Serotonin-Induced Hypercontraction Through 5-Hydroxytryptamine 1B Receptors in Atherosclerotic Rabbit Coronary Arteries

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Background—Augmented vasoconstriction to serotonin (5-hydroxytryptamine [5-HT]) in atherosclerotic vessels plays a crucial role in the development of myocardial ischemia. We investigated mechanisms for serotonin-evoked hypercontraction in atherosclerotic rabbit coronary arteries.

Methods and Results—Contractile responses to serotonergic agents of endothelium-denuded coronary arteries from control and Watanabe heritable hyperlipidemic rabbits (WHHL) were examined. WHHL coronary arteries exhibited hypercontraction to 5-HT1 receptor agonists; the constrictor threshold concentrations and $E_{50}$ to serotonin, 5-carboxamidotryptamine, and sumatriptan in WHHL were significantly lower, and the $E_{max}$ in WHHL to these agents were increased 55% to 59% above those of the control. Serotonin-evoked contractions in both groups were inhibited by GR127935 (5-HT1B/1D antagonist; 0.1 to 1 nmol/L) and pertussis toxin but not by ketanserin (5-HT2 antagonist; 0.01 to 1 μmol/L), suggesting that the hypercontraction is most likely mediated by 5-HT1B/1D receptors through a pertussis toxin–sensitive pathway. Furthermore, simultaneous measurements of $[Ca^{2+}]_i$ and isometric tension of fura-2–loaded arteries revealed that the hypercontraction was concomitant with the augmented elevation of $[Ca^{2+}]_i$ in the smooth muscle. The 5-HT1B mRNA levels in WHHL coronary arteries increased to 2.5-fold over those in control arteries, whereas neither 5-HT1D nor 5-HT2A mRNA was detected in either group.

Conclusions—Atherosclerotic rabbit coronary arteries exhibited the enhancement in contraction and Ca$^{2+}$ mobilization in response to serotonin. The 5-HT1B receptor, which is upregulated by atherosclerosis, most likely mediates the augmenting effects of serotonin. (Circulation. 2001;103:1289-1295.)

Key Words: atherosclerosis • vasoconstriction • receptors • muscle, smooth

Coronary vasoconstriction plays a pivotal role in the pathogenesis of myocardial ischemia in patients with stable angina, vasospastic angina, and acute coronary syndrome.1,2 Serotonin (5-hydroxytryptamine; 5-HT), which is locally released from aggregated platelets, is a candidate for the intrinsic stimulator of coronary vasoconstriction.2 It has been demonstrated that serotonin concentrations are elevated in the coronary sinus in patients with coronary artery disease.2–4 Ergonovine is a powerful and widely used agent for provocation of coronary spasm in susceptible patients with variant angina, and ergonovine-induced attacks are remarkably similar to spontaneous episodes. The action of ergonovine is believed to be mediated mainly through activation of 5-HT receptors on smooth muscle.5 Furthermore, augmented constrictor responses to intracoronary administration of serotonin have been demonstrated in patients with coronary atherosclerosis and variant angina.6–8 This agent can be used to provoke coronary spasm.9 These clinical findings serve to strengthen the pathogenic involvement of serotonin and its receptor in coronary vasoconstriction.

Coronary spasm is well known to occur at the site of atherosclerotic lesions.10 Isolated coronary arteries from patients with variant angina are associated with atherosclerosis and exhibit enhanced susceptibility to the constrictor effect of serotonergic agents.11,12 In addition, an increase in the response to serotonergic agents has been documented in vessels isolated from animal models of vasospasm or atherosclerosis.13–15 These lines of evidence suggest that the augmented vasoconstriction to serotonin, in relation to atherosclerosis, may principally contribute to the genesis of myocardial ischemia. However, the precise mechanism of the serotonin-evoked hypercontraction in atherosclerotic vessels is unclear. The aim of this study was to clarify the mechanism responsible for the altered vasoactivity to serotonin in atheroscle-
rotic coronary arteries by using Watanabe heritable hyperlipidemic rabbits (WHHL), the established animal model of atherosclerosis.16

Methods

Animals and Tissue Preparation
All animal preparations were performed within the Institutional Guidelines of Kobe University School of Medicine. WHHL (8 to 15 months of age, of either sex) and age-matched Japanese White rabbits (control) were anesthetized by administration of sodium pentobarbital (30 mg/kg). Coronary arteries were immediately isolated, cleaned of surrounding tissue, and cut into 2-mm-wide and 15-mm-long helical strips. The endothelium was denuded by rubbing the vessels with wet filter paper and at the end of the study, we verified histologically that endothelium had been removed from the vessel strips. Coronary arteries from WHHL exhibited smooth muscle cell–rich atherosclerotic lesions, as characterized previously.16 Macroscopic study of WHHL coronary arteries revealed distinct atherosclerotic lesions in all segments used in this study.

