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Hirata, Ken-ichi
Yokoyama, Mitsuhiro

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NITRIC OXIDE IS PRODUCED VIA 5-HT_{1B} AND 5-HT_{2B} RECEPTOR ACTIVATION IN HUMAN CORONARY ARTERY ENDOTHELIAL CELLS

Tatsuro ISHIDA, Seinosuke KAWASHIMA, Ken-ichi HIRATA,
and Mitsuhiro YOKOYAMA

First Division, Department of Internal Medicine,
Kobe University School of Medicine

INDEXING WORDS

nitric oxide; 5-HT_{1B} receptor; 5-HT_{2B} receptor; serotonin; human coronary artery endothelial cell

SYNOPSIS

The aim of this study is to clarify the serotonin (5-HT) receptor subtypes responsible for 5-HT-induced nitric oxide (NO) production in cultured human coronary artery endothelial cells (HCAECs). Reverse transcription-polymerase chain reactions revealed that HCAECs possessed only 5-HT_{1B} and 5-HT_{2B} receptor mRNAs. 5-HT (0.001-10 μmol/L) induced nitrite production from HCAEC. A selective 5-$HT_{1B/1D}$ agonist sumatriptan (0.01-10 μmol/L) caused nitrite production. A selective 5-HT_2 agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI, 0.01-10 μmol/L) also mimicked the effect of 5-HT. The 5-HT-induced nitrite production was completely inhibited by the nonselective 5-$HT_{1/2}$ antagonist methiothepin (0.1 μmol/L) , but not by the 5-$HT_{2A/2C}$ antagonist ketanserin (0.1 μmol/L). Each of these agonists evoked the elevation of intracellular Ca^{2+} concentration in HCAECs. These findings suggest that 5-HT-induced NO production is mediated by both of 5-HT_{1B} and 5-HT_{2B} receptor activation in HCAECs. Thus, the 5-HT-evoked endothelium-dependent relaxation of human coronary arteries in vivo might be evoked by net effects of the activation of these receptor subtypes.

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT), which plays an important role in the regulation of vascular tone, can evoke vasodilatation as well as vasoconstriction of a variety of blood vessels including human coronary arteries.[18,29,32,43] 5-HT-evoked vasodilatation is caused by

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Authors' names in Japanese : 石田達郎、川嶋成乃亮、平田健一
横山光宏

endothelium-dependent and endothelium-independent mechanisms. The former is mediated via the endothelium-derived relaxing factor (EDRF),[4,10] which is considered to be identical to nitric oxide (NO) or related substance,[30,31] and the latter is considered to be direct action of 5-HT mediated through smooth muscle relaxant (probably 5-HT_7) receptors.[24,39] Angiographically normal human coronary arteries in vivo dilate in response to 5-HT.[13] This is attributed to EDRF/NO released by activation of 5-HT receptors on the endothelial cells.[3,42] Atherosclerotic coronary arteries constrict in response to 5-HT by activation of constrictor 5-HT receptors on the smooth muscle cells.[28] Therefore, it is suggested that the loss of functional endothelium in diseased arteries results in a loss of 5-HT-receptor-mediated vasodilatation. Indeed, accumulating evidence supports that the impaired release of NO, which endangers the protective role of the endothelium against platelet adhesion/aggregation and monocyte/macrophage infiltration, plays a pivotal role in the development of atherosclerosis and ischemic heart diseases.[2,12,27,38]

A large number of pharmacological studies using various kinds of vessels has suggested that two distinct 5-HT receptors on vascular endothelium, "5-HT_1-like" or "atypical/5-HT_2" receptor types, are involved in 5-HT-evoked endothelium-dependent vasorelaxation (reviewed in ref.18 and 29). Recently, we have reported that human coronary artery medial smooth muscle cells possessed 5-HT_{1B} (previously referred to 5-$HT_{1D\beta}$[16]) and 5-HT_{2A} receptor subtypes by molecular analysis.[21] In addition, Ullmer et al demonstrated that 5-HT_{1B} and 5-HT_{2B} receptor mRNAs were predominantly expressed in human coronary endothelial cells.[40] However, to our knowledge, there is no evidence which demonstrated that activation of these 5-HT receptor subtypes actually induces NO release with elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Furthermore, 5-HT receptor subtypes which mediate endothelium-dependent relaxation in human coronary artery still remain speculative. The aim of this study is to characterize the 5-HT receptor subtypes in NO production by 5-HT in human coronary artery endothelial cells.

