Full Paper

Serotonin Potentiates High-Glucose–Induced Endothelial Injury: the Role of Serotonin and 5-HT2A Receptors in Promoting Thrombosis in Diabetes

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Abstract. To clarify the involvement of 5-hydroxytryptamine (5-HT) in promotion of thrombogenesis in diabetes, we examined the inhibitory effect of sarpogrelate, a 5-HT2A receptor antagonist, on thrombus formation in diabetic rats. In streptozotocin-induced diabetic rats, polyethylene tube–induced thrombus formation was enhanced compared with that in normal rats. The thrombogenesis was inhibited by sarpogrelate; cilostazol, a PDE3 inhibitor; and aspirin, a COX inhibitor, by 75.8%, 42.3%, and 34.3%, respectively. The inhibition by sarpogrelate was more pronounced in diabetic rats than normal ones. High glucose and 5-HT increased the expression of vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs) and combination of both high glucose and 5-HT further potentiated the effect. Sarpogrelate but not aspirin inhibited the increase in VCAM-1 expression induced by high glucose and 5-HT. These findings suggest that 5-HT mediates the enhanced thrombogenesis in diabetes and suggests that a 5-HT2A receptor antagonist may have novel therapeutic potential for the treatment of diabetic complications.

Keywords: 5-hydroxytryptamine, 5-HT2A receptor, human umbilical vein endothelial cell (HUVEC), vascular cell adhesion molecule-1 (VCAM-1), sarpogrelate

Introduction

Diabetes mellitus increases 2- to 4-fold the risk of cardiovascular events (1). There is a growing epidemic of macrovascular complications such as coronary artery diseases, cerebrovascular diseases, and peripheral artery diseases, against the backdrop of an increasing number of diabetic patients. Thrombogenesis is strongly associated with the occurrence of cardiovascular events in patients with diabetes. Along with hyperglycemia, excess free fatty acid release, and insulin resistance, oxidative stress is increased in endothelial cells, underlying the mechanism for promotion of thrombogenesis (2).

5-Hydroxytryptamine (5-HT) in the cardiovascular system is mostly stored in platelets and released to plasma when activated at sites of injury (3 – 5) and also in patients with coronary artery disease (6). Released 5-HT accelerates arteriosclerosis by promoting platelet aggregation (7), vasoconstriction (8), and proliferation of vascular smooth muscle cells (9) via 5-HT2A receptors. High plasma 5-HT levels are associated with accelerated cardiovascular events (10) and likely to play an important role in thrombogenesis.

In patients with diabetes, plasma 5-HT level is elevated (11), and 5-HT-induced platelet aggregation is enhanced (12). In addition, the expression of vascular 5-HT2A receptors is upregulated (13, 14). These results suggest that 5-HT-induced vasoconstriction might be potentiated in diabetic animals compared to non-diabetic ones (15). However, the pathophysiological mechanisms by which 5-HT contributes to the development of thrombogenesis remain to be determined.

To clarify the involvement of 5-HT and 5-HT2A receptors in atherosclerosis in diabetes, we examined the effect of sarpogrelate, a selective 5-HT2A receptor antagonist (16, 17), on thrombogenesis in a model of thrombosis...
in diabetic rats, on collagen- and 5-HT-induced platelet aggregation, and on 5-HT-induced endothelial injury in human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Drugs and chemicals

Sarpogrelate (Anplag®, Mitsubishi Tanabe Pharma, Osaka), cilostazol [extracted from Pletaal® (Otsuka Pharmaceutical, Tokyo)], a PDE3 inhibitor, and aspirin (Wako Pure Chemical Industries, Ltd., Osaka), a COX inhibitor, were used. Streptozotocin (STZ), gum tragacanth, serotonin hydrochloride, and ε-amino-n-caproic acid (ε-ACA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL, USA) and Collagen Reagent Horm (Nycomed Arzneimittel, Munich, Germany) were also used.

Test substances were suspended in 0.5% gum tragacanth solution and administered orally. Sarpogrelate (30 mg/kg) was administered 3 times daily (t.i.d.); cilostazol (50 mg/kg), twice daily (b.i.d.) in the morning and evening; and aspirin (100 mg/kg), once a day (q.d.) in the morning. For control animals without drug treatment, 0.5% gum tragacanth solution was administered. All animals received oral administration of either a drug or vehicle 3 times a day repeatedly. Experimental procedures and measurements were performed 15 min after the administration of sarpogrelate or 1 h after the administration of cilostazol or aspirin.

