5-Hydroxytryptamine 2A receptor signaling cascade modulates adiponectin and plasminogen activator inhibitor 1 expression in adipose tissue

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Abstract Knowledge of the regulatory factors associated with down-regulation of adiponectin gene expression and up-regulation of PAI-1 gene expression is crucial to understand the pathophysiological basis of obesity and metabolic diseases, and could establish new treatment strategies for these conditions. We showed that expression of 5-HT\textsubscript{2A} receptors was up-regulated in hypertrophic 3T3-L1 adipocytes, which exhibited decreased expression of adiponectin and increased expression of PAI-1. 5-HT\textsubscript{2A} receptor antagonists and suppression of 5-HT\textsubscript{2A} receptor gene expression enhanced adiponectin expression. Activation of Gq negatively regulated adiponectin expression, and inhibition of mitogen-activated protein kinase reversed the Gq-induced effect. Moreover, the 5-HT\textsubscript{2A} receptor blockade reduced PAI-1 expression. These findings indicate that antagonism of 5-HT\textsubscript{2A} receptors in adipocytes could improve the obesity-linked decrease in adiponectin expression and increases in PAI-1 expression.

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1. Introduction

The number of overweight individuals worldwide has reached 2.1 billion, leading to an explosion of obesity-related health problems associated with a high mortality rate. Obesity is considered central to the metabolic syndrome and is associated with increases in the risk of an array of diseases, including insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, cardiovascular disease, degenerative disorders, and some cancers [1–4].

Adipose tissue participates in the regulation of energy homeostasis, immune responses, and hemostasis as an important endocrine organ that secretes adipokines [5,6]. Adiponectin is an antidiabetic and antiatherogenic adipokine [7–11]. In obesity, hypertrophic adipocytes decrease their expression and secretion of adiponectin and increase insulin-resistance causing hormones, and lead to insulin resistance and diabetes [12–14].

Plasminogen activator inhibitor (PAI)-1 plays important roles in the pathogenesis of cardiovascular events, promoting both thrombosis and fibrosis [15]. Human adipose tissue contributes to the elevation of plasma PAI-1 concentrations observed in obese subjects [16]. Knowledge of the regulatory factors associated with down-regulation of adiponectin gene expression and up-regulation of PAI-1 gene expression is crucial for understanding the pathophysiological basis of obesity and metabolic diseases and could establish new treatment strategies for these conditions.

Sarpogrelate is a selective antagonist of 5-HT\textsubscript{2A} receptors, widely used for patients with arteriosclerosis obliterans (ASO) because of its vasodilating and antiplatelet effects [17]. Sarpogrelate has recently been reported to increase plasma concentrations of adiponectin in diabetic patients with ASO by 2.6-fold at 3 months [18]. Sarpogrelate also increased circulating adiponectin by 2.5-fold in patients with type 2 diabetes who had elevated soluble E-selectin concentrations, platelet activation, and endothelial injury markers [19]. Sarpogrelate increases adiponectin concentrations in non-diabetic and non-medicated diabetic patients with peripheral artery disease [20]. The 5-HT\textsubscript{2A} receptor has been described as involved in 5-HT-induced platelet activation and contraction of vascular smooth muscle [21]. Neither the distribution of 5-HT receptor subtypes in white adipose tissue nor the function of the 5-HT signaling cascade in adipocytes has been determined.

It has been reported that sarpogrelate decreases serotonin-induced PAI-1 mRNA expression in vascular endothelial cells [22].

To verify the hypothesis that the expressions of adiponectin and PAI-1 are modulated by 5-HT receptor stimuli, we examined the expression of 5-HT receptor subtypes in the 3T3-L1 preadipocyte, 3T3-L1 small adipocytes (Day 10 after induction of adipocyte differentiation) and hypertrophic 3T3-L1 adipocytes (Day 17 after induction). Expression of 5-HT\textsubscript{2A} receptor mRNA was increased in hypertrophic adipocytes and in mesenteric adipose tissue of diabetic-obese mice, db/db mice, which exhibit decreased expression of adiponectin and...
increased expression of PAI-1. Moreover, we clarified the involvement of the 5-HT_{2A} receptor signaling cascade via mitogen-activated protein kinase (MAPK)-dependent pathways in the regulation of adiponectin and PAI-1 expression.

2. Materials and methods

2.1. Chemicals
Sarpogrelate was synthesized at Mitsubishi Tanabe Corporation. Ketanserin, isobutylmethylxanthine, and dexamethasone, GW9662 were purchased from Sigma. Spiperone and PNU22394 were from TOCRIS. Pasteurella Multocida Toxin (rPMT), Ro31-8220, Go6850, Go6983 and PD98059 were from Merck Calbiochem.