Isometric Tension Measurement of Coronary Arterial Strips
Isometric tension of coronary strips was measured as previously described.13,17 Briefly, coronary strips were suspended in organ baths containing Krebs buffer, and an initial preload of 0.5 g was applied. After 2 hours for equilibration, test contractions were induced by adding 20 mmol/L KCl. When the developed tension attained its peak value, strips were relaxed by rinsing with buffer. The concentration–response relations were determined by cumulative additions of serotonin, 5-carboxamidotryptamine (5-CT; 5-HT1 receptor antagonist), sumatriptan (SUM; 5-HT1D receptor antagonist), phenylephrine (PE; 43-adrenoceptor agonist), and histamine (HIS). Constrictor threshold concentrations, one-half maximally effective dose (ED50) values, and maximum responses (Emax) were determined from the log concentration–response curve for each agonist. In the antagonist studies, vessel strips were treated with indicated concentrations of GR127935 (5-HT1D receptor antagonist) or ketanserin (5-HT1D receptor antagonist) for 30 minutes before addition of agonists.

To verify the technique for functional denudation of endothelium, vasodilator responses were examined. Vessel rings with or without endothelium were precontracted by addition of 1 μmol/L PE. After the contraction reached a plateau, substance P was added in a cumulative manner. In a subset of assays with pertussis toxin (PTX), after initial concentration-response curves to serotonin and HIS were obtained, coronary strips were incubated at 37°C for 12 hours in Krebs buffer containing 200 ng/mL PTX or vehicle. The PTX-containing buffer was changed every 3 hours and kept oxygenated. Coronary strips showed comparable contractions to 20 mmol/L KCl before and after treatment with PTX. Concentration–response curves to serotonin and HIS obtained after PTX treatment were compared with those obtained after vehicle treatment.

Simultaneous Measurement of Muscle Tension and [Ca2+]i in Coronary Strips
Isometric tension and cytosolic Ca2+ concentration ([Ca2+]i) in coronary strips were measured with a CAF110 fluorometer (JASCO), as described previously.18 Coronary strips were treated with 6 μmol/L of the acetoxymethyl ester of fura-2 in the presence of 0.02% cremophor EL for 3 to 4 hours at room temperature. The muscle strip was illuminated alternately by a xenon lamp with 2 excitation wavelengths (340 and 380 nm). The intensity of fluorescence induced by excitation at 340 nm (F340) and 380 nm (F380) was measured, the ratio (F340/F380) was calculated automatically, and absolute [Ca2+]i was obtained according to the previously reported method.18 After the tissue was conditioned by application of high K+ (72.7 mmol/L), the concentration–response relations for serotonin and SUM were determined. We measured muscle tension and F340/F380 when the level had reached the sustained phase for each dose of the agent. Muscle tension and [Ca2+]i values in the resting state were taken as 0%, and those in the high K+-stimulated state were taken as 100%.

Ribonuclease Protection Assay
With the use of rabbit brain cDNA as a template, the cDNA regions of rabbit 5-HT1A, 5-HT1D receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by polymerase chain reactions for 35 cycles at 93°C, 56°C, and 72°C for 1, 2, and 3 minutes, respectively, with the following primers (GenBank accession number in parenthesis).

\[ 5'-\text{GCCGAATTCTCCAGATCGAAGTTCCTCAAGGCCAACCCTC-3'} \]
\[ 5'-\text{GTTTGTCCAGACCACTACATTCTCAGTTAGCAGGATTAGCAGTAATAGGGTGTGCA-3'} \]
\[ 5'-\text{ATGTCCCCATCAAACCGATTCGCGA-3'} \]
\[ 5'-\text{AGGACCAAAGGTTGAGAGCCCGT-3'} \]
\[ 5'-\text{GCCGAATTATCCATTGTGACCTCCAC-3'} \]
\[ 5'-\text{TTCGTGAGCATGCCTCACCAGTACGGG-3'} \]

The amplified cDNA fragments were cloned with TA cloning vectors and sequenced by the dyeodeoxy method. Rabbit 5-HT1A receptor was cloned from a rabbit brain cDNA library (T. Ishida et al, unpublished data, 2000) and the S region of this gene was ligated in the pGEM-4Z vector. Ribonuclease protection assay was as performed with the radiolabeled antisense riboprobes.19 The relative signal intensity of 5-HT1A receptor mRNA expression was standardized with that of GAPDH mRNA.