Animals and in vivo thrombosis model

Male Sprague-Dawley (SD) rats (6-week-old) were obtained from Charles River Laboratories, Japan, Inc. (Yokohama). Food and water were provided ad libitum. All experiments were conducted in compliance with guidelines (Regulations on Caretaking of Experimental Animals and Animal Experiments at Mitsubishi Tanabe Pharma, Research Center). Diabetes was induced by injection of STZ (60 mg/kg) dissolved in 0.05 M citrate buffer (pH 4.5) into the tail vein. One week after the start of administration of STZ, blood glucose level was measured; and aspirin (100 mg/kg, q.d.). The thrombus formation was initiated by inserting a polyethylene tube 11 days after the start of administration. The models were prepared 15 min after the administration of sarpogrelate or 1 h after the administration of cilostazol or aspirin; each drug or vehicle was then administered to the animals at 3-h intervals. A predetermined number of doses were administered for each drug on the day after model preparation. After two days, the polyethylene tube was removed 15 min after the administration of sarpogrelate. As a control, the same procedures were performed in age-matched normal rats.

Experiment 1: One week after the induction of diabetes with STZ, rats were administered with oral doses of sarpogrelate (30 mg/kg, t.i.d.). On the 11th day after the start of administration a polyethylene tube was inserted 15 min after the administration of sarpogrelate, and sarpogrelate was then additionally administered twice with an interval of at least 3 h. Sarpogrelate was administered 3 times a day thereafter. Two days later, the polyethylene tube was removed 15 min after the administration of sarpogrelate. As a control, the same procedures were performed in age-matched normal rats.

Effect of platelet aggregation

Blood samples were collected from the abdominal aorta using 3.8% trisodium citrate (9:1 v/v) under sodium
pentobarbital anesthesia (50 mg/mL per kg, i.p.). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation (CT13R; Hitachi Koki Co., Ltd., Tokyo) at 1,100 and 3,000 rpm, respectively, at room temperature for 10 min.

Repeated oral administration of each drug or 0.5% gum tragacanth was started 3 days before the measurement of platelet aggregation as described above. Sarpogrelate was administered 3 times daily; cilostazol, twice daily in the morning and evening; and aspirin, in the morning. For animals receiving no drug administration, 0.5% gum tragacanth was administered; thus, all animals underwent oral administration 3 times daily. On the day of measurement, blood samples were collected 15 min after the administration of sarpogrelate or 1 h after the administration of cilostazol or aspirin. Platelet aggregation was induced by collagen (final concentration: 2, 3, 4, and 5 μg/mL) and measured in an aggregometer (MCM HEMA TRACER 313M; MC Medical, Inc., Tokyo).

In the experiments designed to evaluate the effects of 5-HT, platelet aggregation was induced by 3 μM 5-HT in the presence of 2 or 3 μg/mL collagen.

Cell culture and experimental protocol in vitro

HUVECs (LONZA Walkersville, Inc., Petit-Rechain, Belgium) were cultured in type I collagen–coated dishes using BulletKit EGM-2.

HUVECs (passages, 10 – 11; 9 × 10^4 cells/mL) were treated with 5-HT under a normal (5.5 mM) or high (27.8 mM) concentration of glucose. After 24 h, the expression of vascular cell adhesion molecule-1 (VCAM-1) on the cell surface was measured by flow cytometry. Briefly, cells were gently exfoliated and centrifuged at 3,000 × g for 3 min. Culture medium was replaced and cells were centrifuged at 15,000 × g for 5 min. Cells were then suspended in 0.2 mg/mL Alexa Fluor 488–labeled anti-VCAM-1 antibody (sc-13160AF488; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then allowed to react for 1 h at an ice-cold temperature under light shielding. The fluorescence intensity of 10,000 viable cells from each group was then measured with a flow cytometer (BD-LSR; Becton, Dickinson and Company), and the distribution chart was prepared and analyzed (Cell Quest software, ver. 3.3).

The “high-glucose concentration” is a concentration of glucose that causes endothelial injury (21). Sarpogrelate was used at concentrations that inhibit aggregation of human and rat platelets (22), and aspirin was used at a concentration that inhibits aggregation of human platelets (23).

It is reported that 5-HT and sarpogrelate concentrations of 1 and 1 – 10 μM, respectively, were used in experiments to determine inhibition of rat aortic smooth muscle proliferation (24), IL-6 production in human aortic smooth muscle (25), inhibition of the rat mesangial cells proliferation (26) and human platelet aggregation (27). The concentrations of 5-HT and sarpogrelate in the above experiments were similar to those of VCAM-1 expression in HUVECs.

