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Serotonin 5-HT2B receptor antagonism is fundamental for protecting PC12 cells exposed to hydrogen peroxide

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Abstract

Objectives: According to our previous work, aripiprazole exerted a protective effect on hydrogen peroxide (H_2O_2)-treated PC12 cells; haloperidol did not. Because aripiprazole has distinct affinities to a set of neurotransmitter receptor subtypes, this study aimed to clarify which subtype is responsible for rescuing cells from 0.25 mM H_2O_2 exposure.

Methods: A set of compounds, which are more specific to each subset of G-protein coupled receptors, were examined for their ability to mimic the pharmacological effects of aripiprazole or haloperidol, including their K_i values. The viability of PC12 cells cultured with test compounds with or without H_2O_2 was assessed using WST-8 reagent.

Results: Results from *in vitro* studies using PC12 cells showed that agonism at serotonin 5-HT2C-receptors based on the antagonism against 5-HT2B-receptors played a significant role in resisting H_2O_2-induced cell death. However, the use of a specific 5-HT2B-receptor agonist instead of a 5-HT2B-receptor antagonist completely negated the effect of a specific 5-HT2C-receptor agonist. Furthermore, unlike the dopamine D1-receptor specific antagonist, none of the agonists of dopamine D2-, D3-, and D4-receptors ameliorated the cytopathic effects of H_2O_2.

Conclusion: Antagonism at 5-HT2B-receptors is fundamental for the protection of PC12 cells against the cytopathic effects caused by 0.25 mM H_2O_2. However, the role of negatively regulated cyclic adenosine monophosphate in this phenomenon requires further investigation.

Keywords: hydrogen peroxide, PC12 cells, aripiprazole, serotonin 5-HT2B-receptor, dopamine D1-receptor

Introduction

Aripiprazole is a dopamine receptor partial agonist antipsychotic (dopamine-serotonin system stabilizers)[1-3] with a lower serotonin 5-HT2A-receptor/dopamine D2-receptor affinity ratio.[4] A comprehensive and precise pharmacological profile of aripiprazole has already been provided for a large number of G protein-coupled receptors (GPCRs).[5,6] Aripiprazole has the highest affinity for 5-HT2B-, D2L-, and D3-receptors, but also has significant affinity (average K_i = 5-30 nM) for several other 5-HT-receptors (5-HT1A, 5-HT2A, 5-HT7), as well as adrenergic α1A- and histaminergic H1-receptors. It should be noted that aripiprazole possesses an inversely agonistic action against 5-HT2B-receptors. Aripiprazole has less affinity (average K_i = 30-200 nM) for other GPCRs, including the 5-HT1D-, 5-HT2C-, α1B-, α2A-, α2B-, α2C-, adrenergic β1- and β2-, and H3-receptors. Functionally, the modest 5-HT2A antagonism and 5-HT2C partial agonism, along with D2, D3, D4, and 5-HT1A partial agonism, of aripiprazole may result in its ability to stabilize the disturbed dopamine-serotonin levels, which leads to the moderate intervention observed in the diseases such as schizophrenia.[7] Clinically, aripiprazole provides an effective, more cost-effective, and well-tolerable treatment than other atypical antipsychotics.[8]

Oxidative stress, a state arising from an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanism, is implicated in the pathophysiology of schizophrenia.[9,10] Ideally, antipsychotic drugs should restore the balance between pro-oxidant and antioxidant cellular processes. However, some typical antipsychotic drugs including haloperidol cause or promote oxidative injury.[11-16] Contrary to haloperidol, aripiprazole has been shown to have antioxidant activity in both *in vitro* and *in vivo* studies.[17-19] Recently, we found that aripiprazole recovers the viability of PC12 cells exposed to hydrogen peroxide (H_2O_2); however, haloperidol does not.[20] We also found that aripiprazole does not increase ROS generation in PC12 cells; while haloperidol does.[21] As described above, unlike haloperidol, aripiprazole can act as an agonist or antagonist against neurotransmitter receptors. Therefore, the aim of the present study was to understand which neurotransmitter receptor subtype in PC12 cells was the main target of aripiprazole to maintain cell viability following H_2O_2 exposure. To perform this experiment, specific agonists/antagonists towards a large number of GPCRs were examined for their ability to mimic the effect of aripiprazole or haloperidol with respect to agonism/inverse agonism (or silent antagonism) and differences in K_i values. The first group compounds used in the experiment were chosen according

