Chapter 9

Serotonin Effects on Expression of the LDL Receptor Family Member LR11 and 7-Ketocholesterol–Induced Apoptosis in Human Vascular Smooth Muscle Cells

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Additional information is available at the end of the chapter

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Abstract

We previously confirmed the effect of sarpogrelate hydrochloride (sarpogrelate), 5-hydroxytryptamine (5-HT) 2A receptor antagonist on cardio-ankle vascular index (CAVI) as a marker of systemic arterial stiffness. After 6 months of treatment with sarpogrelate for 35 type 2 diabetic patients, decreased CAVI, indicating the ameliorated arterial stiffness, was observed. Therefore, via 5-HT2A receptor blockade, sarpogrelate might effect as a vasoactive agent, as well as an inhibitor of platelet aggregation. 5-HT is a known mitogen for vascular smooth muscle cells (VSMCs). In addition, the pathogenic change of VSMCs such as dedifferentiation and proliferation/apoptosis represents one of the atherosclerotic changes. On the other hand, LR11, a mosaic LDL receptor family member, may involve in the invasion of VSMCs into neointimal thickening. We therefore investigated an *in vitro* study to clarify whether 5-HT was concerned to LR11 expression and apoptosis of human VSMCs induced by 7-ketocholesterol (7KCHO), a major oxidation product of cholesterol involved in plaque destabilization. Resultantly, 5-HT accelerated the proliferation of VSMCs, and this effect was suppressed by simultaneous addition of sarpogrelate. Sarpogrelate also attenuated the 5-HT–induced LR11 mRNA expression in VSMCs. Additionally, 5-HT attenuated the 7KCHO-induced apoptosis of VSMCs through caspase-dependent pathway. These results suggest new knowledge on the modification of human VSMCs induced by 5-HT.

Keywords: arterial stiffness, vascular smooth muscle cells, LR11, apoptosis, 7-ketocholesterol

1. Introduction

Invasion of vascular smooth muscle cells (VSMCs) to intima takes a principle finding in the progression of atherosclerosis and the incidence of restenosis after vascular intervention [1–3]. Lately, LR11, a mosaic low-density lipoprotein (LDL) receptor family member, is known to exist largely in VSMCs of the hyperplastic intima, but not the media and induce the invasion and migration potential of intimal VSMCs, speculated to originate from medial VSMCs [4–6]. Meanwhile, reduced extracellular matrix, reduced number of VSMCs, thin fibrous cap, and extracellular oxysterol accumulation have been observed in unstable plaques [7–9]. We have previously shown that 7-ketocholesterol (7KCHO), a major oxidation product of cholesterol, revealed as an apoptosis inducer on VSMCs [10, 11], and attenuated the migration of VSMCs [12]. These reports propose that the presence of 7KCHO may reduce the number of VSMCs and contribute to unstable plaque. Generally, proliferation/apoptosis and dedifferentiation of VSMCs in the arterial intima identify one of the pathological findings observed in atherogenesis [2, 13, 14]. Meanwhile, the factors modulating proliferation/apoptosis of VSMCs and the potential cellular mechanisms are not fully elucidated.

Serotonin (5-hydroxytryptamine, 5-HT), secretion from activated platelets, is recognized to be a naturally occurring vasoactive mediator involved in vascular inflammation and atherogenesis [15]. 5-HT has multiple receptor subtypes [16] and induces platelet aggregation, vasoconstriction, and VSMC proliferation [17, 18]. In addition, the plasma level of 5-hydroxyindole-3-acetic acid (5-HIAA; a derivative end product of 5-HT) is relatively high in subjects with visceral adiposity, revealing that 5-HT is one of the potential mediators for atherogenesis in lifestyle diseases [19].

Sarpogrelate hydrochloride (sarpogrelate), a selective 5-HT2A receptor antagonist, is used for diabetic patients with chronic arterial occlusive diseases [20] and is known to suppress platelet aggregation, vascular endothelial dysfunction, and smooth muscle contraction mediated via 5-HT2A receptor [21, 22]. The restorative effects of sarpogrelate on cardiovascular disturbance in experimental diabetic rats were also reported [23]. Furthermore, we investigated prospectively the effect of sarpogrelate on systemic arterial stiffness assessed by cardio-ankle vascular index (CAVI) in type 2 diabetic patients [24]. After 6 months of treatment with sarpogrelate for 35 Japanese type 2 diabetic patients, decreased CAVI, indicating the ameliorated arterial stiffness, was observed (**Table 1**). Sarpogrelate is known to inhibit 5-HT–induced vascular smooth muscle contraction and/or cell proliferation [25, 26]. Moreover, Shirai et al. have reported that CAVI might be affected by change in contractility of vascular smooth muscle [27]. Therefore, these results suggest that sarpogrelate may ameliorate arterial stiffness through inhibiting vascular smooth muscle contractility. However, the effects of 5-HT on vascular composition are not fully understood. We hypothesized that 5-HT was concerned to the invasion and migration of VSMCs through the regulation of LR11, besides the apoptosis of VSMCs.