2.2. Cell culture, triglyceride content of adipocytes and glucose uptake
3T3-L1 cells (ATCC) were cultured on gelatin-coated plates in Dulbecco Modified Eagle Medium containing 10% fetal bovine serum (basal medium). Induction of differentiation into adipocyte phenotypes was performed by treating confluent cells with 0.5 mM isobutyl-methylxanthine, 0.25 μM dexamethasone, and 5 μg/ml insulin in basal medium for 2 days, followed by treatment with 5 μg/ml insulin in basal medium for 2 days. The cells were then maintained in basal medium. Culture of cells on gelatin-coated plates allowed for long-term culture of adipocytes. Culture of 3T3-L1 adipocytes for 35 days after induction of differentiation was possible. Cellular lipid accumulation on Day 10 and Day 17 was determined by oil red 0 staining. Magnification of microscope is 400×. Triglycerides were extracted with hexane/2-propanol (3:2, v/v). Triglyceride content resolved in 2-propanol was determined using determiner LTGII (KYOWA MEDEX Co. Ltd, Tokyo). Cells were solubilized with 0.05 N NaOH to determine protein concentrations using the Bradford method. Glucose uptake was determined as follows. Cells were washed three times with PBS, and were serum-deprived with serum-free DMEM for 2–4 h. After the incubation with PBS containing 0.1% BSA with or without 100 nM insulin for 20 min, 0.3 μCi of [3H]2-deoxy-glucose was added, then incubated for 10 min. Transport reaction was terminated with two washes of ice-cold 0.3 mM phloretin. Insulin responses were shown in % to basal glucose uptake.

2.3. Suppression of gene expression and RNA analysis
5-HT_{2A} modulators were incubated in basal medium for 24 h on Day 10 or Day 17. siRNA constructs were designed by DHARMA-CON. Co. siRNA of pGL2, negative control for siRNA, or siRNA of 5-HT_{2A} receptor were transfected into 3T3-L1 adipocytes for 24 h using Express-si Delivery (Genospectra Inc.). Sarpogrelate was added to the basal medium for an additional 24 h. GW9662 were pre-incubated in basal medium for 24 h before co-incubation of GW9662 and 5-HT_{2A} antagonists for additional 24 h. Total RNA was extracted using the 6100 system (Applied BioSystems Co.). Quantitative analysis of adiponectin and PAI-1 by real-time PCR with cDNA was on an ABI 7500 system. The primer sets and probes for adiponectin were
as follows: for adiponectin: forward primer 5'-TGCCGAAGATGACGGTACTACAA-3', reverse primer, 5'-CATCCAACCTGCAAAATC-3' and the probe 5'-CGCTTTGGTCCCTCCACC-3'. For 36B4: forward primer 5'-CCCTGAAGTGCTCGACATCA-3', reverse primer, 5'-TGGGACACCCCTCAGAA-3' and the probe 5'-AGACGAGGCGTCCACTCTCCG-3'. The primers and probes for 5-HT receptor subtypes and PAI-1 were from Applied Biosystems. The relative abundance of transcripts was normalized to constitutive expression of 36B4.

2.4. Animals
All experiments in animal study were performed on 9–13 week-old male db/db mice and wild-type C57BL/6 mice (Charles River Laboratories, Japan, Inc.). The animal care and procedures of the experiments were approved by the Animal Care Committee of Mitsubishi Tanabe Pharma Corporation.

3. Results

3.1. Changes in expression levels of adiponectin and PAI-1 and lipid accumulation and insulin-dependent glucose uptake in adipocytes during adipocyte differentiation and hypertrophy
To clarify whether the expression of adiponectin and PAI-1 is modulated by 5-HT receptor stimuli in adipocytes, we first examined the expression of adiponectin and PAI-1 genes during differentiation and hypertrophy of 3T3-L1 adipocytes. Cellular lipid accumulation was greater on Day 17 (hypertrophic adipocytes) than on Day 10 (small adipocytes), and on Day 17 (Fig. 1A and D). Expression of adiponectin continued to increase until Day 10, and was reduced on Day 17 compared with on Day 10 (Fig. 1B). Expression of PAI-1 continued to increase during differentiation and was significantly elevated on Day 17 compared with on Day 10 (Fig. 1C). Insulin-dependent glucose uptake was most active on Day 10 and was decreased on Day 17 compared with on Day 10 (Fig. 1E). The triglyceride content of the cells continued to rise after the induction of differentiation (Fig. 1D), appeared to correlate inversely with expression of the adiponectin gene and insulin-dependent glucose uptake between Days 10 and 17, and was proportional to expression of the PAI-1 gene and triglyceride accumulation between Days 10 and 17. To determine whether the 5-HT signaling cascade is involved in the regulation of adiponectin and PAI-1, adipocytes on Day 10 were used as adiponectin-abundant cells. Adipocytes on Day 17 were used as hypertrophic cells to examine the effects of 5-HT receptor antagonism on down-regulated adiponectin.

