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Role of serotonin in angiogenesis: Induction of angiogenesis by sarpogrelate via endothelial 5-HT1B/Akt/eNOS pathway in diabetic mice

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) plays a crucial role in peripheral artery disease (PAD) and diabetes mellitus (DM). In these conditions, the balance between the 5-HT2A receptor in smooth muscle cells and the 5-HT1B receptor in endothelial cells (ECs) regulates vascular tonus. In the present study, we focused on the role of 5-HT in endothelial dysfunction using a selective 5-HT2A receptor blocker, sarpogrelate.

In human EC, 5-HT markedly stimulated eNOS expression and the phosphorylation of eNOS, Akt and ERK1/2. In addition, a dose-dependent increase in tubule-formation on Matrigel was observed after 5-HT treatment. In contrast, high glucose significantly inhibited tubule formation and eNOS expression through inactivation of Akt, while 5-HT significantly attenuated these actions of high glucose ($P < 0.01$). These results indicate that 5-HT stimulated angiogenesis through activation of Akt in ECs. However, in clinical situations, 5-HT seems to act as the "devil". To examine the role of 5-HT in diabetic PAD, a hindlimb ischemia model was created in diabetic mice. The blood flow ratio of the ischemic to non-ischemic limb was significantly lower in DM mice than in normal mice, while sarpogrelate significantly attenuated the decrease in the blood flow ratio compared to control ($P < 0.01$). Consistently, the decrease in eNOS expression and Akt activity in DM mice was significantly attenuated by sarpogrelate.

Overall, the present study demonstrated that selective inhibition of 5-HT2A by sarpogrelate significantly restored ischemic limb blood perfusion in a severe diabetic mouse model through stimulation of the eNOS/Akt pathway via the endothelial 5-HT1B receptor. Enhancement of vasodilation and angiogenesis by sarpogrelate might provide a unique treatment for PAD and DM patients.

1. Introduction

Progression of atherosclerosis results in the occlusion of major limb arteries and diminishes limb perfusion, leading to peripheral artery disease (PAD). Diabetes mellitus (DM) is a strong risk factor for cardiovascular disease [1,2]. The prevalence of PAD in patients with DM is 5-fold higher than that in patients without DM [3]. In the pathological conditions of PAD [4] and DM [5], it is well known that platelet activation occurs and platelet aggregation in the vascular wall is increased. As activated platelets in atherosclerotic lesions release large amounts of serotonin (5-hydroxytryptamine, 5-HT), plasma 5-HT level is elevated [6]. Released 5-HT binds to the 5-HT2A receptor in vascular smooth muscle cells (VSMC), and accelerates calcium influx and VSMC proliferation [7,8], leading to vascular constriction. Alternatively, 5-HT initiates autocrine activation, and aggregation of platelets through its receptor, 5-HT2A, on the cell surface, occurs [9]. It is reported that vascular sensitivity to 5-HT is significantly enhanced in patients with DM [10], suggesting this vicious cycle is tightly linked to the pathogenesis and development of PAD. Under such conditions, inhibition of 5-HT2A in both VSMC and platelets might be beneficial to treat such patients. Indeed, a selective 5-HT2A receptor blocker, sarpogrelate, has been widely used clinically to treat PAD patients [11,12], as sarpogrelate suppressed 5-HT-mediated vasoconstriction [13] and increased limb blood perfusion [14,15] in experimental animal models.

However, in the endothelium, 5-HT is known to mediate NO production through 5-HT1B on endothelial cells, leading to vasodilation [16,17]. Nevertheless, the role of 5-HT in the vasculature is thought to lean towards vascular constriction, as

