

## Original Article

### Effects of serotonin on expression of the LDL receptor family member LR11 and 7-ketocholesterol-induced apoptosis in human vascular smooth muscle cells

Daiji Nagayama<sup>1</sup>, Noriko Ishihara<sup>1</sup>, Hideaki Bujo<sup>2</sup>, Kohji Shirai<sup>3</sup>, Ichiro Tatsuno<sup>1\*</sup>

<sup>1</sup>Center of Diabetes, Endocrinology and Metabolism, <sup>2</sup>Department of Clinical

Laboratory Medicine and <sup>3</sup>Department of Vascular Function, Toho University,

Sakura Medical Center, 564-1, Shimoshizu, Sakura-City, Chiba 285-8741, Japan

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Address all correspondence and reprint requests to \*Dr. Ichiro Tatsuno, Center of Diabetes, Endocrinology and Metabolism, Toho University, Sakura Medical Center, 564-1, Shimoshizu, Sakura-City, Chiba 285-8741, Japan. Tel: +81-43-462-8811; Fax: +81-43-489-9770; E-mail: [ichiro.tatsuno@med.toho-u.ac.jp](mailto:ichiro.tatsuno@med.toho-u.ac.jp)

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## Abstract

Serotonin (5-HT) is a known mitogen for vascular smooth muscle cells (VSMCs). The dedifferentiation and proliferation/apoptosis of VSMCs in the arterial intima represent one of the atherosclerotic changes. LR11, a member of low-density lipoprotein receptor family, may contribute to the proliferation of VSMCs in neointimal hyperplasia. We conducted an *in vitro* study to investigate whether 5-HT is involved in LR11 expression in human VSMCs and apoptosis of VSMCs induced by 7-ketocholesterol (7KCHO), an oxysterol that destabilizes plaque. 5-HT enhanced the proliferation of VSMCs, and this effect was abolished by sarpogrelate, a selective 5-HT<sub>2A</sub> receptor antagonist. Sarpogrelate also inhibited the 5-HT-enhanced LR11 mRNA expression in VSMCs. Furthermore, 5-HT suppressed the 7KCHO-induced apoptosis of VSMCs via caspase-3/7-dependent pathway.

These findings provide new insights on the changes in the differentiation stage of VSMCs mediated by 5-HT.

Key words: Serotonin (5-HT), Vascular smooth muscle cells, LR11, Apoptosis

## 1. Introduction

Proliferation of intimal vascular smooth muscle cells (VSMCs) plays a major role in the development of atherosclerosis and restenosis after angioplasty [1-3]. Recently, LR11, a member of the low-density lipoprotein (LDL) receptor family, has been shown to be expressed at high level in VSMCs of the hyperplastic intima but not the media, and enhance the migration and invasion activities of intimal VSMCs that are thought to originate from medial VSMCs [4-6]. On the other hand, extracellular oxysterol accumulation, reduced number of VSMCs, thin fibrous cap and reduced extracellular matrix have been demonstrated in vulnerable plaques [7-9]. We have reported that 7-ketocholesterol (7KCHO), an oxysterol, has an apoptosis-inducing effect on VSMCs [10, 11], and inhibits the migration of VSMCs [12]. These findings suggest that accumulation of 7KCHO in atherosclerotic plaque may decrease the number of VSMCs in the plaque and render atherosclerotic plaques unstable.

The dedifferentiation and proliferation/apoptosis of VSMCs in the arterial intima represent one of the changes found in atherosclerotic lesions [2, 13, 14]. However, the potential cellular mechanisms and the factors modulating proliferation/apoptosis of VSMCs are not fully understood.

Serotonin (5-hydroxytryptamine, 5-HT) released from activated platelets is considered to be a naturally occurring vasoactive substance involved in vascular inflammation and atherogenesis [15]. 5-HT has various receptor subtypes [16], and promotes vasoconstriction, VSMC proliferation, and platelet aggregation [17, 18]. The plasma concentration of 5-hydroxyindole-3-acetic acid (5-HIAA; a

derivative end product of 5-HT) is high in subjects with metabolic syndrome, suggesting the potential importance of 5-HT as one of the underlying mechanisms of atherosclerosis in metabolic syndrome [19]. In addition, we reported that sarpogrelate hydrochloride, a selective 5-HT<sub>2A</sub> receptor antagonist, decreases arterial stiffness assessed by cardio-ankle vascular index in type 2 diabetic patients [20]. Nevertheless, the effects of 5-HT on vascular structure remain controversial. We hypothesized that 5-HT is involved in the migration and invasion of VSMCs regulated by LR11 expression, as well as the apoptosis of VSMCs.

This study was conducted to determine the influence of 5-HT on LR11 expression in VSMCs. Furthermore, we investigated whether there was an interaction between 5-HT and 7KCHO in inducing VSMC apoptosis.