Statistical analyses

Results are expressed as means ± S.E.M. The occlusion ratios of the treatment group and vehicle-control group were compared by Fisher’s exact probability test. For other experiments, statistical analysis was performed to compare treatment groups and the vehicle-control group using Student’s t-test or Dunnett’s multiple comparison test. P-values less than 0.05 were considered significant. Statistical analysis was performed with a SAS system (SAS 9.1.3; SAS institute, Inc., Cary, NC, USA).

Results

Antithrombotic effects

Experiment 1: Figure 1 shows the protein contents in the thrombus produced by placing a polyethylene tube in the carotid artery. The protein content in thrombus seemed to be higher in diabetic rats than normal ones. The fluorescence intensity of 10,000 viable cells from each group was then measured with a flow cytometer (BD-LSR; Becton, Dickinson and Company), and the distribution chart was prepared and analyzed (Cell Quest software, ver. 3.3).

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Fig. 1. Effect of sarpogrelate (30 mg/kg, t.i.d.) on arterial thrombosis in implanted polyethylene tube (A) and occlusion ratio (B) in normal and streptozotocin-induced diabetic rats (DM). A) The data are represented as the mean ± S.E.M. of the number of measurements shown in parentheses. **P < 0.01, as compared to vehicle (Student’s t-test). B) Fraction shown in parentheses is the number of occlusion / number of experiments. *P < 0.05, as compared to vehicle (Fisher’s exact test).
(504.0 ± 47.8 vs. 388.6 ± 40.6 μg) (P = 0.08). Sarpogrelate (30 mg/kg, t.i.d.) significantly inhibited the thrombogenesis in both the normal and diabetic rats. The inhibition by sarpogrelate was greater in diabetic rats (73.0%) than normal ones (61.0%).

Inserting the polyethylene tube in the artery occluded it and ceased the blood flow in 9 out of 11 (occlusion ratio of 81.8%) and 11 out of 11 animals (occlusion ratio of 100%) in vehicle-treated normal and diabetic rats, respectively. Sarpogrelate suppressed the occlusion ratio to 55.6% (5/9) and 42.9% (3/7) in normal and diabetic rats, respectively.

The plasma glucose of diabetic rats was higher than that of normal-glucose rats. Sarpogrelate decreased the blood glucose but did not affect the body weight (Table 1).

Experiment 2: Figure 2A shows the protein content in thrombus produced by placement of a polyethylene tube in the carotid artery. Sarpogrelate (30 mg/kg, t.i.d.), cilostazol (50 mg/kg, b.i.d.), or aspirin (100 mg/kg, q.d.) significantly inhibited the thrombus formation by 75.8%, 42.3%, and 34.3%, respectively. Sarpogrelate was the most potent among the tested drugs in terms of inhibitory activity.

Sarpogrelate, cilostazol, and aspirin all significantly reduced the occlusion ratio to 40% (8/20), 80% (16/20), and 70% (14/20), respectively, in Fig. 2B. On the last day of the test, there were no significant differences between vehicle and drugs (sarpogrelate, cilostazol, or aspirin) in blood glucose and body weight (Table 2).

Effects on platelet aggregation

Effects on collagen-induced platelet aggregation were examined after repeated administration of sarpogrelate (30 mg/kg, t.i.d.), cilostazol (50 mg/kg, b.i.d.), or aspirin (100 mg/kg, q.d.) for 3 days. Collagen concentration-dependently induced platelet aggregation at 2, 3, 4, and 5 μg/mL (Fig. 3). Sarpogrelate, cilostazol, and aspirin significantly inhibited the ex vivo platelet aggregation induced by 3, 4, and 5 μg/mL collagen. The maximum aggregation induced by collagen (4 μg/mL) was 65.5% ± 3.2%; and sarpogrelate, cilostazol, and aspirin reduced the maximum aggregation by 43%, 55%, and 88%, respectively.

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<th>Table 1. Effects of sarpogrelate on body weight and plasma glucose in thrombosis model of control and DM rats</th>
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Each value is expressed as a mean ± S.E.M. **P < 0.01, as compared to vehicle control or sarpogrelate control (Student’s t-test). *P < 0.05, as compared to vehicle of DM (Student’s t-test). DM: diabetes mellitus, NT: not tested.

