Biochemical and Biophysical Research Communications 446 (2014) 906–910

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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 [a], Noriko Ishihara [a], Hideaki Bujo [b], Kohji Shirai [c], Ichiro Tatsuno [a,*]

[a] Center of Diabetes, Endocrinology and Metabolism, Toho University, Sakura Medical Center, 564-1, Shimoshizu, Sakura-City, Chiba 285-8741, Japan
[b] Department of Clinical Laboratory Medicine, Toho University, Sakura Medical Center, 564-1, Shimoshizu, Sakura-City, Chiba 285-8741, Japan
[c] Department of Vascular Function, Toho University, Sakura Medical Center, 564-1, Shimoshizu, Sakura-City, Chiba 285-8741, Japan

ARTICLE INFO

Article history:
Received 5 March 2014
Available online 17 March 2014

Keywords:
Serotonin (5-HT)
Vascular smooth muscle cells
LR11
Apoptosis

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.

* Corresponding author. Fax: +81 43 489 9770.
E-mail address: ichiro.tatsuno@med.toho-u.ac.jp (I. Tatsuno).

http://dx.doi.org/10.1016/j.bbrc.2014.03.031
0006-291X/

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.

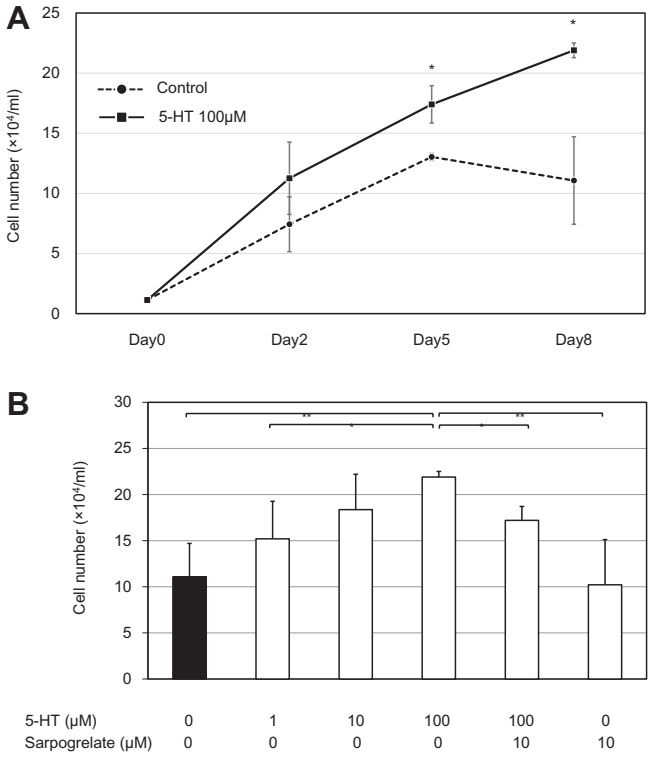

Fig. 1. Effects of serotonin (5-HT) and sarpogrelate on proliferation of vascular smooth muscle cells (VSMCs). (A) Changes in cell number over time. After seeding VSMCs in 12-well microplates (1×10^4/well, in triplicate) and culturing for 72 h, 5-HT (100 μM) was added. Cell number was counted from days 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.

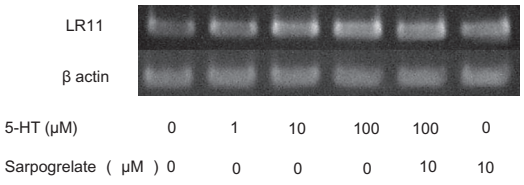

Fig. 2. Effects of serotonin (5-HT) and sarpogrelate on LR11 mRNA expression in vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (6×10^4/well, in triplicate) and culturing for 72 h, 5-HT and sarpogrelate at indicated concentrations were added and cultured for another 72 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.

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 (Fig. 4). Apoptotic rate was significantly increased by the addition of 7KCHO (50 μM) alone, but this effect of 7KCHO was abolished by the addition of 5-HT at 100 μM.