## **2. Materials and methods**

### **2.1. Cell cultures and reagents**

Human VSMCs isolated from the femoral artery were cultured in a growth medium of Dulbecco's modified Eagle's minimal essential medium supplemented with 5 to 10% (v/v) delipidated fetal calf serum (FCS) or delipidated calf serum mixture, 2 mmol/L L-glutamine and 40 µg/mL gentamicin, at 37 °C under 5% CO<sub>2</sub>.

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

### **2.2. Cell number**

Cells were seeded in triplicate into 12-well microplates at a density of  $10^4$  cells/well. After culturing for 72 h, the growth medium was changed to Dulbecco's modified Eagle's minimal essential medium containing 5% FCS, and 5-HT and/or sarpogrelate was added. Cell numbers were counted using a hemocytometer from days 0 to 8 after the addition of 5-HT and/or sarpogrelate.

### 2.3. Reverse transcription PCR for LR11 mRNA

Total cellular RNA was extracted from VSMCs using an RNeasy kit (Qiagen, Courtaboeuf, France), and complementary DNA was synthesized using a reverse transcription PCR kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. RNA concentrations were determined by measuring absorbance at 260 nm. Then, reverse transcription PCR was performed using 1  $\mu$ g of reverse transcribed total RNA. Expression of the housekeeping gene  $\beta$ -actin was used as an internal standard. LR11 mRNA levels were detected using primers for LR11 (sense 5'-AGGAGGGCATCCTGCAGTATTGCCAAGAAG-3', antisense 5'-TGGCGACGGTGTGCCAGTGA-3') and  $\beta$ -actin (sense 5'-CTCTTCCAGCCTTCCTTCCT-3' and antisense 5'-AGCACTGTGTTGGCGTACAG-3'). Polymerase chain reaction was run on a Gene Amp PCR System 9700 (Applied Biosystems, Foster city, CA) for 35 cycles both for LR11 and  $\beta$ -actin. Denaturation, annealing, and extension were performed at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds, respectively. The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV irradiation. The images were photographed with an Olympus digital camera (Tokyo, Japan).

#### 2.4. Analysis of caspase activity

We used two methods to evaluate caspase activity in VSMCs. The first was flow cytometric analysis using the fluorescein-5-isothiocyanate (FITC) Active Caspase-3 Apoptosis Kit (BD Pharmingen, La Jolla, California) as described below. The other was a luminescent assay that measures caspase-3 and -7 (caspase-3/7) activities. VSMCs incubated in 96-well microplates were washed twice with phosphate-buffered saline, and caspase activities were determined using the Caspase-Glo® 3/7 Assay (Promega, Wisconsin) according to the manufacturer's protocol.

We used the WST-8 cell counting kit (Dojindo Laboratories, Kumamoto, Japan) to determine cell numbers. When serial dilutions of VSMCs were seeded into 96-well microplates and assayed, a linear response in absorbance at 460 nm was observed (data not shown). Cell number was calculated using the regression equation. Caspase-3/7 activity was corrected for mean cell number calculated for each group.

#### 2.5. Analysis of apoptosis by flow-cytometry

Cells were collected following brief trypsin treatment, and transferred into 5-mL FACS tubes in phosphate-buffered saline containing 5% FBS. Then, samples were run on a Becton Dickinson FACScalibur (Immunocytometry Systems, San Jose, Calif.) equipped with a 15-mW, 488-nm argon laser and filter configuration. Active caspase-3 was detected by the FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen). Cellular DNA content was quantitated using BD™ Biosciences

Propidium Iodide Staining Solution. Cell samples (20,000 cells) were analyzed on a FACSort cytometer using Cell Quest Pro software (BD Biosciences). All results were confirmed by manual counting of adherent VSMCs using fluorescence microscopy.

## 2.6. Statistical analysis

All data are expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using SPSS software (version 11.5, Chicago, IL, USA). Treatment effects were evaluated using a one-way ANOVA followed by Bonferroni multiple comparison test, and *p*-values less than 0.05 were considered significant.

## 3. Results

### 3.1. Effects of 5-HT and sarpogrelate on VSMCs proliferation

Figure 1A shows the 8-day time course of VSMC proliferation in the absence or presence of 5-HT. Exposure of VSMCs to 5-HT (100  $\mu$ M) significantly accelerated proliferation at days 5 and 8. Figure 1B shows the cell counts of VSMCs on day 8 after the addition of 5-HT (1, 10, or 100  $\mu$ M) with or without sarpogrelate (10  $\mu$ M). The addition of 5-HT resulted in a dose-dependent increase in cell number of VSMCs, and sarpogrelate suppressed the effect of 5-HT.

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

Reverse transcription PCR analysis showed that LR11 mRNA expression was enhanced dose-dependently by 5-HT at 1 to 100  $\mu$ M in VSMCs. In addition,

sarpogrelate at 10  $\mu$ M suppressed the effect of 5-HT (Figure 2).