3.2. Expression of 5-HT receptor subtypes in 3T3-L1 preadipocytes, adipocytes and mesenteric adipose tissue of obese-diabetic mice
To address the possibility that the 5-HT signaling cascade regulates adiponectin and PAI-1 mRNA, we studied the expression of 5-HT receptors in 3T3-L1 preadipocytes before induction of differentiation (Day 0) and during differentiation. Expression of 5-HT2A receptors increased during differentiation and remained high. It was significantly elevated in Day 17 hypertrophic adipocytes compared with in Day 10 (Fig. 2A). Expression of 5-HT1A,1B, and 1D receptors was abundant in preadipocytes but decreased after induction of differentiation (Fig. 2B–D). Expression of all other 5-HT1A,1B, and 1D receptors did not differ between Day 10 and Day 17 (Fig. 2B and C). Expression of all other 5-HT2B,2C, 4,5A,5B, and 7 receptors we examined was undetectable at Day 0, Day 10, and Day 17. Among the active 5-HT receptors (1A, 1B, 1D, and 2A), the 5-HT2A receptor was more abundant in hypertrophic adipocytes that exhibited lower adiponectin expression.

Fig. 2. Changes gene expression of 5-HT2A receptor (A), 5-HT1A receptor (B), 5-HT1B receptor (C), and 5-HT1D receptor (D) during differentiation of 3T3-L1 adipocytes. Maximum values are presented as 100%. *P < 0.05; **P < 0.01 versus Day 10. n.s., not significant versus Day 10. Values are means ± S.E. (n = 6).
To bolster the significance of these in vitro findings, we examined the expression levels of 5-HT receptor subtypes, adiponectin and PAI-1 in mesenteric adipose tissue of db/db mice and wild-type C57BL/6 mice. The 5-HT2A receptor mRNA levels in the mesenteric adipose tissues of db/db mice were increased as compared with in lean control mice (Fig. 3A). In the mesenteric adipose tissues of db/db mice, adiponectin expression levels were decreased (Fig. 3B), and PAI-1 expression levels were increased (Fig. 3C) as compared with in lean control mice. Expression of the 5-HT1A and the 5-HT1B receptors appeared to have tendency to be decreased in db/db mice, but they did not differ significantly ($P = 0.25, 0.34$, respectively) (Fig. 3D). Expression levels of the 5-HT1D receptor did not differ between db/db mice and lean mice (Fig. 3D).

These findings, together with the observation that the 5-HT was indeed present at the concentration of 0.64 $\mu$M equivalent to human 5-HT in cell-culture serum, prompted us to attempt to examine the 5-HT2A receptor in relation to its involvement in adiponectin expression and PAI-1 expression.

3.3. 5-HT2A receptor signals modulate adiponectin expression in 3T3-L1 adipocytes

PNU22394, a 5-HT2A/2B partial agonist, reduced adiponectin gene expression (Fig. 4A), whereas sarpogrelate (Fig. 4B), spiperone (Fig. 4C), and ketanserin (Fig. 4D), 5-HT2A receptor antagonists, increased expression on Day 10. The effects of sarpogrelate on adiponectin mRNA were inhibited by suppression of 5-HT2A receptor gene expression with siRNA (Fig. 4E). Expression of adiponectin mRNA was increased by siRNA-5-HT2A (Fig. 4E). Expression of the 5-HT2A receptor was decreased by 70% by siRNA (data not shown). Sarpogrelate (Fig. 5A), spiperone (Fig. 5B), ketanserin (Fig. 5C), which are 5-HT2A antagonists, increased adiponectin expression on Day 17.

3.4. Downstream of activation of the 5-HT2A receptor in regulation of adiponectin expression

Adiponectin mRNA was reduced to 10% of control level by rPMT, a Gq activator (Fig. 6A), U-73122, a PLC inhibitor, Ro31-8220, Go6850, Go6983, which are PKC inhibitors, had
no significant effect on the Gq-activated decrease in adiponectin mRNA (data not shown). PD98059, a MAPK inhibitor, partially reversed the effects of rPMT (Fig. 6A). PNU22394, a 5-HT2A agonist, had no effect on adiponectin mRNA in hypertrophic 3T3-L1 adipocytes on Day 17 (Fig. 6B), at which point, adiponectin mRNA was down-regulated (Fig. 1B). Ro31-8220, Go6983, PKC inhibitors, did not increase adiponectin mRNA (data not shown). PD98059 significantly ameliorated down-regulation of adiponectin mRNA on Day 17 (Fig. 6B).