**Fig. 2.** Effects of sarpogrelate (30 mg/kg, t.i.d.), cilostazol (50 mg/kg, b.i.d.), and aspirin (100 mg/kg, q.d.) on arterial thrombosis in implanted polyethylene tube (A) and occlusion ratio (B) in streptozotocin-induced diabetic rats. A) The data are represented as the mean ± S.E.M. of the number of measurements shown in parentheses. **P < 0.01, as compared to vehicle (Student’s t-test). ***P < 0.01, as compared to the sarpogrelate group (Dunnett’s multiple comparison test). B) Fraction shown in parentheses is the number of occlusion / number of experiments. *P < 0.05, **P < 0.01, as compared to vehicle (Fisher’s exact test). #P < 0.05, as compared to the sarpogrelate group (Fisher’s exact test).
Figure 4 shows the effects of antiplatelet drugs on ex vivo platelet aggregation induced by combination of collagen and 5-HT. In the vehicle group, collagen (2 μg/mL) induced platelet aggregation to 13.1% ± 8.1% and additional 5-HT (3 μM) enhanced the collagen-induced aggregation to 27.9% ± 11.1%. Similarly, the platelet aggregation by collagen (3 μg/mL: 48.8% ± 10.6%) was enhanced by 5-HT (3 μM) to 57.3% ± 4.4%. Sarpogrelate and aspirin but not cilostazol significantly inhibited the induction of platelet aggregation induced by combination of collagen and 5-HT (3 μM) at both concentration regimens.

Effects on VCAM-1 expression on vascular endothelial cells

Figure 5 represents the distribution chart of fluorescence intensities due to VCAM-1 expression by 5-HT in normal (5.5 mM) and high glucose (27.8 mM) conditions. 5-HT (0.5 μM) at the high glucose concentration shifted the distribution of the fluorescence to the right. The rightward shift of the distribution indicates the increase of VCAM-1 expression. Sarpogrelate reversed the shift in the distribution.

In the group loaded with high glucose (27.8 mM), VCAM-1 expression was increased to 1.2-fold (Fig. 6). 5-HT (0.5 μM) significantly increased the VCAM-1 expression to 1.7 times and 5-HT (0.5 μM) with high glucose (27.8 mM) increased the expression to 1.9 times. Sarpogrelate at 3 and 10 μM suppressed the 5-HT-increased VCAM-1 expression in high glucose conditions by 43.2% and 87.2%, respectively. In contrast, the addition of aspirin (10 μM) did not inhibit the increase in expression of VCAM-1.

Discussion

In diabetes, enhanced thrombogenesis due to vascular endothelial injury as well as activation of platelet function leads to pathological events (28). In this study, the protein content in thrombus induced by placing a polyethylene tube in the artery was higher in STZ-induced diabetic rats than normal ones. The ratio to develop thrombotic occlusion in the polyethylene tube was also

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<th>Table 2. Effects of sarpogrelate, cilostazol, and aspirin on body weight and plasma glucose in thrombosis model of diabetic rats</th>
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Each value is expressed as a mean ± S.E.M. Not significantly different from vehicle (Student’s t-test).
higher in diabetic rats than normal rats. These findings indicate that thrombogenesis is more advanced in diabetic rats than in normal ones. Sarpogrelate decreased the blood glucose in Experiment 1 (Table 1) of the thrombosis model. In Experiment 2 (Table 2), sarpogrelate slightly decreased the blood glucose but the difference was not significant. Sarpogrelate possibly showed the tendency to decrease blood glucose in STZ-induced diabetic rats such as KK-Ay mice (29).

Treatment with sarpogrelate, a 5-HT2A receptor antagonist, reduced the thrombus formation to 61.0% and 73.0% in normal and diabetic rats, respectively. Sarpogrelate seemed to suppress both the thrombus formation and the occlusion ratio more effectively in diabetic rats than normal ones. In STZ-induced diabetic rats, sarpogrelate, cilostazol, and aspirin inhibited the thrombus formation by 75.8%, 42.3%, and 34.3%, respectively. It has been reported that 5-HT2A receptor antagonists are more effective than COX inhibitors in preventing cardiovascular events in patients with diabetes (30). Our experimental results demonstrate the involvement of 5-HT in enhanced thrombogenesis in diabetic rats.