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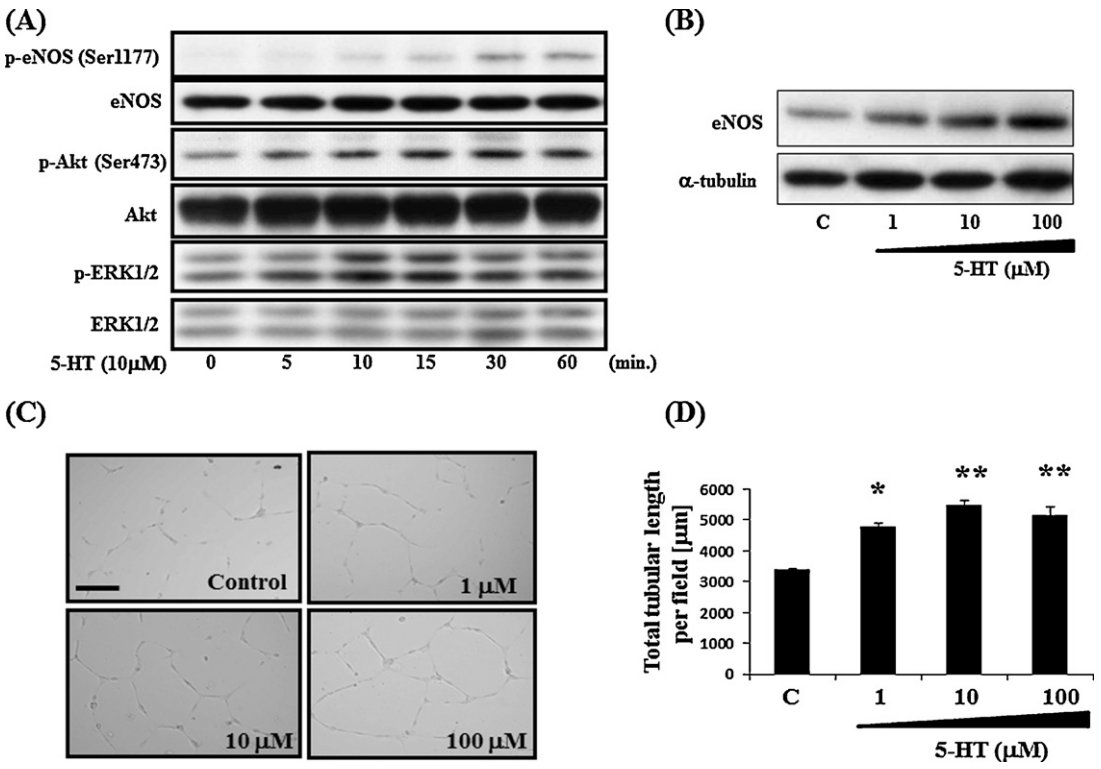

Fig. 1. Effects of 5-HT on HAEC. (A) Effect of 5-HT (10 μM) on the phosphorylation of Akt and eNOS in HAECs. (B) Effect of 5-HT (0–100 μM) on protein expression of eNOS in HAECs at 24 h after treatment. (C) Representative photographs of tubule formation in HAEC on Matrigel at 24 h after treatment. Scale bar = 200 μm. (D) Quantitative analysis of tubule length after 5-HT stimulation. 1×10^5 cells were seeded on Matrigel-coated 24-well plates. Tubule length was determined at 24 h after stimulation with various concentrations of 5-HT. $n = 3$, $^*P = 0.0040$, $^{**}P < 0.001$ vs. control. Tubule length was determined in 5 random fields.

hypersensitivity to 5-HT-induced vascular constriction is increased in advanced atherosclerosis [13]. Thus, the precise molecular mechanisms of how 5-HT causes progression of vascular dysfunction are still an enigma. In the present study, we focused on the role of 5-HT in endothelial function both *in vivo* and *in vitro* using a selective 5-HT2A receptor blocker, sarpogrelate. Here, we demonstrated that the selective inhibition of the 5-HT2A receptor by sarpogrelate enhanced therapeutic angiogenesis in a severe diabetic mouse model.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) purchased from LONZA (Portsmouth, NH) were cultured in endothelial basal medium-2 (Clonetics, Walkersville, MD, USA) supplemented with EGM and 5% fetal bovine serum (FBS). Prior to stimulation, cells were starved with 0.5% FBS-containing medium for 24 h.

2.2. Western blotting

Protein extracts (10 μg) were fractionated on 8% SDS–PAGE gel and transferred to a PVDF membrane (Millipore, USA) [18].

2.3. Matrigel tubule formation assay

The detailed procedure is described in Supplemental information section.

2.4. Animal model

The detailed procedure is described in Supplemental information section.

2.5. Materials

The materials used in this study are described in Supplemental information section.

2.6. Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) using JMP9.0 (SAS Institute, Inc., Cary, NC, USA). Normality and homoscedasticity were checked visually. Analysis was performed after log-transformation for non-normally distributed data. Within each ANOVA, the *P*-values used to assess the statistical significance of pairwise comparisons were adjusted for multiplicity by the Tukey–Kramer method. Differences were regarded as significant at the $P < 0.05$ level. All data were reported as mean ± standard error (SE).

3. Results

3.1. Induction of angiogenesis by 5-HT in HAEC

To examine the role of 5-HT in endothelial function, we initially examined the effects of 5-HT on eNOS and Akt activity using an *in vitro* culture model. As shown in Fig. 1, 5-HT markedly stimulated the phosphorylation of eNOS (Ser1177) and Akt (Ser473) (Fig. 1A). Similarly, 5-HT also stimulated the phosphorylation of ERK1/2. Also, an increase in the expression of eNOS was still observed at 24 h after 5-HT treatment (Fig. 1B). As an increase in eNOS is reported