We examined adiponectin mRNA using the PPAR (peroxisome proliferator-activated receptor) gamma antagonist GW9662 to determine whether 5-HT2A antagonists can induce adiponectin in the absence of significant PPAR gamma activity in Day 17 adipocytes. Co-incubation of PPARgamma significantly reduced the effects of sarpogrelate, spiperone, and ketanserin, which are 5-HT2A antagonists, on adiponectin mRNA, at least partially, raising the possibility that the mechanism of 5-HT2A antagonists-induced adiponectin mRNA might be dependent on activity of PPARgamma, at least in part (Fig. 7A–C).

3.5. PAI-1 mRNA was decreased by sarpogrelate through 5-HT2A receptor via MAPK

Sarpogrelate and siRNA of the 5-HT2A receptor each reduced PAI-1 mRNA expression in 3T3-L1 adipocytes (Fig. 8A). The reduction by sarpogrelate was not shown in adipocytes transfected with siRNA of 5-HT2A receptor (Fig. 8A). PAI-1 mRNA was increased with rPMT, whereas PD98059 reversed the rPMT-induced augmentation (Fig. 8B). U-73122 and Ro31-8220 had no effect on the increase of PAI-1 mRNA in response to rPMT (data not shown).
4. Discussion

The adipocyte differentiation program is coordinated by several positive negative adipogenic molecules, including a variety of growth factors, cytokines and hormones [23].

We have reported that adiponectin has insulin-sensitizing actions and that obesity decreases adiponectin sensitivity, thereby leading to insulin resistance, which in turn aggravates hyperinsulinemia [24]. In this study, we focused on modulation of adiponectin and PAI-1 gene expression to explore the role of the 5-HT signaling cascade in obesity-linked changes in expression of these two proteins. Expression of PAI-1 was increased in hypertrophic 3T3-L1 adipocytes, which produced a decrease in adiponectin expression. These results are consistent with the adipocyte dysfunction shown in obesity and type 2 diabetes.

It is reported that sarpogrelate inhibits serotonin and collagen-induced phosphatidic acid formation in rat platelets (IC50 value is 2.8 \mu M, \textit{K}i value is 100 nM for rat 5-HT2 receptor) [17], and spiperone and ketanserin inhibit serotonin-induced inositol phosphate production in rat uterine smooth muscle cells at 1 \mu M (\textit{K}i values of spiperone and ketanserin for 5-HT2A receptor are 3.5 nM and 2 nM, respectively) [25,26]. Together with our results, it is possible that blockade of the 5-HT2A receptor could result in an increase in adiponectin expression. It is also possible that 5-HT2A antagonists have other targets in adipocytes.

The augmentation of adiponectin expression by sarpogrelate was inhibited by suppression of the 5-HT2A receptor gene using siRNA and suppression of this gene also increased adiponectin expression. In agreement with these findings, 5-HT2A stimulation by rPMT, which activates Gq protein coupled to the 5-HT2A receptor, decreased adiponectin expression. These findings indicate that the 5-HT2A receptor signaling cascade negatively regulates adiponectin expression. Moreover, expression of the 5-HT2A receptor was up-regulated in the adipose tissue of db/db mice and 3T3-L1 hypertrophic adipocytes, in which adiponectin expression was down-regulated and PAI-1 expression was up-regulated. These findings raised the possibility that the increase in 5-HT2A receptor expression in
hypertrophic adipocytes is at least partially responsible for the obesity-linked reduction in adiponectin expression.

Adiponectin receptor agonists and adiponectin sensitizers should serve as versatile treatment strategies for obesity-linked diseases such as diabetes and metabolic syndrome [27]. Long-lasting 5-HT2A receptor blockade might increase adiponectin expression down-regulated in obesity.

Transcriptional activity of PPARgamma which increases adiponectin levels has been reported to decrease by MAPK phosphorylation [28]. It has been reported that the 5-HT2A receptor stimulates MAPK in pulmonary artery fibroblasts which causes proliferative signals [29]. 5-HT2A receptor stimulation may decrease the expression of adiponectin through activation of MAPK in adipocytes. These important issues should be addressed in future studies.

The MAPK pathway activation has been reported to increase in PAI-1 gene expression in kidney [30]. The 5-HT2A receptor signaling cascade could modulate PAI-1 expression through MAPK pathway activation in adipocytes.

It has been reported that arrestin binding to GPCR enables MAPK activation [31]. Therefore, arrestin binding to the 5-HT2A receptor might be involved in the regulation of adiponectin and PAI-1 through MAPK.

In conclusion, the results of this study reveal that 5-HT2A receptor antagonism increases expression of adiponectin and decreases PAI-1 expression via the 5-HT2A receptor signaling cascade. Furthermore, obesity-linked up-regulation of 5-HT2A receptor expression could cause down-regulation of adiponectin expression. Antagonism of 5-HT2A receptors has the potential to protect against risk factors for and contribute to the treatment of cardiovascular diseases associated with metabolic syndrome as a result of obesity-related, aberrant adipocytokine metabolism.

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