Statistics
Results are expressed as mean±SEM. An unpaired Student’s t test was used to detect significant differences when 2 groups were compared. Statistical differences among group means were determined by ANOVA with Bonferroni correction. A value of P<0.05 was taken as significant.

Drugs
The following drugs were used: 5-hydroxytryptamine creatinine sulfate, l-phenylephrine hydrochloride, and histamine diphosphate (Sigma); 5-carboxamidotryptamine maleate, sumatriptan, and GR127935 hydrochloride monohydrate (gifts from Glaxo Wellcome); ketanserin tartrate (a gift from Janssen Pharmaceutica); and pertussis toxin (Research Biochemicals International).

Results
5-HT1A Receptor Agonist Causes Hypercontraction in Atherosclerotic Arteries
Concentration–response relations for vasoactive agents are shown in Figure 1. Serotonin (Figure 1A), 5-CT (Figure 1B), and SUM (Figure 1C) induced contractions of control and WHHL coronary arteries in a dose-dependent manner. The rank order of potency of the agonists examined was 5-CT>serotonin>SUM in both groups of arteries. Interestingly, the concentration–response curve for these agents in WHHL exhibited a left upward shift as compared with control. The threshold concentrations and ED50 values for serotonin, 5-CT, and SUM in WHHL were significantly lower than those in control, and the Emax values were 59%, 57%, and 55% greater, respectively, as compared with the responses in control (Table). Serotonin, 5-CT, and SUM were 2.48-fold (P<0.05), 5.17-fold (P<0.01), and 2.05-fold (P<0.05) more potent, respectively, in WHHL as compared with control. In our coronary preparation, endothelial cells were denuded and endothelium-dependent relaxation to sub-
stance P was completely abolished (Figure 1F), suggesting that the influence of functional endothelium did not participate in the increase in potency of serotonergic agonists. On the other hand, WHHL coronary arteries did not exhibit hypersensitivity or hyperreactivity to PE and HIS (Figure 1, D and E). Indeed, the threshold concentration to PE was somewhat higher in WHHL (Figure 1D). These results suggest that augmented contractile responses to serotonergic agonists in WHHL were unlikely to be attributable to the nonspecific alterations in vascular reactivity.

We next examined the effects of GR127935 and ketanserin. As depicted in Figure 2A, low concentrations of GR127935 (0.1 to 1 nmol/L) inhibited the serotonin-evoked contraction in a dose-dependent manner. The high antagonist potency of GR127935 in both coronary preparations strongly implies that 5-HT1B and/or 5-HT1D receptors mediate the contractile responses. As is the case with previous studies, a significant reduction of E_{max} (P<0.05) by GR127935 was observed. On the other hand, low concentrations of ketanserin (0.01 to 0.1 μmol/L) did not affect the serotonin-evoked (Figure 2B), 5-CT–evoked, or SUM-evoked (data not shown) contractions. Although higher concentrations of ketanserin (1 μmol/L) slightly inhibited the serotonin effects, the inhibition was not statistically significant. Notably, GR127935 inhibited 5-CT–evoked and SUM-evoked contractions (Figure 2C), whereas ketanserin did not (data not shown) in control coronary artery strips. These results strongly support that GR127935-sensitive (i.e., 5-HT1B/1D) receptors mainly contribute to the contraction by serotonin.

PTX Attenuated Serotonin-Evoked Vasoconstriction

To investigate the signaling pathway in the serotonin-evoked contraction, coronary strips were treated with 200 ng/mL PTX for 12 hours. As shown in Figure 3A, the significant reduction of contractile responses to serotonin was observed in the PTX-treated vessels in both control (74.0±7.8% reduction, P<0.01) and WHHL (72.1±5.1% reduction, P<0.01). On the other hand, the histamine-evoked contraction, which is mediated through Gq protein, was not attenuated by treatment with PTX in either group (Figure 3B). Together, this indicates that serotonin-evoked contractions in both WHHL and control are mediated through PTX-sensitive mechanisms.

Serotonin-Evoked Hypercontraction Was Associated With Augmented Ca^{2+} Mobilization

The muscle tension and [Ca^{2+}]i in fura-2–loaded coronary strips were simultaneously measured in response to serotonin and SUM. In the resting state, [Ca^{2+}]i levels in control arteries

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<th>Contractile Threshold Concentrations, ED_{50} Values, and E_{max} Values in Rabbit Coronary Strips</th>
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*P<0.05, †P<0.01 vs corresponding control value.
and WHHL coronary arteries were 212±17 and 225±29 nmol/L, respectively. In the high K⁺–stimulated (72.7 mmol/L) state, [Ca^{2+}]ᵢ levels in control arteries and WHHL coronary arteries were 2206±342 and 2345±351 nmol/L, respectively. There were no significant differences in tension and [Ca^{2+}]ᵢ between control and WHHL in both resting and high K⁺–stimulated states. Therefore, we used the percentage values of tension and [Ca^{2+}]ᵢ of those induced by high K⁺ and compared relative changes in [Ca^{2+}]ᵢ in WHHL with those in control.