MATERIALS AND METHODS

Cell culture

Human coronary artery endothelial cells (HCAECs) were purchased from Clonetics, Inc. (San Diego, USA) and grown according to the supplier's instruction. Cells were used from 5 to 8 passages.

Reverse transcription-polymerase chain reactions (RT-PCR)

Total RNA was extracted from HCAEC using the RNeasy kit (Qiagen, Inc, Hilden, Germany). The RNA samples were treated with 10 U RNase free DNase I (Boehringer Mannheim, Inc, Mannheim, Germany) per 10 μg RNA at 37 °C for 20 minutes in order to eliminate any residual contaminating genomic DNA. After Tris-saturated phenol-chloroform extraction, they were subjected to ethanol precipitation and then dissolved in RNase free water.

An aliquot (5 μg) of total RNA was incubated at 42 °C for 1 hour with oligo(dT)$_{15}$ primers (Promega, Madison, WI, USA) and SuperScript II reverse transcriptase (GIBCO BRL, Life Technologies, Rockbille, MD, USA) in the reaction buffer to synthesize single-stranded cDNA. The reaction buffer contained the followings: 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 10 mmol/L dithiothreitol, and 3 mmol/L $MgCl_2$. After the single-stranded cDNA synthesis, 2 U of RNase H (GIBCO BRL) was added to remove the residual RNA.

We have designed PCR primers which distinguish all known 5-HT$_1$ and 5-HT$_2$ receptors based on the published sequences. Forward and reverse primers for each 5-HT receptor are shown as below:

5-HT$_{1A}$ [22]; acagaattcatggatgtgctcagccctggtca
agagtcgacgtccacttgttgagcacctgatac

5-HT$_{1B}$ [26]; tatggtaccatggaggaaccgggtgctca
tctgagctccatcacaaaggcattgg

5-HT$_{1D}$ [15]; ggggtttgaattcatgtccccactgaaccagtc
tcagtcgaccagggagccaatcaggtagtt

5-HT$_{1E}$ [25]; gtgctgcagatgaacatcacaaactgtacc
atagtcgacttggcaggctggtggagcttctt

5-HT$_{1F}$ [1] ; gcgctgcagatggatttcttaaattcatctg
tcctggcatactcaacagcatc

5-HT$_{2A}$ [34]; gcgggtaccatgcaattaaatgatgacaccaggctc
acagagctcgccatgatgacgagtatgtttcc

5-HT$_{2B}$ [35]; agaggtaccatggctctctcttacagagtgtctgaac
agagagctcatgagtatcagaagagctgcccagtgcag

5-HT$_{2C}$ [34]; ctcgaattcatggtgaacctgaggaatgc
tctgggaatttgaagcgtccaccatcgga

Double-stranded cDNAs were synthesized and amplified using 1 U recombinant Taq polymerase (TAKARA, Japan) and 80 nmol/L primers in 0.05 mL of 10 mmol/L Tris-HCl buffer (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 0.5 mmol/L dNTP, 2 mmol/L dithiothreitol, and 0.01 % gelatin for 40 cycles at 93 °C, 52 °C, and 72 °C for 1, 2 and 3 minutes, respectively. The PCR products were checked with 4 % agarose gel electrophoresis and then identified by dideoxy sequencing.

Measurement of NO release

HCAECs were grown in gelatin-coated 60-mm dishes up to confluency. At the time of study, the HCAEC dishes were rinsed with 1 mL physiological salt solution (PSS) and incubated with 1.5 mL culture medium at 37 °C for 90 minutes with various drugs. After incubation, a 0.5-mL aliquot was used to measure NO release by chemiluminescence. To standardize the protein concentration, HCAECs were solubulized with 1 mL of 1 N NaOH for protein determination.