We confirmed the effect of 5-HT on LR11 expression in human VSMCs. Additionally, whether there was an interaction of 5-HT with 7KCHO in inducing VSMC apoptosis was investigated.

	Baseline	After 6 months	*p* Value
N (male/female)	35 (21/14)	-	-
Age (y)	67.4 ± 9.1	-	-
Height (cm)	159.5 ± 8.1	-	-
Body weight (kg)	61.9 ± 10.1	61.9 ± 10.1	0.982
BMI (kg/m^2)	23.4 ± 2.9	23.4 ± 2.9	0.966
CAVI	10.11 ± 0.92	9.87 ± 0.97	0.013
sBP (mmHg)	128 ± 11	132 ± 12	0.076
dBP (mmHg)	75 ± 8	76 ± 8	0.231
TG (mg/dl)	161 ± 234	178 ± 243	0.068
HDL-C (mg/dl)	58 ± 14	55 ± 14	0.304
LDL-C (mg/dl)	98 ± 14	104 ± 34	0.054
FPG (mg/dl)	154 ± 53	153 ± 60	0.771
HbA1c (%)	7.2 ± 1.2	7.1 ± 1.2	0.463
Medication: n (%)			
Sulfonylurea	11 (31.4)	-	-
Biguanide	6 (17.1)	-	-
Alpha-glucosidase inhibitor	10 (28.6)	-	-
Thiazolidinedione	4 (11.4)	-	-
ARB/ACE-I	8 (22.9)	-	-
Calcium channel blocker	7 (20.0)	-	-
Statin	15 (42.9)	-	-
Fibrate	5 (14.3)	-	-

Data are presented as mean ± standard deviation. Paired *t*-test was used in comparing baseline and 6-month data. BMI, body mass index; CAVI, cardio-ankle vascular index; sBP, systolic blood pressure; dBP, diastolic blood pressure; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; ARB, angiotensin receptor blocker; ACE-I, angiotensin converting enzyme inhibitor.

Table 1. Participant characteristics before and after 6 months of sarpogrelate treatment.

2. Materials and methods

2.1. Cell cultures

VSMCs prepared from human femoral artery were cultured in growth medium of Dulbecco's modified Eagle's minimal essential medium supplemented with delipidated calf serum

mixture or 5–10% (v/v) heat inactivated fetal calf serum (FCS), 40-μg/mL gentamicin, and 2-mmol/L L-glutamine, maintained at 37°C in 5% carbon dioxide.

Sarpogrelate was a gifted reagent from Mitsubishi-Tanabe Pharma Co., Osaka, Japan. 5-HT, 7KCHO, and other reagents were generously provided by Sigma (St. Louis, Missouri).

2.2. Proliferation of VSMCs

VSMCs were plated into 12-well microtiter plates in triplicate (10^4 cells per well). After 72 h of growth, the medium was changed to Dulbecco's modified Eagle medium containing 5% FCS and sarpogrelate and/or 5-HT was administered. Thereafter, proliferation of VSMCs was evaluated by a hemocytometer during 0 and 8 days after the administration of sarpogrelate and/or 5-HT.