By contrast, in response to serotonin, WHHL coronary arteries exhibited enhanced E₅₀ of [Ca^{2+}]ᵢ and tension as compared with the control arteries (Figure 4, A and B). The serotonin-induced elevation of [Ca^{2+}]ᵢ in smooth muscles of WHHL was augmented in association with the enhanced contractile response. Furthermore, SUM mimicked the augmenting effects of serotonin in WHHL. Similar to serotonin, SUM-induced enhanced contraction in WHHL was concomitant with the augmented elevation of [Ca^{2+}]ᵢ in smooth...
muscles (Figure 4, C and D). These findings indicate that stimulation of 5-HT\textsubscript{1B/1D} receptors causes Ca\textsuperscript{2+} mobilization in smooth muscles and that the hypercontraction to serotonin in atherosclerotic rabbit coronary arteries is associated with augmented receptor-mediated Ca\textsuperscript{2+} mobilization in smooth muscles.

**5-HT\textsubscript{1B} Receptor mRNA Levels Are Increased in Atherosclerotic Coronary Arteries**

To evaluate gene expression of 5-HT receptors, we performed a ribonuclease protection assay. Cohybridization of rabbit 5-HT\textsubscript{1B} receptor and GAPDH riboprobes with rabbit coronary artery RNA, followed by digestion with single strand-specific ribonucleases and denaturing polyacrylamide gel electrophoresis, yielded the protected riboprobe fragments of 225 and 142 bases in length, consistent with the 5-HT\textsubscript{1B} receptor and GAPDH, respectively (Figure 5A, lanes 4 to 7). Expression levels of 5-HT\textsubscript{1B} receptor mRNA were increased by 2.5±0.2-fold (P<0.05) in WHHL compared with those in control (Figure 5A, lanes 4, 5 versus lanes 6, 7). In contrast, there were no significant differences in 5-HT\textsubscript{1B} receptor mRNA expression in whole brain between the groups (Figure 5A, lane 8 versus lane 9). On the other hand, neither 5-HT\textsubscript{2A} (Figure 5C) nor 5-HT\textsubscript{1D} mRNA (data not shown) was detected by this assay. Therefore, serotonin, 5-CT, and SUM were considered to selectively stimulate 5-HT\textsubscript{1B} receptors in rabbit coronary arteries. Together, the data indicate that the 5-HT\textsubscript{1B} receptor is functionally expressed and is the major subtype in the rabbit coronary artery.

**Discussion**

In this study, we demonstrated that rabbit coronary arteries with atheroma exhibit hypercontraction in response to serotonergic agonists, which is concomitant with the augmented receptor-mediated Ca\textsuperscript{2+} mobilization in smooth muscles. Threshold concentrations and ED\textsubscript{50} values to serotonin, 5-CT, and SUM in WHHL were reduced, and E\textsubscript{max} to these agents were augmented. In contrast, neither PE- nor HIS-induced contraction was enhanced by the presence of atheroma. These findings reflect that the effect of serotonin is preferentially augmented in atherosclerotic rabbit coronary arteries.

In general, two plausible explanations may account for the increase in potency of serotonin: impaired endothelium-dependent relaxation and hypercontraction of the smooth muscle. In this study, the hypercontraction in atherosclerotic arteries is considered to result from the altered reactivity of vascular smooth muscle cells per se because we verified histologically that endothelium has been removed from the vessel strips and that endothelium-dependent relaxation to substance P was abolished in our coronary preparations. It is likely that atherosclerosis changes the function of 5-HT receptors in vascular smooth muscle. We speculate that this abnormality in serotonin-evoked Ca\textsuperscript{2+} mobilization in the smooth muscle is a key step in the development of enhanced susceptibility to serotonin in atherosclerotic rabbit coronary arteries.