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

Caspase activity in VSMCs exposed to 5-HT and/or 7KCHO was assessed by two methods.

Flow cytometric analysis was performed using VSMCs stained with FITC-conjugated anti-active caspase-3 monoclonal antibody. The histograms in Figure 3 compares the distribution of VSMCs. VSMCs exposed to 5-HT alone showed a slight leftward shift of the peak from control, which indicates a decrease in active caspase-3 expression [21]. In contrast, addition of 7KCHO alone caused an increase in active caspase-3 expression as indicated by a rightward shift of the histogram, and this effect of 7KCHO was suppressed by 5-HT (Figure 3B).

Next, a luminescent assay was performed to measure caspase-3/7 activities by the same protocol as the previous experiment. 5-HT administration did not change the caspase-3/7 activities in VSMCs. However, the addition of 7KCHO alone increased caspase-3/7 activity 9-fold compared to the control, and this effect of 7KCHO was suppressed by 5-HT (Figure 3C). Thus similar results were observed in both methods of evaluating caspase activity in VSMCs.

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

Analysis of DNA fragmentation using propidium iodide fluorescence was conducted to evaluate the apoptosis-inducing effect of 7KCHO on VSMCs incubated with or without 5-HT (Figure 4). Apoptotic rate was significantly



increased by the addition of 7KCHO (50  $\mu$ M) alone, but this effect of 7KCHO was abolished by the addition of 5-HT at 100  $\mu$ M.

#### 4. Discussion

In this study, 5-HT increased the proliferation of VSMCs, and the 5-HT-induced proliferation enhancement was abolished by concomitant addition of sarpogrelate. Furthermore, sarpogrelate also inhibited the 5-HT-induced increase in LR11 mRNA expression in VSMCs. On the other hand, 5-HT decreased the 7KCHO-induced VSMC apoptosis via caspase-3/7-dependent pathway.

5-HT is known to be a mitogen for VSMCs. However, there is so far no evidence regarding the effect of 5-HT on LR11 expression and apoptosis in VSMCs. The present report is the first to demonstrate 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 elucidated. The factors that modulate the migration of VSMCs from the media to the intima should be verified. LR11 expression is known to depend largely on the differentiation of VSMCs. The VSMCs with a contractile phenotype found in the media do not express LR11, whereas the VSMCs in an active synthetic state present in the intima express LR11 [22, 23]. In the present study, 5-HT increased the expression of LR11 mRNA in VSMCs, and sarpogrelate abolished the effect of 5-HT. These results suggest that 5-HT may contribute to neointimal hyperplasia by inducing not only proliferation but also migration of VSMCs through up-regulating LR11 expression. Consequently, sarpogrelate may

exert its pleiotropic vascular effect partially by inhibiting LR11 expression in VSMCs.

Apoptosis of VSMCs observed in atherosclerotic lesion may be related to plaque rupture [24, 25]. In the present study, the results of flow cytometric analysis and luminescent assay indicated that 7KCHO activated the caspase-3/7-dependent apoptotic pathway. In atherosclerotic plaque, 7KCHO is considered to cause the disappearance of VSMCs, which render plaque unstable leading to rupture. On the other hand, 5-HT inhibited 7KCHO-induced VSMC apoptosis. These results suggest that 5-HT may prevent plaque rupture through reducing 7KCHO-induced VSMC apoptosis that is involved in increasing plaque vulnerability.

Whether or not 5-HT is beneficial for the vascular remodeling process is probably a controversial issue. In the setting of vascular injury, endothelial damage and subsequent platelet activation may lead to increased plasma 5-HT concentration. 5-HT induces the contraction, migration and proliferation of VSMCs via the 5-HT<sub>2A</sub> receptor followed by various intracellular signal transduction mechanisms [26-28]. Thus 5-HT initiates the vascular repair process, which results in neointimal hyperplasia and deterioration of peripheral blood flow. Note that exposure of VSMCs to 5-HT seemed to enhance the activities required for cell migration mediated by LR11 up-regulation in this study. Furthermore, the inhibitory effect of 5-HT on VSMC apoptosis might contribute to prevent plaque vulnerability induced by 7KCHO. These effects of 5-HT may be considered vascular protective effects.

However, it remains unclear whether the changes in the differentiation stage of VSMCs accompanied by LR11 up-regulation directly contribute to the suppression

of the apoptosis-inducing effect of 7KCHO. Further elucidation of the cause-effect relationship between LR11 expression and VSMC apoptosis should be elucidated.

In conclusion, we demonstrated that 5-HT up-regulated LR11 mRNA expression in VSMCs, and the effect was abolished by sarpogrelate. Additionally, the apoptosis-inducing effect of 7KCHO on VSMCs was reduced by 5-HT. These findings provide new insights on the changes in the differentiation stage of VSMCs mediated by 5-HT.