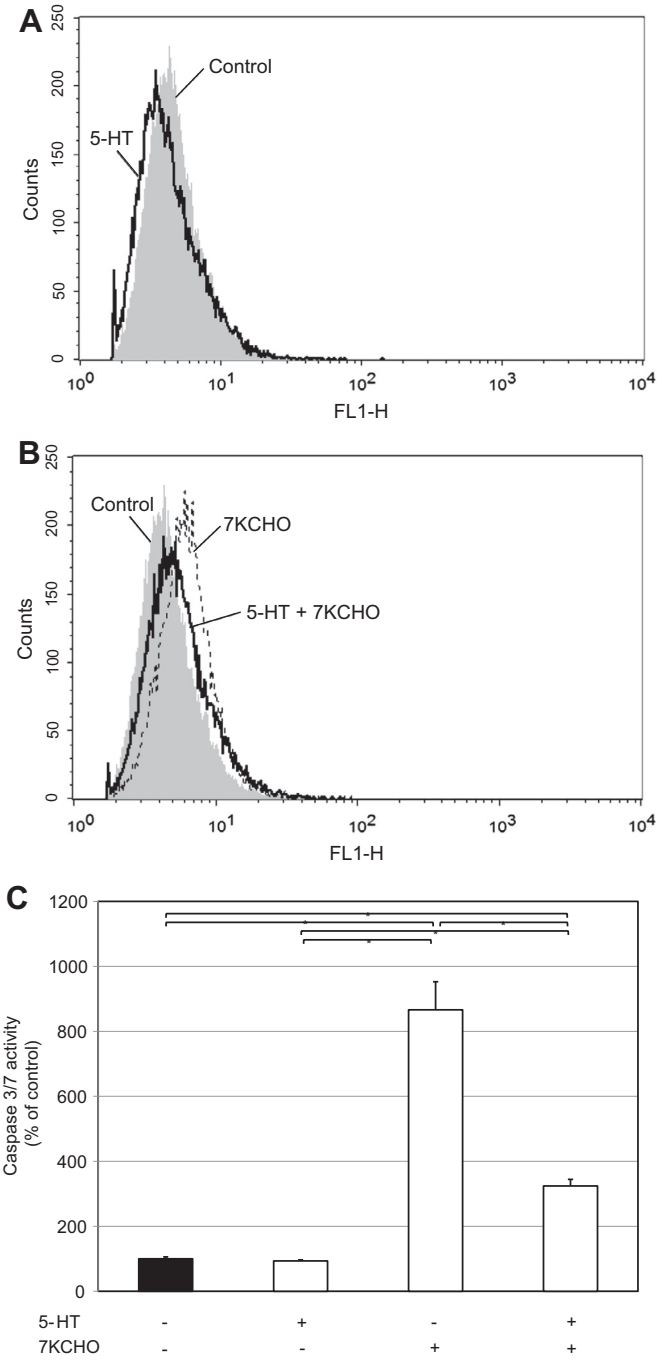

Fig. 3. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on caspase activity in vascular smooth muscle cells (VSMCs). (A and B) Caspase-3 activity assayed by anti-active caspase antibody and flow cytometry. After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing 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.

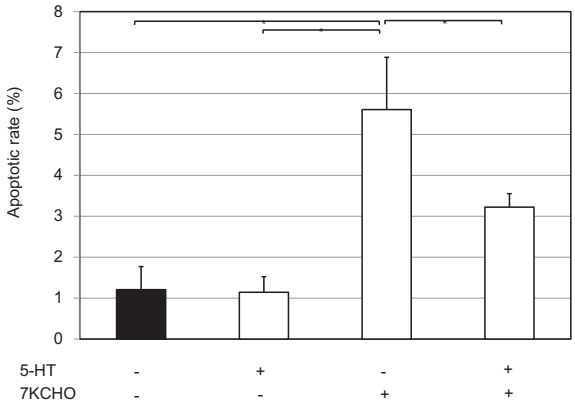

Fig. 4. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on quantitative analysis of apoptosis of vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing for 48 h, VSMCs were incubated with or without the addition of 5-HT (100 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 3 independent experiments. $^*p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

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-HI-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-HT2A 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.

Declaration of conflicting interests

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

Acknowledgment

We would like to thank all the staff members in our departments who contributed to this study.