2.3. Reverse transcription polymerase chain reaction

Total RNA was isolated from VSMCs by RNeasy kit (Qiagen, Courtaboeuf, France), and complementary (c) DNA was prepared by reverse transcription (RT-) polymerase chain reaction (PCR) kit (TaKaRa, Tokyo, Japan) as already described in the manufacturer's instructions. RNA concentrations were evaluated by measuring absorbance at 260 nm. Thereafter, RT-PCR was carried out using 1 μg of reverse transcribed total RNA. Expression of β-actin internal standard was adopted as housekeeping gene for quantifying RNA levels. The specific primers were as follows:

LR11:

sense 5′-AGGAGGGCATCCTGCAGTATTGCCAAGAAG-3′

antisense 5′-TGGCGACGGTGTGCCAGTGA-3′

β-actin:

sense 5′-CTCTTCCAGCCTTCCTTCCT-3′

antisense 5′-AGCACTGTGTTGGCGTACAG-3′

PCR was run on a Gene Amp PCR System 9700 (Applied Biosystems, Foster city, CA) for 35 cycles both for β-actin and LR11. The terminal cycle for denaturation, annealing, and elongation was required at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for each. The amplified products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by UV irradiation. Furthermore, the images were photographed using an Olympus digital camera (Tokyo, Japan).

2.4. Caspase activity of VSMCs

Two methods were used in order to measure caspase activity in VSMCs. First, a luminescent assay for measuring caspase-3 and -7 (caspase-3/7) activities was adopted. The other was flow

cytometric analysis by the fluorescein-5–isothiocyanate (FITC) Active Caspase-3 Apoptosis Kit (BD Pharmingen, La Jolla, California). After two washes in ice-cold phosphate-buffered saline, VSMCs incubated in 96-well microplates were scraped off the tissue culture dish, and caspase activities were evaluated using the Caspase-Glo® 3/7 Assay (Promega, Wisconsin) as described in the manufacturer's protocol.

WST-8 cell counting kit (Dojindo Laboratories, Kumamoto, Japan) was adopted to evaluate bioavailability of VSMCs. When serial dilutions of VSMCs were plated into 96-well microtiter plates and analyzed, an absorbance response at 460 nm was observed linearly (data not shown). The number of VSMCs was measured using the regression equation. Caspase-3/7 activity was corrected for mean cell number measured for each.

2.5. Apoptosis of VSMCs

VSMCs were harvested by trypsinization and kept into 5-mL fluorescence-activated cell sorting (FACS) tubes in phosphate buffered saline (PBS) (pH 7.4) containing 5% FCS. Thereafter, samples were processed in a Becton Dickinson FACScalibur (Immunocytometry Systems, San Jose, California) equipped with a 15 mW, 488 nm argon laser, and filter configuration. BD™ Biosciences Propidium Iodide Staining Solution was adopted to quantify DNA content. FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen) was used to evaluate active caspase-3. Cell samples (20,000 cells) were analyzed on a FACSort cytometer using Cell Quest Pro software (BD Biosciences). The percentage of apoptotic cells in each sample was quantified by manual counting of adherent VSMCs using fluorescence microscopy.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS software (version 11.5, Chicago, IL, USA). The data are presented as mean ± standard deviation (SD). The effects of reagents were compared by one-way ANOVA followed by Bonferroni multiple comparison test and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of 5-HT and/or sarpogrelate on VSMC proliferation

Figure 1A proposes VSMC proliferation in the absence or presence of 5-HT for 8 days. Administration of 5-HT (100 μM) to VSMCs significantly increased proliferation at days 5 and 8. The cell counts of VSMCs on day 8 after the administration of 5-HT (1, 10, or 100 μM) with or without sarpogrelate (10 μM) were shown in **Figure 1B**. The administration of 5-HT to VSMCs induced a dose-dependent increase in cell number and simultaneous addition of sarpogrelate attenuated the effect of 5-HT.