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### **Declaration of Conflicting Interests**

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Figure Captions

**Fig. 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 ( $1 \times 10^4$ /well, in triplicate) and culturing for 72 h, 5-HT (100  $\mu$ M) was added. Cell number was counted from days 0 to day 8 after the addition of 5-HT. Data are presented as mean  $\pm$  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 ( $1 \times 10^4$ /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  $\pm$  SD of triplicate samples. \* $p < 0.05$ , \*\* $p < 0.01$ ; one-way ANOVA followed by Bonferroni multiple comparison test.

**Fig. 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 ( $6 \times 10^4$ /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 show LR11 mRNA expression determined by reverse transcription PCR. Beta-actin expression shown in the lower panel was used as internal standard.

**Fig. 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 anti-active caspase antibody and flow cytometry. After seeding VSMCs in 6-well microplates ( $8 \times 10^4$ /well, in duplicate) and culturing for 48 h, VSMCs were incubated with no addition, addition of 5-HT (100  $\mu$ M) alone, or addition of 5-HT (100  $\mu$ M) and 7KCHO (50  $\mu$ M) 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<sub>2</sub>. 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 ( $1 \times 10^5$ /well, in triplicate), and incubated with or without the addition of 5-HT (100  $\mu$ M) and/or 7KCHO (50  $\mu$ M) for 48 h. Luciferase activity was measured according to the protocol from Promega. Data are presented as mean  $\pm$  SD of triplicate samples. \* $p < 0.01$ ; one-way ANOVA followed by Bonferroni multiple comparison test.

**Fig. 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 ( $8 \times 10^4$ /well, in duplicate) and culturing for 48 h, VSMCs were incubated with or without the addition of 5-HT (100  $\mu$ M) for another 96 h. 7KCHO (50  $\mu$ M) was added to some wells 72 h after the addition of 5-HT. Cells were stained with 50  $\mu$ g/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  $\pm$  SD of 3 independent experiments. \* $p$ <0.01; one-way ANOVA followed by Bonferroni multiple comparison test.