It has been postulated that rabbit coronary artery is an example of a tissue in which contractions to serotonin are mediated mainly by 5-HT\textsubscript{1A}-like receptors.\textsuperscript{17,21} In our study, SUM as well as 5-CT mimicked the serotonin effects, and serotonin-evoked contraction was inhibited by low concentrations of GR127935 in both groups, supporting the involvement of 5-HT\textsubscript{1B} and/or 5-HT\textsubscript{1D} receptors. 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} receptors have been reported to have remarkable pharmacological similarities and are difficult to distinguish from one another despite their low sequence homology. Notably, it has been reported that 1 μmol/L ketanserin blocks the 5-HT\textsubscript{1D} but
not the 5-HT_{1B} subtype on recombinant receptors. Thus, in combination with previous reports,\textsuperscript{17,21} we speculate that the predominant 5-HT receptor in rabbit coronary arteries is most likely the 5-HT_{1B} subtype. Furthermore, detection of 5-HT_{1B} mRNA but not 5-HT_{1D} or 5-HT_{2A} mRNA in the coronary strips is consistent with the involvement of 5-HT_{1B} receptors. From the standpoint of pharmacological analysis, however, we could not completely rule out the involvement of 5-HT_{1D} receptors regarding the effect of atherosclerosis. In this study, we did not use specific antagonists for these receptors, and to make a sharp distinction between 5-HT_{1A} and 5-HT_{1D} receptors, the effects of specific antagonists for these receptors should be examined in the future.

Although activation of 5-HT_{1} receptors is believed to be coupled with an inhibitory G protein, the precise intracellular signal transduction system of this receptor is still obscure. Particularly, there is little evidence that activation of this receptor causes Ca^{2+} mobilization in vascular tissues leading to contraction. Previous studies have demonstrated that activation of both 5-HT_{1B} and 5-HT_{1D} receptors causes [Ca^{2+}], elevation through the PTX-sensitive mechanism in other cells.\textsuperscript{23,24} In this study, we have verified that activation of endogenous vascular 5-HT_{1B/D} receptors by SUM and serotonin causes Ca^{2+} mobilization and subsequent contraction through a PTX-sensitive pathway in vascular tissue preparations. Moreover, this is the first study demonstrating that activation of endogenous vascular 5-HT_{1B} receptors contribute to hypercontractions, concomitant with augmented Ca^{2+} mobilization in atherosclerotic vessels.

Expression levels of 5-HT_{1B} receptor mRNA in WHHL coronary arteries were increased to 2.5-fold of those in control. In contrast, neither 5-HT_{1D} nor 5-HT_{2A} mRNA was detected. Upregulation of the 5-HT_{1B} receptor mRNA may not directly reflect the receptor protein or its function. We could not quantify the receptor protein levels because of low expression levels. However, we speculate that the enhanced responsiveness to serotonin is related to the upregulation of the 5-HT_{1B} receptor mRNA. That is, an increase in 5-HT_{1B} receptor number would account for the increase in the vascular responsiveness to serotonin, particularly in the reduction in threshold serotonin concentrations required for the response. Interestingly, we found an increase in the 5-HT_{1B} receptor mRNA in the human coronary arteries with atheroma. Further studies are required to clarify the mechanism of the upregulation of this gene and its contribution to hypercontraction.

It has been reported that serotonin-evoked contractions of human large coronary arteries are mediated by both 5-HT_{1B} and 5-HT_{2A} receptors.\textsuperscript{19,22,26} Kaumann et al\textsuperscript{22} have demonstrated that the relative contribution of these receptors in vasoconstriction of human coronary arteries was not directly related to the degree of atherosclerosis. On the other hand, some studies have indicated that ketanserin-resistant (or 5-HT\textsubscript{1}) receptors predominantly participate in constrictions of coronary arteries in patients with atherosclerosis or coronary artery diseases.\textsuperscript{8,27,28} Moreover, isolated coronary arteries from patients with variant angina exhibited enhanced susceptibility to SUM as well as serotonin and ergonovine.\textsuperscript{11,12} Recent intravascular ultrasound studies have shown that atherosclerosis is present at sites with coronary vasospasm even in the absence of angiographically significant coronary artery disease.\textsuperscript{29} Although our present findings are based on rabbit coronary arteries and vascular response to serotonin largely varies among species, vascular beds, age, and disease status, we speculate that 5-HT_{1B} receptors could possibly account for the ketanserin-resistant pathogenic constriction in patients with atherosclerosis or coronary spasm.

Conclusions
Atherosclerotic rabbit coronary arteries exhibited enhanced contraction in response to serotonin. Augmented Ca^{2+} mobilization in smooth muscle may be the principal mechanism of the hypercontraction. The responses are mainly mediated by activation of GR127935-sensitive receptors. In particular, the 5-HT_{1B} receptor, which is upregulated by atheroma, most likely mediates the serotonin effects. Our findings might provide a novel role of this receptor in the abnormal modulation of vascular tone in atherosclerotic arteries.

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