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to the differences in agonism/inverse agonism (or silent antagonism) between aripiprazole and haloperidol: *i.e.*, agonists for serotonin 5-HT2C-, dopamine D2-, D3-, and D4-receptors. The second group was chosen based on differences in K_i values between aripiprazole and haloperidol: *i.e.*, agonist for 5-HT1A-receptor; antagonists for 5-HT2A-, 5-HT2B-, D1-, D5-, α2A-, α2C-, and H1-receptors. Catecholaminergic PC12 cells originating from rat pheochromocytoma were used in this study as for our previous studies,[20-24] because these cells exhibit many of the functions observed in primary neuron cultures.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose (catalog no. D5796) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (lot no. 2B0360) and horse serum (lot no. 1C0610) were from JRH Biosciences (Lenexa, KS, USA). The following drugs were purchased from Tocris (Bristol, UK): xaliproden hydrochloride (a specific 5-HT1A-receptor agonist), BW723C86 hydrochloride (a specific 5-HT2B-receptor agonist), 1-methylpsilocin (a specific 5-HT2C-receptor agonist), SKF83566 and LE300 (specific D1-receptor antagonists), SCH39166 hydrobromide (a specific D1/D5-receptor antagonist), sumanirole maleate (a specific D2-receptor agonist), 7-hydroxy-PIPAT maleate (a specific D3-receptor agonist), Ro10-5824 dihydrochloride (a specific D4-receptor agonist), BRL44408 maleate (a specific α2A-adrenoceptor antagonist), JP1302 dihydrochloride (a specific α2C-adrenoceptor antagonist). Ritanserin (a specific 5-HT2A/2C-receptor antagonist), raclopride (a specific D2-receptor antagonist), and pyrilamine maleate (a specific H1-receptor antagonist) were purchased from Sigma-Aldrich. RS127445 (a specific 5-HT2B-receptor antagonist) was obtained from AdooQ BioScience (Irvine, CA, USA). WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt] was from Dojindo (Mashiki, Kumamoto, Japan).

Cell culture

The strain of PC12 cells used in this study, which was generously provided by Dr. Hiroshi Ichinose (Tokyo Institute of Technology), is known to release large amounts of dopamine and very scarce amount of noradrenaline.[25] There is no evidence of serotonin release.[25] Cells were cultured and maintained as monolayers in DMEM containing 25 mM glucose with 5% (v/v) fetal bovine serum and 10% (v/v) horse serum in a 5% CO_2 atmosphere at 37℃ as described previously.[24] The cells from four to eight passages were incubated with drugs in DMEM as described below.

Effects of drugs on PC12 cell viability in the presence of H_2O_2

The genes encoding the receptors examined in this study were identified using mRNA obtained from PC12 cells and RT-PCR except for the α2A-adrenoceptor (data not shown). However, the endogenous expression of the dopamine D2-, serotonin 5HT2A-, and histamine H1-receptors in PC12 cells has already been confirmed.[26-28] All test compounds except for BRL44408 were dissolved in dimethylsulfoxide (DMSO) to prepare 10, 20, or 30 mM stock solutions, and then further diluted to the desired concentration in DMEM containing 25 mM glucose, 5% (v/v) fetal bovine serum and 10% (v/v) horse serum. BRL44408 was dissolved in milli-Q water to be 10 or 20 mM, The volume of vehicle added to media was 0.5% (v/v). PC12 cells (5000 cells/well) cultured in 100 μL DMEM with 5% (v/v) fetal bovine serum and 10% (v/v) horse serum in a 96-well plate were incubated for 3 h with each compound (50 μM and 20 nM), with or without graded doses of H_2O_2 (0.25 and 0.05 mM). Next, 10 μL of WST-8 reagent was added to each well and the cells were transferred to a CO_2 incubator for culture. After 2-h incubation with WST-8 reagent, the absorbance of the samples was measured at 450 nm with an ARVO-X5 2030 Multilabel Reader (Perkin-Elmer Cetus, Norwalk, CT, USA). Simultaneously, a second assay was performed using a cell-free system to eliminate the possibility of any drug interaction with H_2O_2 affecting the results of the WST-8 reducing reaction. All figures for the WST-8 assay show the values after subtraction of corresponding values obtained from the cell-free system. The value for each group was calculated as the percentage of the value for vehicle-treated cells, which was designated as 100%.

Statistical analyses

All data are expressed as the mean ± SD. Cell viability data from the WST-8 assay were analyzed by two-way ANOVA. If ANOVA showed a significant overall effect, the Tukey-Kramer's honest significant difference (HSD) test was used to compare differences between groups, with the comparison-wise type 1 error rate (α) set at 0.05.