Figure  
Figure 1A

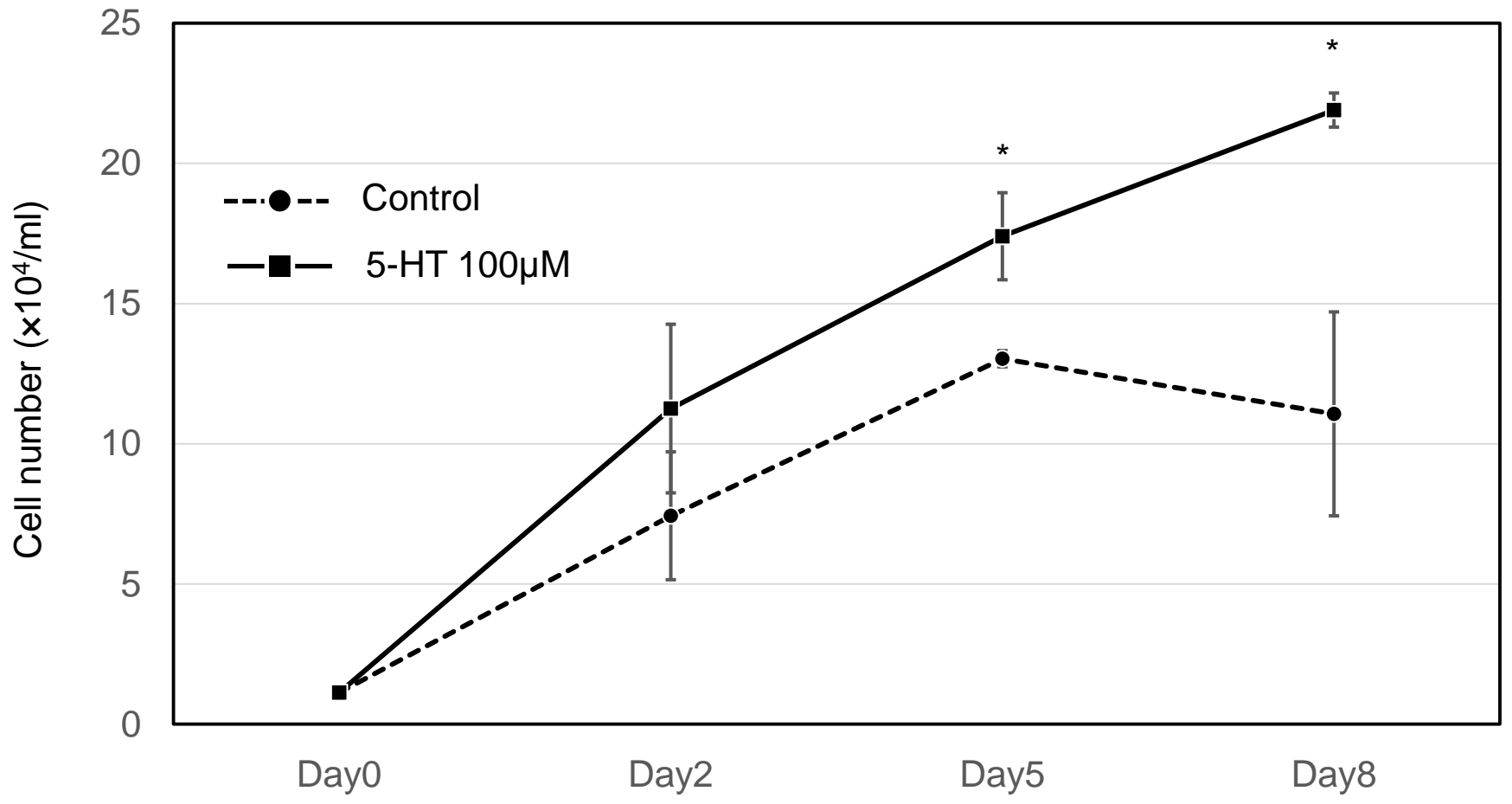


Figure 1B