5-HT alone did not induce platelet aggregation but enhanced collagen-induced platelet aggregation. To investigate the mechanism of action of 5-HT, we compared the inhibitory effects of antiplatelet drugs on platelet aggregation ex vivo. Each drug significantly inhibited the collagen-induced platelet aggregation at any of the tested concentrations of collagen. Sarpogrelate and aspirin but not cilostazol significantly inhibited the platelet aggregation induced by a combination of collagen and 5-HT. Since aspirin inhibited platelet aggregation irreversibly ex vivo, aspirin inhibited platelet aggregation induced by collagen plus 5-HT. In addition, it appears that inhibition of platelets and other factors may contribute to the promotion of thrombogenesis in diabetes.

We therefore examined the involvement of 5-HT in vascular endothelial injury. 5-HT increased the VCAM-1 expression in HUVECs especially when high glucose was loaded. Sarpogrelate inhibited the 5-HT-induced increase of VCAM-1 expression in both low and high glucose conditions. The inhibition by sarpogrelate was more pronounced at higher glucose conditions. On the other hand, aspirin did not inhibit the 5-HT-induced VCAM-1 expression. 5-HT appears to enhance thrombogenesis by acting on endothelial cells to elevate the production and activity of tissue factor (TF), a potent trigger of the coagulation cascade, and plasminogen activator inhibitor-1 (PAI-1), which inhibits the fibrinolytic system.
by inhibiting the activity of tissue plasminogen activator (31). The endothelium-dependent relaxation (EDR) induced by high glucose is improved by 5-HT$_{2A}$ receptor antagonists in isolated rat aorta by decreasing superoxide anion ($O_2^-$) production and by increasing superoxide dismutase (SOD) activity and nitric oxide (NO) release (32). Furthermore, a 5-HT$_2$ receptor antagonist not only inhibits the release of intercellular adhesion molecule-1 (ICAM-1) and VCAM-1, both of which are markers of vascular endothelial injury in patients with type 2 diabetes (33), but also improves endothelial function in patients with peripheral arterial diseases (34). While elevation of glucose concentration alone does not increase the expression of VCAM-1 or ICAM-1 (35), we have demonstrated in this study that 5-HT increases vascular endothelial injury in the presence of high glucose. These findings suggest that promotion of 5-HT-induced endothelial injury in high glucose conditions leads to exacerbation of thrombogenesis in diabetic animals.

The present study demonstrated the involvement of 5-HT in the development of atherothrombosis in diabetes. Previous studies have shown increases in plasma concentration of 5-HT in patients with diabetes (11) and enhanced expression of 5-HT$_3$ receptors in tissues of diabetic peripheral occlusion model animals (14). 5-HT-induced platelet aggregation is enhanced by advanced glycation endproducts (AGEs), which are involved in the development of microvascular complications (36). It has been suggested that 5-HT is associated with the development of diabetic microvascular complications (37–39). In male patients with type 2 diabetes, the plasma 5-HT level is positively correlated with carotid intima-media thickness and pulse wave velocity, which are markers of subclinical atherosclerosis, and with urinary albumin excretion (38). The presence of 5-HT$_{2A}$ receptors in glomerular mesangial cells (40) suggests the involvement of 5-HT in the development of diabetic nephropathy through proliferation and matrix synthesis in mesangial lesions (41).

The existence of endothelial 5-HT$_{2A}$ receptors was reported but there is little work about the expression of 5-HT$_{2A}$ receptors in endothelium (31). On the other hand, a 5-HT$_{2A}$ receptor antagonist showed endothelium-dependent vasodilatation via 5-HT$_1$-like or 5-HT$_{2B}$ receptors (42) and angiogenesis via the 5-HT$_{1D}$/Akt/eNOS pathway in diabetic mice (43). In ischemia–reperfusion injury in isolated rat heart sarpogrelate induced coronary vasodilatation via endothelial NO production (44). Sarpogrelate improves endothelial function through a reduction of glomerular platelet activation In STZ-induced diabetic rats (45).

Sarpogrelate enhanced eNOS expression in diabetic mice (43), so it is possible that sarpogrelate directly enhanced eNOS expression or enhanced the 5-HT$_{1B}$ receptors pathway via blockade of 5-HT$_{2A}$ receptors, but the mechanism by which sarpogrelate improved the function of endothelium is unknown. Further investigations are necessary to elucidate the precise mechanism.

In conclusion, we have shown that 5-HT, through acting on 5-HT$_{2A}$ receptors, aggravates thrombogenesis in diabetes by exacerbation of vascular endothelial injury in addition to enhanced platelet aggregation. Involvement of 5-HT in thrombogenesis in diabetes suggest that a 5-HT$_{2A}$ antagonist may have a novel potential for treatment of diabetic complications.

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