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

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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-HT2A 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.

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-HT2A 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.

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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 5–10% (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_2.

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 μg of reverse transcribed total RNA. Expression of the housekeeping gene β-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 β-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 β-actin. Denaturation, annealing, and extension were performed at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, 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 fluoresein-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 ± 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

Fig. 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 μM) significantly accelerated proliferation at days 5 and 8. Fig. 1B shows the cell counts of VSMCs on day 8 after the addition of 5-HT (1, 10, or 100 μM) with or without sarpogrelate (10 μM). 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 1–100 μM in VSMCs. In addition, sarpogrelate at 10 μM suppressed the effect of 5-HT (Fig. 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 Fig. 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 (Fig. 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 (Fig. 3C). Thus similar results were observed in both methods of evaluating caspase activity in VSMCs.