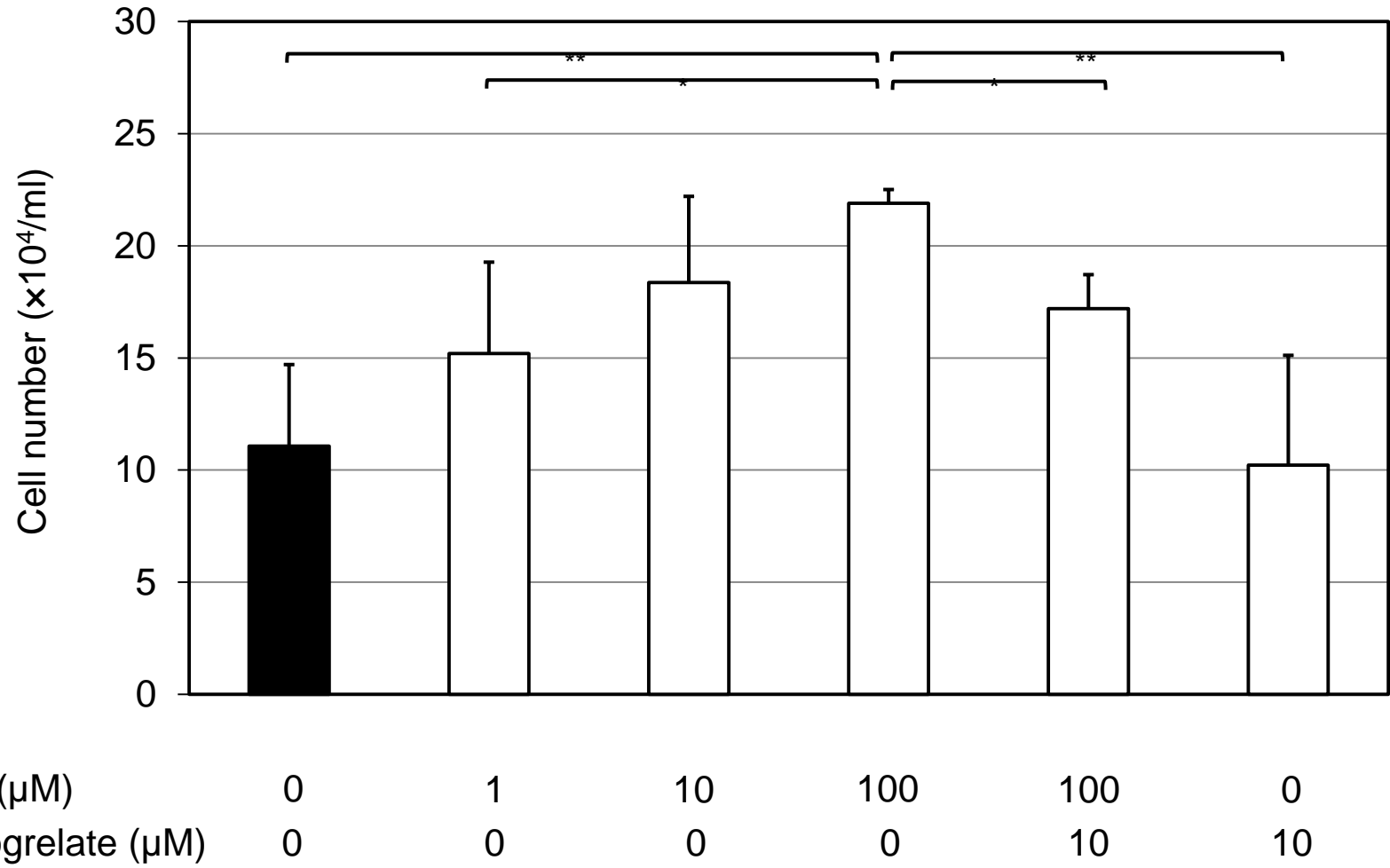


Figure 2

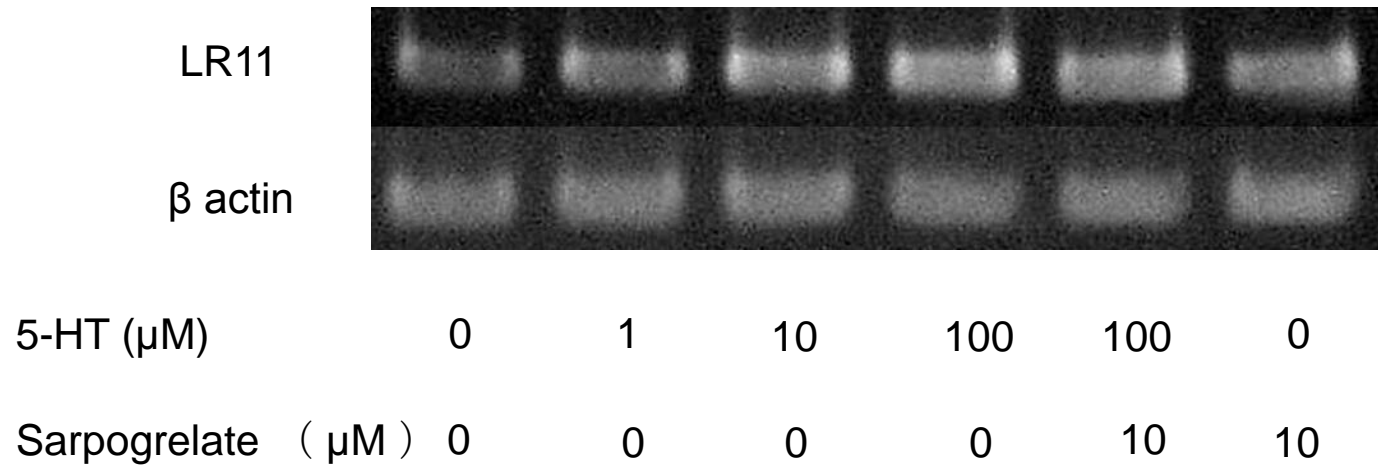