Results

Cell viability under H_2O_2 challenge

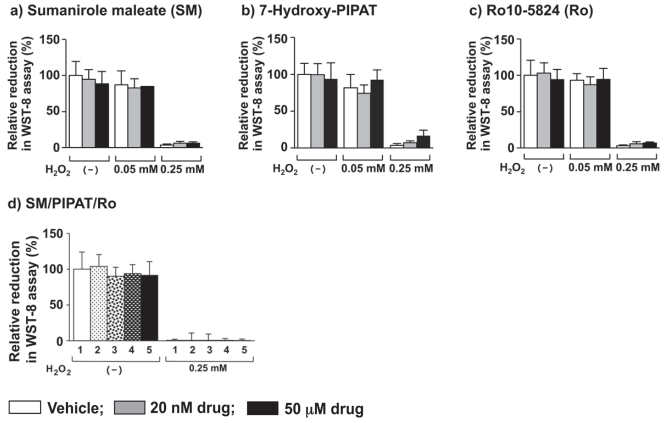
The results of the WST-8 reduction reaction in PC12 cells are shown in Figure 1, 2, 3, and 4. As shown in Figure 1, 50 μM sumanirole maleate, 7-hydroxy-PIPAT maleate, and Ro10-5824 dihydrochloride, all of which are D2 class receptor agonists, did not exert any enhancement of cellular metabolism; all combinations of these compounds (each a final concentration of 50 μM) were also ineffective in promoting cell viability. In contrast, 50 μM SKF83566 and LE300, both of which are D1 antagonists, and SCH39166 hydrobromide (D1/D5 antagonist) effectively inhibited the H_2O_2-induced cell death (Figure 2). However, 50 μM raclopride (D2 antagonist) did not show any protective effect.

The effects of 5-HT-receptor agonists/antagonists are shown in Figure 3. Xaliproden hydrochloride (50 μM), a 5-HT1A agonist, alone was the only compound that effectively reduced H_2O_2-induced cell death in PC12 cells; exclusive use of the single compounds (ritanserin [5-HT2A/2C antagonist], RS127445 [5-HT2B antagonist], BW723C86 hydrochloride [5-HT2B agonist], and 1-methylpsilocin [5-HT2C agonist]) was unable to inhibit H_2O_2-induced cell death. However, a combination of these compounds, *i.e.*, ritanserin and RS127445, xaliproden hydrochloride and 1-methylpsilocin, RS127445 and 1-methylpsilocin, at a final concentration of 50 μM each, induced a reduction in H_2O_2-induced cell death in PC12 cells. The combination of xaliproden hydrochloride and RS127445 did not show such an effect. The replacement of RS127445 with BW723C86 hydrochloride (5-HT2B agonist) completely negated the effect of RS127445.

The antagonists of α2A- and α2C-adrenoceptors and the histamine H1-receptor were examined, because the K_i values of aripiprazole and haloperidol differ markedly to those receptors (Figure 4). BRL44408 maleate (α2A antagonist) did not show any advantageous effect on inhibiting H_2O_2-induced cell death.

JP1302 dihydrochloride (50 μM), an α2C antagonist, markedly decreased the cell viability of PC12 cells, but showed no effects in reducing H_2O_2-induced cell death at all. However, the combination of BRL44408 maleate and JP1302 dihydrochloride at 50 μM each reduced H_2O_2-induced injury. Pyrilamine maleate (50 μM), H1 antagonist, did not exert any effect on H_2O_2-induced cell death (Figure 4).

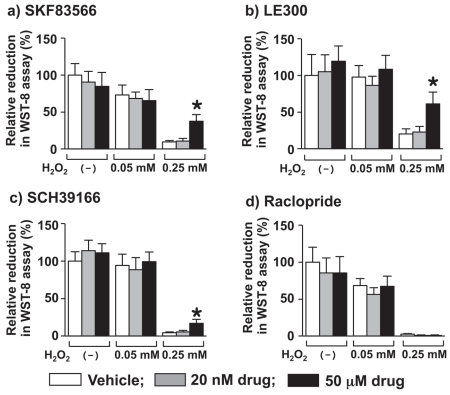
Figure 1



Dopamine D2-. D3-, and D4-receptor agonists did not exert any inhibitory effects against H_2O_2-induced cell death in PC12 cells. Cells were incubated with 50 μM sumanirole maleate (SM), 7-hydroxy-PIPAT maleate (PIPAT), or Ro10-5824 dihydrochloride (Ro) mixed with 0.25 mM or 0.05 mM H_2O_2 for 3 h, and then incubated for a further 2 h after addition of WST-8 reagent. 1, vehicle; 2, SM + PIPAT; 3, SM + Ro; 4, PIPAT + Ro; 5, SM + PIPAT + Ro. The absorbance of the samples was measured at 450 nm. Each group comprised of eight samples.