Agonist-induced NO release from cultured HCAEC was measured by examining the production of nitrite, the stable degradation product of NO, with an NOx analyzer as described previously.[17]

Measurement of intracellular calcium

HCAECs seeded on gelatin-coated glass coverslips were loaded for 1 hour with 10 μmol/L fura 2-acetoxymethylester prior to experiments. Coverslips were mounted on an inverted microscope (Diaphots, Nikon, Japan) and perfused with PSS at 37 °C. Each serotonergic agonist was added to the monolayer of fura 2-loaded HCAECs in PSS containing 1 mmol/L $CaCl_2$, and signals of intracellular Ca^{2+} from two or three cells were recorded. The details about the measurement of $[Ca^{2+}]_i$ were described previously.[19]

Determinations

Protein concentrations were determined with bovine serum albumin as a standard protein as described.[7] Results are expressed as mean±SEM. Statistical analysis of the data was performed by Student's *t* test for unpaired observation. Values were considered to be statistically different at a value of $p<0.05$.

Drugs used

The following drugs were used: 5-hydroxytryptamine creatinin sulfate and ionomycin (Sigma Chemical Co, St Louis, MO, USA); sumatriptan (gifts from Glaxo); (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) and methiothepin mesylate (Research Biochemicals, Inc., Natik, MA, USA); ketanserin tartrate (a gift from Janssen Pharmaceutica); fura 2-acetoxymethylester (Dojindo Laboratories, Kumamoto, Japan).

RESULTS

Analysis of 5-HT receptor mRNA expression in HCAECs

To examine the expression of 5-HT receptor mRNAs in HCAECs, we performed RT-PCR using the specific primers flanking each of the known 5-HT_1 and 5-HT_2 receptors. Contamination with genomic DNA was excluded in all RNA preparations by performing the same RT-PCR procedure without the addition of reverse transcriptase. By RT-PCR, we obtained 232 and 204 base-pair-PCR products which were consistent with the 5-HT_{1B} and 5-HT_{2B} receptors, respectively. The PCR products were identified by dideoxy sequencing. A representative result of agarose gel electrophoresis is shown in Fig.1. Among the 5-HT receptor subtypes, only 5-HT_{1B} and 5-HT_{2B} receptor mRNAs were detected in HCAECs. None of the 5-$HT_{1A/1D/1E/1F/2A/2C}$ receptor mRNA was detected by RT-PCR (data not shown).

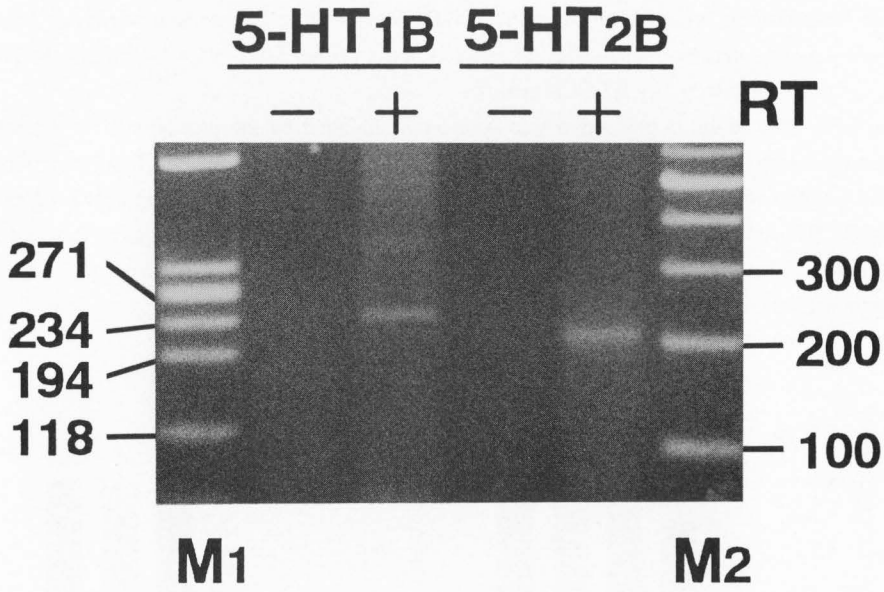

Fig. 1. Representative results of RT-PCR analysis for 5-HT receptor mRNAs in HCAECs. Only 5-HT_{1B} (232 bp) and 5-HT_{2B} (204 bp) mRNAs were detected among all 5-HT_1 and 5-HT_2 subtypes. To evaluate DNA contamination, all RT-PCRs were performed with or without the addition of reverse transcriptase (+ or - RT) to the single-strand cDNA synthesis reaction. M1 (ϕX174/Hae III digest) and M2 (100 bp DNA ladder) are molecular size markers. The identical results were obtained in other independent experiments.