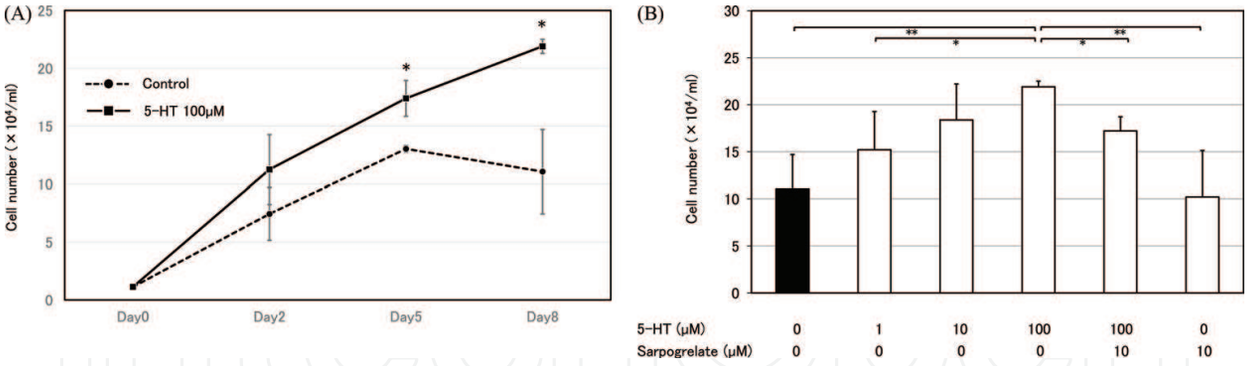

Figure 1. Effects of serotonin (5-HT) and sarpogrelate on proliferation of vascular smooth muscle cells (VSMCs). (A) Changes in cell number over time. After seeding VSMCs in 12-well microplates (1 × 10^4/well, in triplicate) and culturing for 72 h, 5-HT (100 μM) was added. Cell number was counted from day 0 to day 8 after the addition of 5-HT. Data are presented as mean ± SD of triplicate samples. *Significantly higher ($p < 0.05$, unpaired *t*-test) cell count compared with control. (B) Effects of 5-HT concentration and sarpogrelate on cell proliferation. After seeding VSMCs in 12-well microplates (1 × 10^4/well, in triplicate) and culturing for 72 h, 5-HT and sarpogrelate at indicated concentrations were added. Cell numbers were counted on day 8 after addition of 5-HT and/or sarpogrelate. Data are presented as mean ± SD of triplicate samples. *$p < 0.05$, **$p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

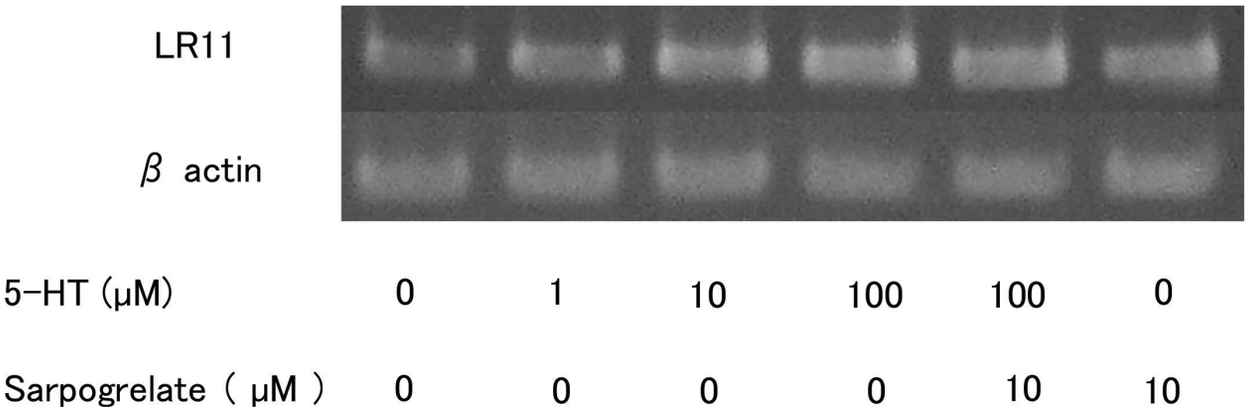

Figure 2. Effects of serotonin (5-HT) and sarpogrelate on LR11 mRNA expression in vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (6 × 10^4/well, in triplicate) and culturing for 72 h, 5-HT and sarpogrelate at indicated concentrations were added and cultured for another 72 h. Upper panel shows LR11 mRNA expression determined by reverse transcription PCR. Beta-actin expression shown in the lower panel was used as internal standard.

3.2. Effects of 5-HT and/or sarpogrelate on LR11 mRNA expression in VSMCs

RT-PCR showed that LR11 mRNA expression was enhanced dose dependently by administration of 5-HT at 1–100 μM in VSMCs. Additionally, simultaneous addition of sarpogrelate at 10 μM attenuated the effect of 5-HT (**Figure 2**).

3.3. Effects of 5-HT and/or 7KCHO on caspase activity in VSMCs

The effects of 5-HT and/or 7KCHO on caspase activity in VSMCs were examined using two methods. Flow cytometric analysis was carried out by VSMCs stained with FITC-conjugated

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antiactive caspase-3 monoclonal antibody. The histograms in **Figure 3A** propose the distribution of VSMCs. Administration of 5-HT to VSMCs alone caused a slight leftward shift of the peak from control, revealing an attenuation in active caspase-3 expression [28]. Contrastingly, administration of 7KCHO alone caused an enhancement in active caspase-3 expression as revealed by a rightward shift of the histogram, and this effect of 7KCHO was attenuated by simultaneous addition of 5-HT (**Figure 3B**).