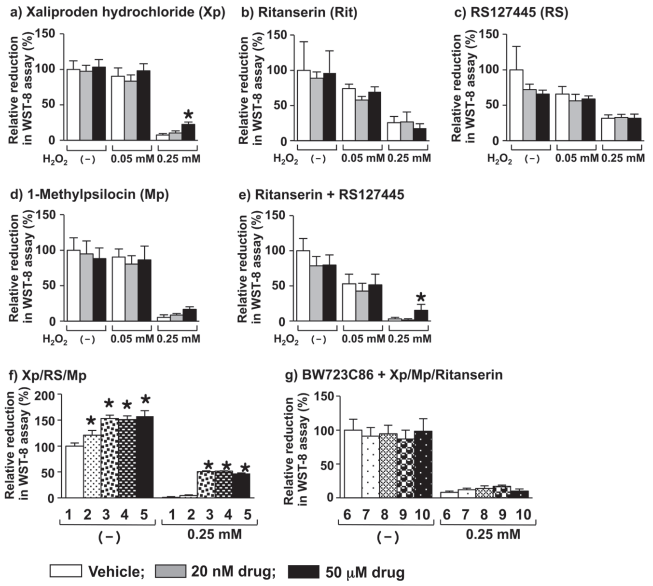
For all figures: Values were normalized to those of vehicle-treated samples (100% cell viability) and are shown as the mean (columns) ±SD (bars). Data were analyzed by Tukey-Kramer HSD test *post hoc*. Asterisks indicate significant difference ($p < 0.05$) between samples with or without drugs under 0.25 mM H_2O_2 challenge.

Figure 2



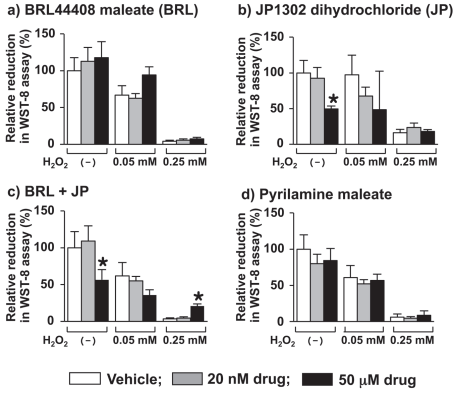
Dopamine D1- and D1/D5-receptor antagonists reduced H_2O_2-induced cell 351 death in PC12 cells. Cells were incubated with 50 μM SKF83566, LE300, SCH39166 hydrobromide, or raclopride mixed with 0.25 mM or 0.05 mM H_2O_2 for 3 h and assayed using WST-8 reagent. Each group comprised of eight samples.

Figure 3



A combination of 5-HT receptor agonists/antagonists partly mimicking the action of aripiprazole readily reduces H_2O_2-induced cell death in PC12 cells. Cells were incubated with 50 μM xaliproden hydrochloride (Xp), ritanserin (Rit), RS127445 (RS), BW723C86 (BW), 1-methylpsilocin (Mp), alone or in combination, and mixed with 0.25 mM H_2O_2 for 3 h prior to the WST-8 assay. The combination in panel f): 1, vehicle; 2, Xp + RS; 3, Xp + Mp; 4, RS + Mp; 5, Xp + RS + Mp. The combination in panel g): 6, vehicle; 7, BW alone; 8, BW + Xp; 9, BW + Mp; 10, BW + Rit. Each group comprised of eight samples.

Figure 4



A combination of α2A- and α2C-adorenoceptor antagonists reduces H2O2-induced cell death in PC12 cells. Cells were incubated with a combination of 50 μM BRL44408 maleate (BRL) and JP1302 dihydrochloride (JP) mixed with 0.25 mM or 0.05 mM H2O2 for 3 h and assayed using WST-8 reagent. Pyrilamine maleate was ineffective in reducing H2O2-induced cell death. Each group comprised of eight samples.

Discussion

In this study, we found that agonism at serotonin 5-HT2C-receptors based on the antagonism of 5-HT2B-receptors plays a substantial role in the pharmacological effects of aripiprazole on the protection of PC12 cells against H_2O_2-induced cell death. In addition, we revealed that the antagonism against the dopamine D1-receptor readily reduces H_2O_2-induced cell death. The combination of 5-HT1- and 5-HT2-receptor agonists/antagonists and the single use of dopamine D1-receptor antagonists were used to partly mimic the action of aripiprazole.