Nitrite release from HCAECs

Since RT-PCR analysis provides the molecular evidence that HCAECs mainly posses 5-HT_{1B} and 5-HT_{2B} receptor mRNAs, we examined the effect of 5-HT and the selective agonists for these receptors on nitrite release in HCAECs.

Fig.2 shows the agonists-stimulated nitrite release in culture medium from HCAECs. 5-HT (0.001-10 μmol/L) induced nitrite production from cultured HCAEC in a dose-dependent manner. A selective 5-$HT_{1B/1D}$ agonist sumatriptan (SUM; 0.01-10 μmol/L), which is devoid of agonist properties on 5-HT_{2B} receptor,[9] caused nitrite production in HCAEC. A selective 5-HT_2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; 0.01-10 μmol/L), which does not stimulate endothelial 5-HT_{1B} receptor,[41] also mimics the effect of 5-HT and the effective concentration of DOI is relatively higher than those of 5-HT and SUM (Fig.2C).

In the present study, effects of SUM and DOI were considered to be mediated selectively by 5-HT_{1B} and 5-HT_{2B} receptor in HCAEC, respectively, since neither 5-HT_{1D} nor 5-$HT_{2A/2C}$ receptor mRNAs were not detected by RT-PCR analysis..

5-HT-induced nitrite production was completely inhibited by the pretreatment of the cells with a nonselective 5-$HT_{1/2}$ antagonist methiothepin (Fig.2D). However, 5-HT-induced nitrite production was not significantly inhibited by a selective 5-$HT_{2A/2C}$ receptor antagonist ketanserin (Fig.2D). These findings suggest that 5-HT_{1B} and 5-HT_{2B} receptors are functionally expressed in HCAECs and that 5-HT-induced NO production is mediated by both of 5-HT_{1B} and 5-HT_{2B} receptor activation in HCAECs.

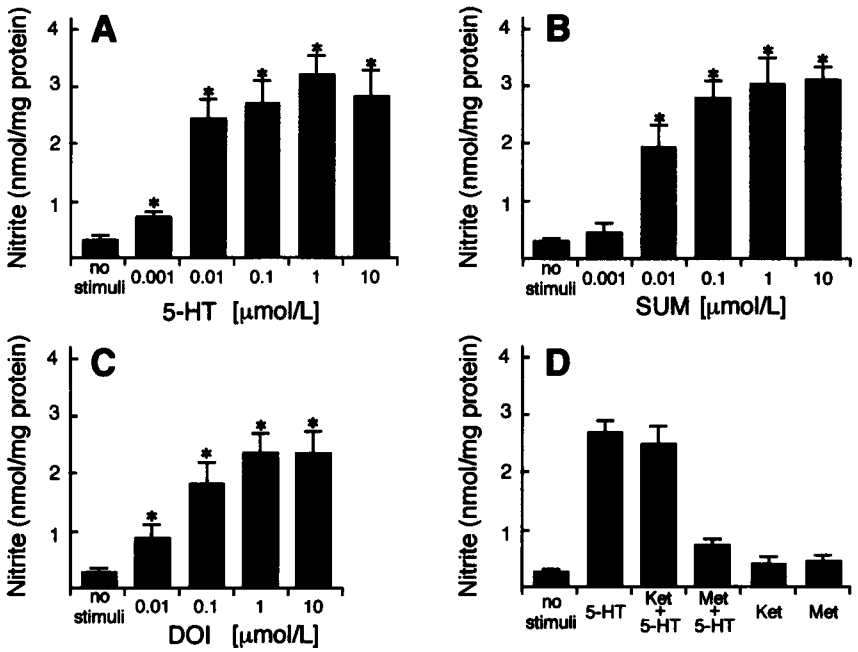

Fig. 2. A-C; Bar graphs show concentration responses of 5-HT-induced (A), SUM-induced (B), and DOI-induced (C) accumulation of nitrite release from HCAECs. HCAECs were incubated for 90 minutes with each agonist at the indicated concentration. D; The effect of methiothepin and ketanserin on 5-HT-induced nitrite production. HCAECs were incubated for 30 minutes with methiothepin (0.1 μmol/L) or ketanserin (0.1 μmol/L) and then incubated with 5-HT (1 μmol/L). Results are expressed as mean±SEM of five (for A-C) or three (for D) independent experiments. *$p<0.05$ compared with nitrite release without each agonist.