Next, a luminescent assay was carried out in order to evaluate caspase-3/7 activities using the same protocol shown in the previous experiment. Administration of 5-HT alone did not change the caspase-3/7 activities in VSMCs. On the other hand, administration of 7KCHO alone enhanced caspase-3/7 activity ninefold compared to the control, and this

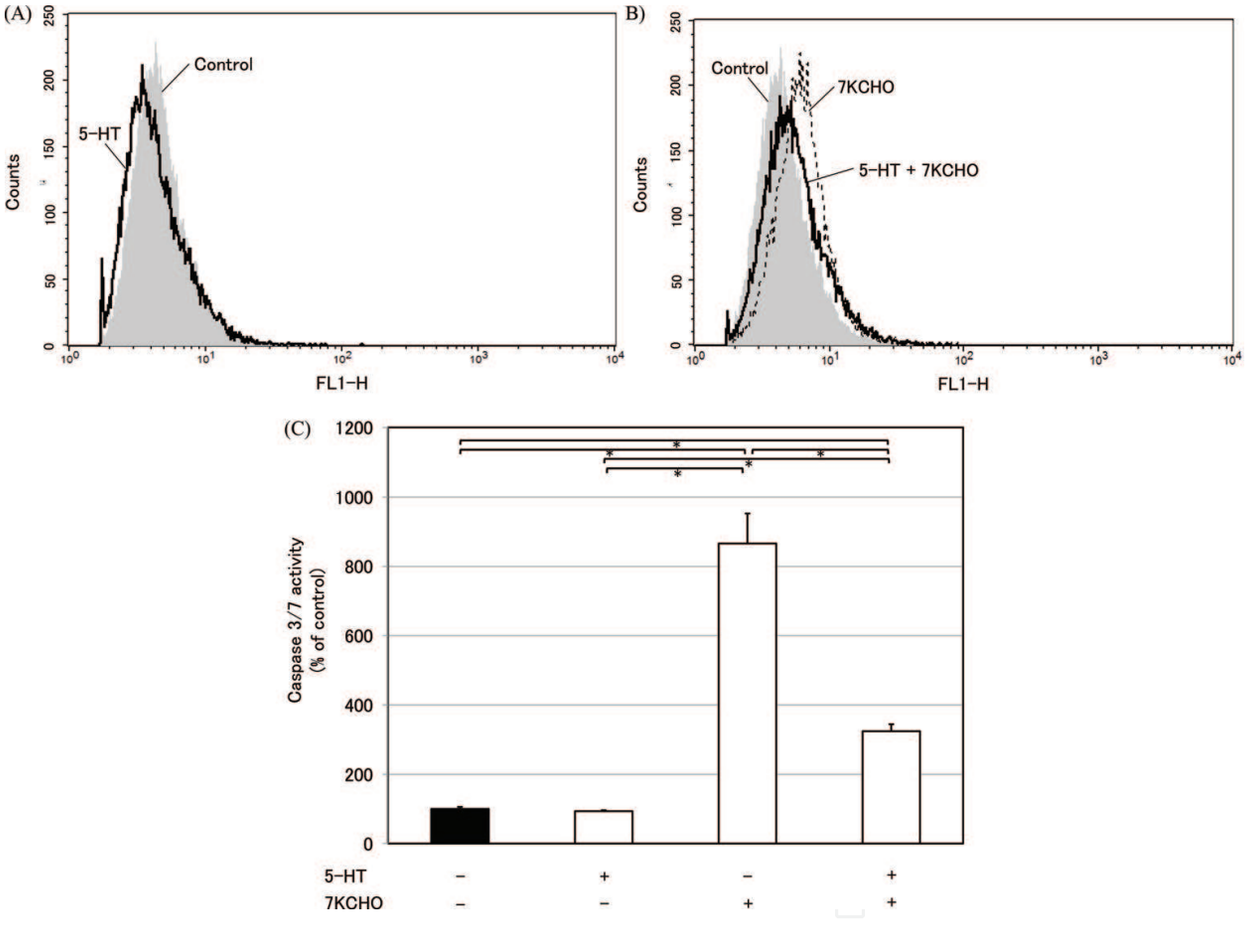

Figure 3. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on caspase activity in vascular smooth muscle cells (VSMCs). (A and B) Caspase-3 activity assayed by antiactive caspase antibody and flow cytometry. After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing for 48 h, VSMCs were incubated with no addition, addition of 5-HT (100 μM) alone, or addition of 5-HT (100 μM) and 7KCHO (50 μM) for another 48 h. The cells were maintained in Dulbecco's modified Eagle's minimal essential medium containing 10% FBS and 1% non essential amino acid, and incubated at 37°C, 5% CO_2. Caspase-3 expression was analyzed using FITC-conjugated monoclonal anti active caspase-3 antibody followed by flow cytometry. Changes in active caspase-3 activity are shown in FL1 histograms. (C) Caspase-3/7 activities assayed by luminescent assay. VSMCs were seeded into 96-well microplates (1×10^5/well, in triplicate), and incubated with or without the addition of 5-HT (100 μM) and/or 7KCHO (50 μM) for 48 h. Luciferase activity was measured according to the protocol from Promega. Data are presented as mean ± SD of triplicate samples. $^*p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