Figure 3A

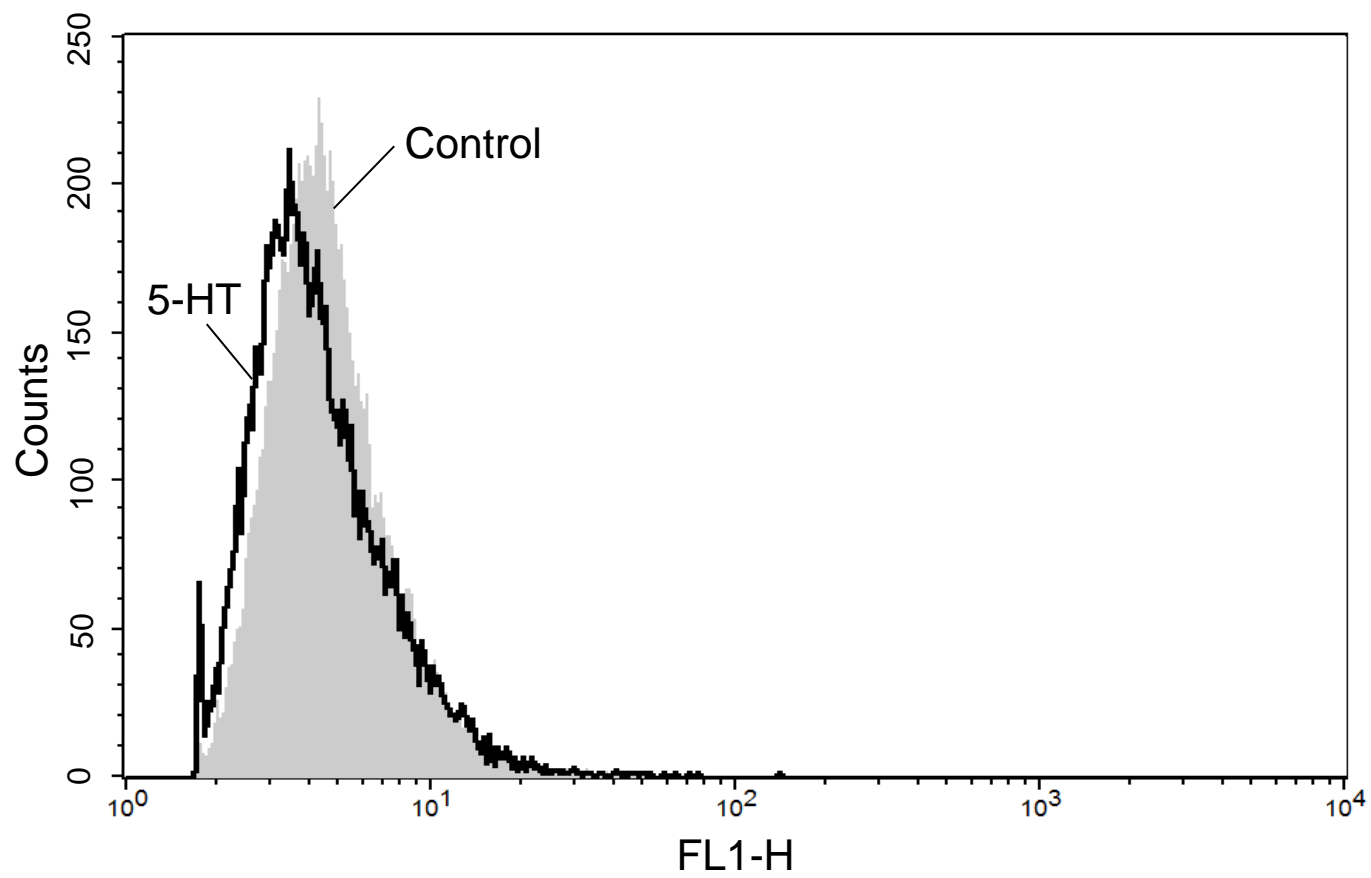


Figure 3B

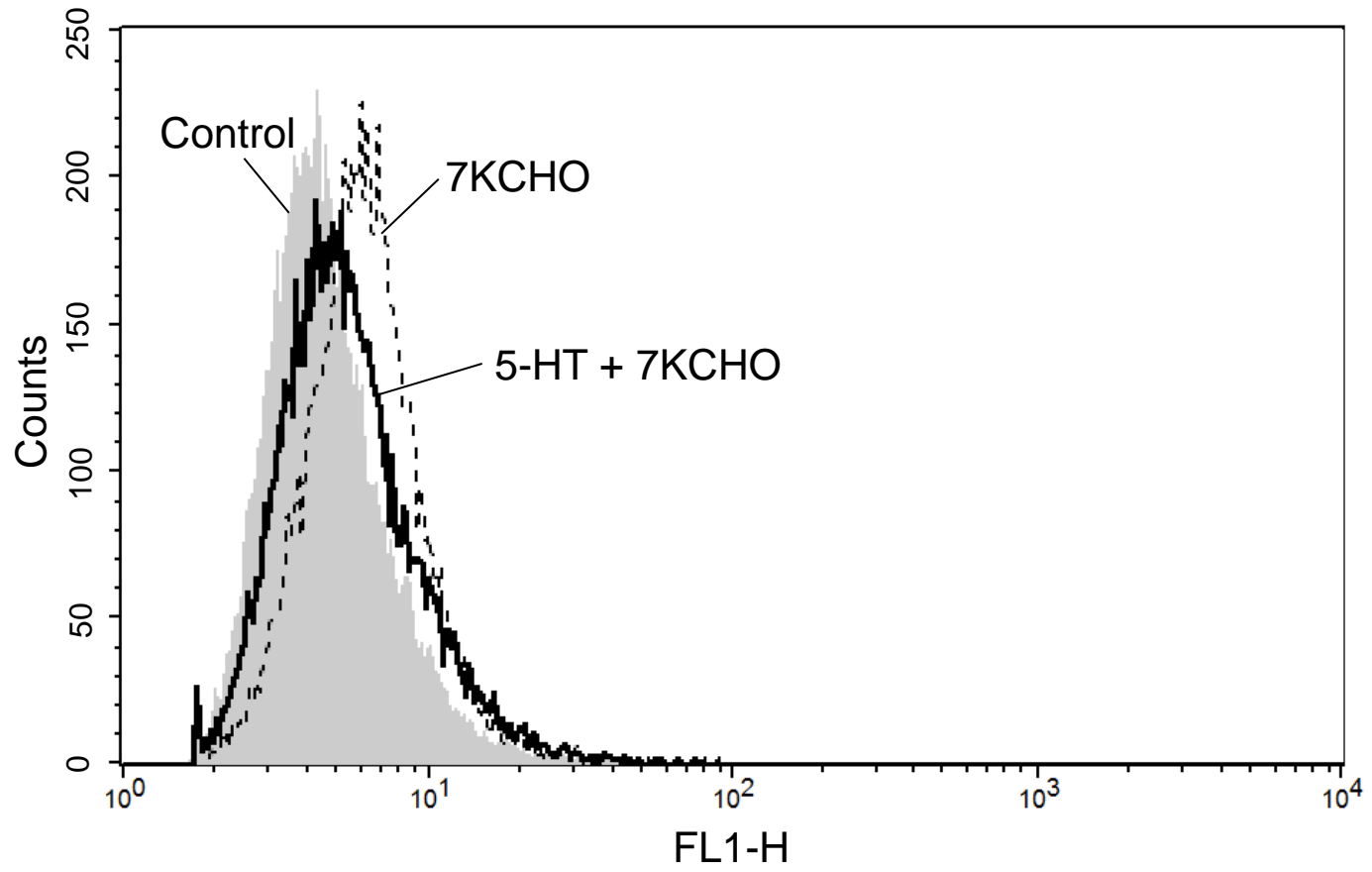


