelet reactivity (H1 and H2) but not coagulation (CT1 and CT2) [7]. In addition, shear-induced haemostasis tests such as haemostatometry are physiologically relevant to platelet reactivity in vivo [6, 27] and haemostatometry is very sensitive to physiological agonists compared to conventional tests. For example, unlike conventional platelet aggregation, haemostatometry responds to physiological, nanomolar concentrations of norepinephrine [8]. As with the majority of in vitro platelet function tests, however, this method lacks the ability to examine the role of endothelial dysfunction in thrombogenesis.

An FMV test has been described to evaluate endothelial function in humans [14–16] in which vascular responses are mediated by endothelial derived NO [19]. In animals, endothelial function is classically assessed using excised aortic rings to which vasoactive agents may be added in culture chambers [17, 18]. There are few reports using blood vessels in situ for evaluating endothelial function in animal models [19, 20]. In the present study we have adapted the principles of FMV to examine endothelial function in SHRSP, a well-characterized prothrombotic model. Using exposed femoral arteries, we demonstrated that vasodilatation occurred after the release of temporary stasis, and that this vasodilation lasted more than 15 min. This appeared to be different from the results of human FMV tests in which the responses are transient and last for 180 s at most. These differences remain to be fully investigated but may be related to the surgical procedure performed in animals. Nevertheless, vasodilatation consistently occurred in control animals (WKY) but not in SHRSP. In contrast, the NO donor, NOC 5, promoted vasodilatation in SHRSP as well as in WKY, and it seems likely that this was an effect of NOC 5 on the smooth muscle layer rather than on the endothelium. These results were in keeping with our previous report that acetylcholine was not antithrombotic in SHRSP [12] and confirm the presence of endothelial dysfunction in the hypertensive animal. Similar results were also obtained in another hypertensive model (SHR) [28, 29], and the findings are supported by the observation that production of NO is decreased in hypertensive rats and human patients [11, 30, 31].

NO inhibits platelet reactivity (adhesion and aggregation) [32, 33] and there is evidence that impaired endothelial NO synthesis contributes to the development of arterial disease [34–36]. In the present study, the effects of an exogenous NO donor (NOC 5), an NO scavenger (carboxy-PTIO) and an NO synthase inhibitor (L-NAME) were assessed using haemostatometry. Carboxy-PTIO and L-NAME did not affect these global haemostasis measurements. It may be that although the tests were completed within 4 min of blood collection, the short half-life of NO prevented detection of the effects of the NO modulators. In contrast, however, NOC 5 inhibited platelet reactivity in WKY, but not in SHRSP. These findings may reflect platelet-vessel wall reactions in vivo, and suggest that platelets in SHRSP are non-responsive to NO in the absence of endogenous endothelial NO release. Reduced platelet reactivity was observed in SHRSP. This might represent a protective phenomenon against the high thrombogenicity of the SHRSP vessel wall. There are reports showing the presence of NO synthase in platelets [37]. The present results, however, question the importance of platelet-derived NO in thrombogenesis.

In conclusion, we have established a new technique for evaluating endothelial function in situ in an animal model by adapting the principles of the well-characterized, FMV test previously used in humans. The present findings extend earlier data on the thrombotic tendency in the presence of endothelial disturbances observed in SHRSP. The method is especially useful to investigate the vascular endothelial contribution to thrombotic mechanisms in vivo.

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