effect of 7KCHO was attenuated by simultaneous addition of 5-HT (**Figure 3C**). To sum up, almost same effects of 5-HT were shown in both methods for measuring caspase activity in VSMCs.

3.4. Effects of 5-HT and/or 7KCHO on quantitation of apoptosis in VSMCs

Apoptotic DNA fragmentation in VSMCs was evaluated by propidium iodide fluorescence to clarify the apoptosis-inducing effect of 7KCHO in the absence or presence of 5-HT (**Figure 4**). The apoptotic rate was elevated after administration of 7KCHO (50 μM) alone, but this effect of 7KCHO was suppressed by simultaneous addition of 5-HT at 100 μM.

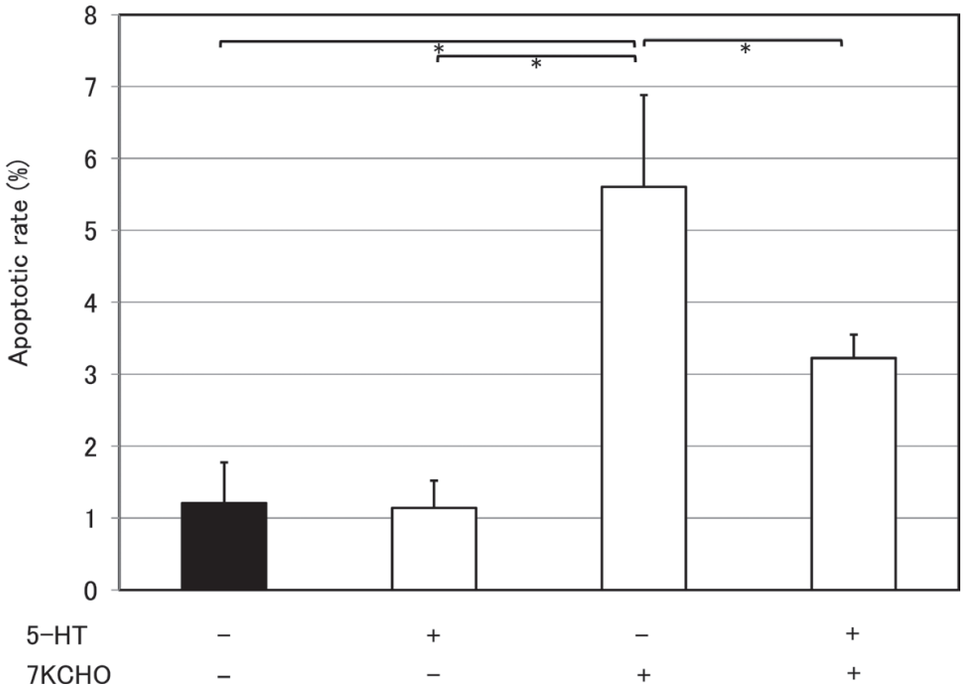

Figure 4. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on quantitative analysis of apoptosis of vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing for 48 h, VSMCs were incubated with or without the addition of 5-HT (100 μM) for another 96 h. 7KCHO (50 μM) was added to some wells 72 h after the addition of 5-HT. Cells were stained with 50 μg/ml of propidium iodide after cell lysis and analyzed by flow cytometry. Apoptotic rate is the percentage of nuclei in the sub-G1 population representing DNA fragmentation as shown in FL2 histograms. Data are presented as mean ± SD of three independent experiments. $^*p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