Figure 3C

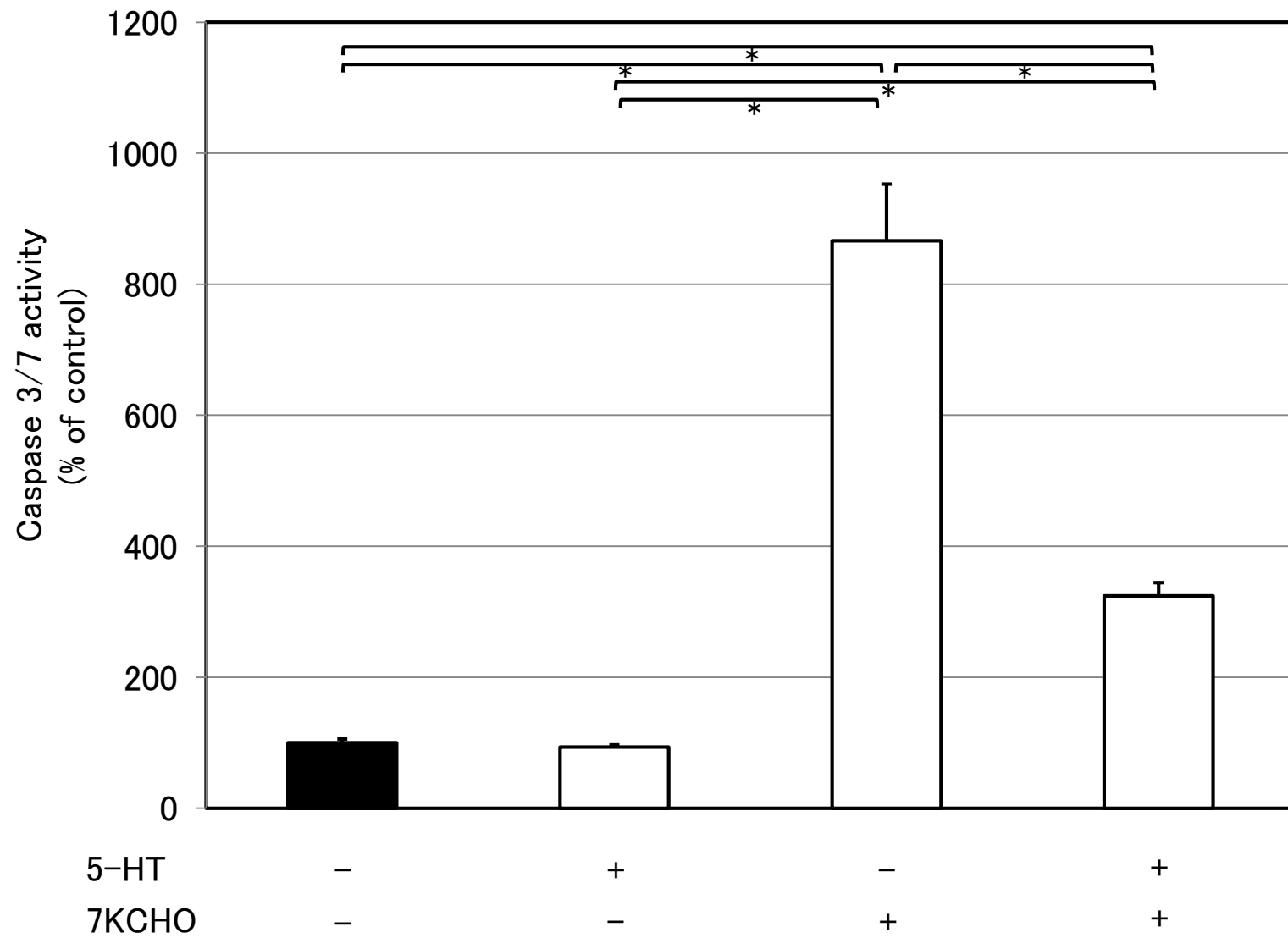




Figure 4