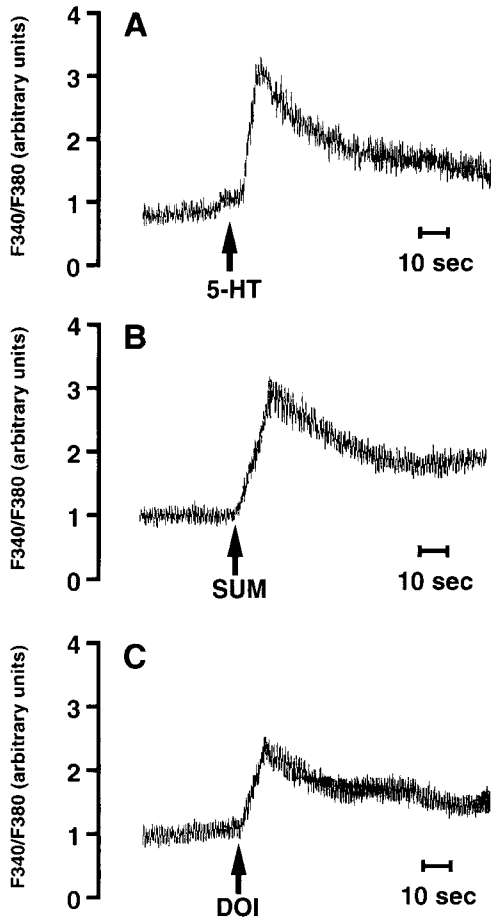

Fig. 3. Tracings show the effects of 5-HT (A), SUM (B), and DOI (C) on $[Ca^{2+}]_i$ in HCAECs. In physiological salt solution containing 1 mmol/L $CaCl_2$, 1 μmol/L of each agonist was added to the monolayer of fura 2-loaded HCAECs. Each tracing is a representative of three independent experiments.

Ca^{2+} mobilization by serotonergic agonists

It is generally accepted that 5-HT_{1B} receptor inhibits adenylate cyclase in various cell types including human endothelial cells,[36,37,41] while 5-HT_{2B} receptor stimulates phosphoinositide turnover and increase $[Ca^{2+}]_i$.[23,35] It is unclear whether activation of these receptors by the

selective agonists can increase $[Ca^{2+}]_i$ in HCAECs. Therefore, we examined the agonists-induced Ca^{2+} mobilization in HCAECs.

,The tracings of $[Ca^{2+}]_i$ levels in HCAEC were recorded in response to serotonergic agonists. Each of 5-HT, SUM, and DOI induced a biphasic rise of $[Ca^{2+}]_i$, namely, a rapid increase followed by a sustained increase in a dose-dependent manner in HCAECs (Fig.3). We show the representative tracings of $[Ca^{2+}]_i$ induced by 5-HT, SUM and DOI with the dose (1 μmol/L) which each agonists evokes nearly maximum nitrite production. These findings suggest that NO production mediated through both of $5\text{-}HT_{1B}$ and 5-HT2B receptors is associated with the increased $[Ca^{2+}]_i$ in HCAECs.

DISCUSSION

In the present study, we confirmed by RT-PCR that HCAECs express $5\text{-}HT_{1B}$ and $5\text{-}HT_{2B}$ receptor mRNAs. The data are consistent with the study by Ullmer et al.[40] No other mRNAs for $5\text{-}HT_1$ and $5\text{-}HT_2$ receptor subtypes were detected. Although very low expression of $5\text{-}HT_4$ receptor mRNA was detected in the Ullmer's study, we could not detected it in our HCAEC preparations (data not shown). Thus, $5\text{-}HT_{1B}$ and $5\text{-}HT_{2B}$ receptors are considered to be predominant subtypes among known 5-HT receptors in HCAECs.

5-HT evoked NO release from HCAECs in a dose-dependent manner. 5-HT-induced nitrite production was inhibited by methiothepin, but not by ketanserin. The results agree well with the putative pharmacological profile of 5-HT receptors mediating endothelium-dependent vasorelaxation, which have been reported in the previous pharmacological studies.[14,18,29] SUM and DOI, which is a selective agonist for $5\text{-}HT_{1B/1D}$ and $5\text{-}HT_2$ receptors, respectively, also caused Ca^{2+} mobilization and subsequent NO production in HCAEC. The data suggest that both of these two agonists, which do not cross-react with one another, can induce NO production in HCAEC independently. Therefore, it is postulated that 5-HT-induced NO production in HCAECs is evoked by net effects of the activation of these two receptors.