4. Discussion

In the present study, 5-HT accelerated the proliferation of human VSMCs and this effect of 5-HT was attenuated by simultaneous addition of sarpogrelate. Moreover, sarpogrelate also suppressed the 5-HT–induced enhancement of LR11 mRNA expression in VSMCs. 5-HT attenuated the 7KCHO-induced VSMC apoptosis through caspase-3/7–dependent pathway

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besides. There is so far no evidence providing the effect of 5-HT on LR11 expression and apoptosis in VSMCs. The present report demonstrates the effects of 5-HT on such pathogenic changes in VSMCs.

The mechanism by which 5-HT regulates the number of intimal VSMCs has not been fully clarified, so that the modulators for migration of VSMCs from the arterial media to the intima should be examined. Previous reports have shown that LR11 expression was largely involved in the differentiation of VSMCs. Furthermore, the VSMCs with a contractile phenotype observed in the arterial media do not express LR11, whereas the VSMCs in an active synthetic phenotype located in the intima highly express LR11 [29, 30]. Additionally, circulating soluble form of LR11 concentrations in serum is known to correlate with the degree of coronary organic stenosis, carotid intima-media thickness, and pulmonary arterial hypertension [31–33]. These findings suggest that LR11 may play key role of modification in VSMCs during atherogenesis. Our data suggested that 5-HT enhanced the expression of LR11 mRNA in VSMCs, and simultaneous addition of sarpogrelate attenuated this effect of 5-HT. These results reveal that 5-HT may participate to neointimal thickening through stimulating not only proliferation, but also invasion of VSMCs accompanied by upregulation of LR11. Therefore, sarpogrelate may show the pleiotropic effect on vascular tissue, such as decreased systemic arterial stiffness, partially through down-regulation of LR11 in VSMCs. Additionally, sarpogrelate was recently reported to ameliorate the development of chronic hypoxic pulmonary hypertension through the occurrence of increased apoptosis and decreased proliferation of VSMCs [34]. However, we cannot definitely consider that the effect of 5-HT on VSMCs is absolutely malignant to human body.

Apoptosis of VSMCs located in atheroma is known to be associated with vulnerable plaque ruptures [35, 36]. In this study, luminescent assay and flow cytometric analysis revealed that 7KCHO enhanced the caspase-3/7–dependent apoptotic pathway. In a phase of progressive atherosclerotic plaque formation, 7KCHO is speculated to induce the absence of VSMCs, which make plaque unstable leading to rupture. Meanwhile, simultaneous addition of 5-HT suppressed 7KCHO-induced VSMC apoptosis. These findings reveal that 5-HT may reduce the occurrence of plaque rupture through the attenuation of 7KCHO-induced VSMC apoptosis.

Whether 5-HT is favorable or not for the vascular remodeling process is still controversial. In state of vascular injury, subsequent platelet activation accompanied by endothelial damage provides increasing local plasma level of 5-HT. Furthermore, 5-HT causes the proliferation, contraction, and migration of VSMCs through the 5-HT2A receptor amplified by various intracellular signaling pathways [37–39]. Thus, 5-HT plays the basic principle of vascular repair, which spreads to neointimal thickening and decrease of peripheral blood flow. Note that the administration of 5-HT to VSMCs resulted in the enhancement of potential for cell migration caused by up-regulation of LR11 in the present study. Moreover, the suppressive effect of 5-HT on VSMC apoptosis might concern to the suppression of vulnerability in atheromatous plaque caused by 7KCHO. These effects of 5-HT can be protective for vascular composition.

It is still controversial whether modification of VSMCs along with upregulation of LR11 directly concern to the attenuation in apoptosis-inducing effect of 7KCHO. Further elucidation about the causal relationship of LR11 expression with VSMC apoptosis should be examined.

Resultantly, 5-HT accelerated the proliferation of VSMCs, and this effect was suppressed by simultaneous addition of sarpogrelate. Sarpogrelate also attenuated the 5-HT–induced LR11 mRNA expression in VSMCs. Additionally, 5-HT attenuated the 7KCHO-induced apoptosis of VSMCs through caspase-dependent pathway. These results suggest new knowledge on the modification of human VSMCs induced by 5-HT.

Declaration of conflicting interests

Potential conflicts of interest with any of the authors: None.

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