References

[1] S.M. Schwartz, M.A. Reidy, E.R.M. O'Brien, et al., Assessment of factors important in atherosclerotic occlusion and restenosis, Thromb. Haemost. 74 (1995) 541–551.
[2] J.H. Campbell, G.R. Campbell, The role of smooth muscle cells in arteriosclerosis, Curr. Opin. Lipidol. 5 (1994) 323–330.
[3] Y. Zhu, H. Bujo, S. Yamazaki, et al., Enhanced expression of the LDL receptor family member LR11 increases migration of smooth muscle cells in vitro, Circulation 105 (2002) 1830–1836.
[4] A.W. Clowes, S.M. Schwartz, Significance of quiescent smooth muscle migration in the injured rat carotid artery, Circ. Res. 56 (1985) 139–145.
[5] R. Ross, The pathogenesis of atherosclerosis – an update, N. Engl. J. Med. 314 (1986) 488–500.
[6] N. Koyama, N. Morisaki, Y. Saito, S. Yoshida, Regulatory effects of platelet-derived growth factor-AA homodimer on migration of vascular smooth muscle cells, J. Biol. Chem. 267 (1992) 22806–22812.
[7] M.J. Davies, P.D. Richardson, N. Woolf, et al., Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content, Br. Heart J. 69 (1993) 377–381.
[8] M.J. Davies, Stability and instability: two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995, Circulation 94 (1996) 2013–2020.
[9] M.J. Davies, The composition of coronary-artery plaques, N. Engl. J. Med. 336 (1997) 1312–1314.
[10] Y. Miyashita, K. Shirai, Y. Ito, et al., Cytotoxicity of some oxysterols on human vascular smooth muscle cells was mediated by apoptosis, J. Atheroscler. Thromb. 4 (1997) 73–78.
[11] Y. Urano, K. Shirai, H. Watanabe, Y. Miyashita, et al., Vascular smooth muscle cell outgrowth, proliferation, and apoptosis in young and old rats, Atherosclerosis 146 (1999) 101–105.
[12] T. Oyama, Y. Miyashita, K. Kinoshita, et al., Effect of deposited lipids in atheromatous lesions on the migration of vascular smooth muscle cells, J. Atheroscler. Thromb. 9 (2001) 109–113.
[13] A. Linares, S. Perales, R.J. Palomino-Morales, et al., Nutritional control, gene regulation, and transformation of vascular smooth muscle cells in atherosclerosis, Cardiovasc. Hematol. Disord.: Drug Targets 6 (2006) 151–168.
[14] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, Nature 362 (1993) 801–809.
[15] M.F. Katz, H.W. Farber, Z. Dodds-Stitt, et al., 5-HT stimulated aortic endothelial cells secrete a novel T lymphocyte chemotactic and growth factor, J. Leukoc. Biol. 55 (1994) 567–573.

[16] T. Nagatomo, M. Rashid, H. Abul Muntasir, et al., Functions of 5-HT2A receptor and its antagonists in the cardiovascular system, Pharmacol. Ther. 104 (2004) 59–81.
[17] D. Hoyer, D.E. Clarke, J.R. Fozard, et al., International union of pharmacology classification of receptors for 5-hydroxytryptamine (5-HT), Pharmacol. Rev. 46 (1994) 157–203.
[18] G.M. Nemecek, S.R. Coughlin, D.A. Handley, et al., Stimulation of aortic smooth muscle cell mitogenesis by 5-HT, Proc. Natl. Acad. Sci. USA 83 (1986) 674–678.
[19] M. Fukui, M. Tanaka, H. Toda, et al., High plasma 5-hydroxyindole-3-acetic acid concentrations in subjects with metabolic syndrome, Diabetes Care 35 (2012) 163–167.
[20] D. Nagayama, M. Ohira, A. Saiki, et al., Sarpogrelate hydrochloride decreases cardio-ankle vascular index accompanied by increased serum lipoprotein lipase mass in type 2 diabetic patients, Int. Heart J. (2014) [Epub ahead of print].
[21] C.G. Yedjou, P.B. Tchounwou, In vitro assessment of oxidative stress and apoptotic mechanisms of garlic extract in the treatment of acute promyelocytic leukemia, J. Cancer Sci. Ther. 3 (2012) 6.
[22] M. Jiang, H. Bujo, Y. Zhu, et al., Pitavastatin attenuates the PDGF-induced LR11/uPA receptor-mediated migration of smooth muscle cells, Biochem. Biophys. Res. Commun. 348 (2006) 1367–1377.
[23] K. Ohwaki, H. Bujo, M. Jiang, et al., A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates formation of lipid-laden macrophages, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1050–1056.
[24] W. Martinet, D.M. Schrijvers, J.P. Timmermans, et al., Interactions between cell death induced by statins and 7-ketocholesterol in rabbit aorta smooth muscle cells, Br. J. Pharmacol. 154 (2008) 1236–1246.
[25] G. Palladini, G. Finardi, G. Bellomo, Disruption of actin microfilament organization by cholesterol oxides in 73/73 endothelial cells, Exp. Cell Res. 223 (1996) 72–82.
[26] T. Watanabe, R. Pakala, T. Katagiri, et al., Lipid peroxidation product 4-hydroxy-2-nonenal acts synergistically with serotonin in inducing vascular smooth muscle cell proliferation, Atherosclerosis 155 (2001) 37–44.
[27] K. Tamura, T. Kanzaki, Y. Saito, et al., Serotonin (5-hydroxytryptamine, 5-HT) enhances migration of rat aortic smooth muscle cells through 5-HT2 receptors, Atherosclerosis 132 (1997) 139–143.
[28] A. Banes, J.A. Florian, S.W. Watts, Mechanisms of 5-hydroxytryptamine (2A) receptor activation of the mitogen-activated protein kinase pathway in vascular smooth muscle, J. Pharmacol. Exp. Ther. 291 (1999) 1179–1187.