Ullmer et al demonstrated that the 5-HT-induced $[Ca^{2+}]_i$ increase in human pulmonary artery endothelial cells is mediated by activation of $5\text{-}HT_{2B}$ receptor, although in their study, no evidence for a receptor-stimulated release of NO was obtained.[41] Notably, they described that activation of $5\text{-}HT_{1B}$ receptor by sumatriptan did not cause Ca^{2+} mobilization despite the expression of $5\text{-}HT_{1B}$ receptor mRNA in human pulmonary artery endothelial cells.[41] In contrast, we showed in the present study the direct evidence that activation of the endogenous $5\text{-}HT_{1B}$ receptor by sumatriptan actually evokes Ca^{2+} mobilization and NO production in HCAEC. These discrepancies remain unexplained but are most likely due to the methodological difference or to the heterogeneity and multiplicity of 5-HT receptor profile among vascular beds.

It is generally accepted that $5\text{-}HT_{1B}$ receptor couples Gi protein and inhibits adenylate cyclase,[18] whereas $5\text{-}HT_{2B}$ receptor hydrolyzes phosphatidylinsitol 4,5-bisphosphate to produce

diacylglycerol, a specific activator of protein kinase C, and inositol 1,4,5-triphosphate, a compound that mobilizes Ca^{2+} from intracellular Ca^{2+} stores.[18,23,35] Indeed, activation of 5-HT_{1B} receptor inhibits forskolin-stimulated cAMP accumulation in human vascular endothelial cells.[36,41] On the other hand, there are some studies which demonstrated the coupling of the endogenous or stably transfected 5-HT_{1B} receptor to an increase in $[Ca^{2+}]_i$ through a pertussis toxin-sensitive mechanism in CHO-K1 cells[11] or murine fibroblasts.[44] However, although activation of these two receptors produced $[Ca^{2+}]_i$ elevation and NO release in HCAECs in the present study, little is known about the mechanisms for 5-HT_{1B} receptor-mediated Ca^{2+} mobilization and NO production in vascular endothelial cells. It must be clarified how activation of 5-HT_{1B} receptor can cause Ca^{2+} mobilization in endothelial cells.

In porcine coronary artery, endothelium-dependent relaxation is reported to be mediated by pertussis-toxin-sensitive (5-HT, α_2-adrenergic, leukotriens, thrombin) and insensitive (bradykinin, adenosine diphosphate) receptors, which suggests that both Gi and Gq proteins can couple receptor activation to the increase in $[Ca^{2+}]_i$ required to stimulate endothelial cell nitric oxide synthase. Interestingly, there are several studies showing that, in arteries with regenerated endothelium and in cultured endothelial cells, NO release mediated through pertussis-toxin-sensitive receptors is severely reduced or absent, while the response through other pertussis-toxin-insensitive receptor is relatively maintained.[6,33] In this term, our present data may imply that 5-HT_{1B} receptor-mediated vasodilatation could be preferentially impaired in diseased vessels. We and others have shown that there are 5-HT_{1B} receptor mRNAs in medial smooth muscle cells in human coronary arteries,[5,21] and 5-HT_{1B} receptor-mediated vasoconstriction is augmented in diseased coronary arteries.[8,20] Therefore, we speculate that changes in 5-HT_{1B} receptor function or expression in both endothelium and smooth muscle might play an important role in the abnormal vascular tone in diseased human coronary arteries. Further studies are needed to clarify the role of these receptors in various pathological conditions.

In summary, HCAECs possess 5-HT_{1B} and 5-HT_{2B} receptor mRNA and produce NO release in response to 5-HT. SUM and DOI, known as a selective agonist on 5-$HT_{1B/1D}$ and 5-HT_2 receptor, respectively, can induce NO release associated with $[Ca^{2+}]_i$ elevation. Taken together, HCAECs produce NO by two distinct pathway, via activation of 5-HT_{1B} and 5-HT_{2B} receptors. The 5-HT-evoked endothelium-dependent relaxation of human coronary arteries in vivo might be evoked by net effects of the activation of these two receptors.

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