In our previous study, we showed that aripiprazole was the antipsychotic examined able to protect PC12 cells against H_2O_2-induced cell death.[20] Here, we reveal that the ability of aripiprazole to inhibit H_2O_2-induced PC12 cell death is because of agonism at serotonin 5-HT2C-receptors. In fact, the agonism of aripiprazole at serotonin 5-HT2C-receptors is distinguishable from other atypical and typical antipsychotics. Atypical antipsychotics, *i.e.*, clozapine, olanzapine, risperidone, and ziprasidone display potent inverse agonist activity at rat and human 5-HT2C-receptors; haloperidol was devoid of any inverse agonist activity at 5-HT2C-receptors.[29] Several drugs without significant antipsychotic actions such as ritanserin, ketanserin, and amitriptyline were potent inverse agonists at the 5-HT2C-receptors as shown with transfection experiments in HEK-293 cells.[30] However, contradictory reports against the main role of serotonin 5-HT2C agonism have been presented. Clozapine is another antipsychotic that displayed an anti-cytotoxic effect against H_2O_2 exposure.[20] The combination of ritanserin (5-HT2A/2C inverse agonist) and RS127445 (5-HT2B antagonist) ameliorated H_2O_2-induced cell death to a small but significant level (Figure 3, panel e). Conversely, antagonism at 5-HT2B-receptors may be crucial for PC12 cells to resist the cytotoxic effect of 0.25 mM H_2O_2. The idea of the importance of antagonism against 5-HT2B-receptors is reinforced by the observation that the use of BW723C86 hydrochloride (5-HT2B agonist) instead of RS127445 as the combination partner completely negates the additive effects of RS127445 to 1-methylpsilocin (5-HT2C agonist) or ritanserin (5-HT2A/2C antagonist, Figure 3, panel g). It should be noted that aripiprazole has highest affinity to 5-HT2B-receptors.[5,6]

Dopamine binds to and activates GPCRs that belong to two subclasses; the D1 receptor (D1- and D5-receptor) class and the D2 receptor (D2-, D3-, and D4-receptor) class. The D2 receptor class mediates the major part of its signaling and functions by coupling to $G_{i/o}$ proteins to negatively regulate cyclic adenosine monophosphate (cAMP) production; It is believed that the clinical efficacy of almost all antipsychotic drugs (including traditional and newer antipsychotics) directly correlates with dopamine D2-receptor binding affinity and the capacity to antagonize this receptor.[31-34] The D1 receptor class is coupled to the G_s protein, which subsequently activates adenylate cyclase, increasing the intracellular amount of cAMP. Our results show that none of the D2-receptor agonists induce any inhibitory effect against H_2O_2-induced PC12 cell death. This result was unexpected, because aripiprazole possesses agonistic action at dopamine D2 class receptors, which is strikingly different from those of typical and atypical antipsychotics.[1] Contrary to the agonism at the D2 receptor class, the antagonists against dopamine D1-receptors readily reduced H_2O_2-induced cell death in PC12 cells. This result may be because antagonism at dopamine D1-receptors decreases the intracellular amounts of cAMP. In contrast, aripiprazole is a potent partial agonist of 5-HT1A-receptors.[2,5] The agonistic action at 5-HT1A-receptors also activates $G_{i/o}$ proteins to inhibit cAMP production. Xaliproden hydrochloride (5-HT1A agonist) readily inhibited H_2O_2-induced cell death. Collectively, negatively regulated cAMP may be one of the protective mechanisms involved in inhibiting H_2O_2-induced cytotoxicity. However, there were no obvious changes in PC12 intracellular cAMP levels following exposure to 50 μM aripiprazole.[24] Therefore, we must be careful in attributing these protective observations to negatively regulated cAMP.

In our experimental scheme, the combination of BRL44408 maleate (α2A antagonist) and JP1302 dihydrochloride (α2C antagonist) at 50 μM each readily reduced H_2O_2-induced cell death. Because α_2-adrenoceptors are known to couple to $G_{i/o}$ proteins, it has been reported that the actions of α_2-adrenoceptors agonists may result in an inhibition of cAMP production.[35] Aripiprazole and clozapine are silent antagonists and do not interact potently with the human α2A- and α2C-adrenoceptors.[36] As for dopamine receptors, more precise experiments are also needed to fully clarify the roles of α2-adrenoceptors in resisting H_2O_2-induced cell death of PC12 cells.

Overall, the difficulty in interpreting the results presented in this paper can be attributed to the fact that both activators and inhibitors of cAMP generation can protect PC12 cells from H_2O_2 injury. Therefore, it may be necessary to interpret the data from another point of view. In our recent study,[20] aripiprazole increased NAD(P)H-quinone oxidoreductase-1 and heme oxygenase-1 in PC12 cells; haloperidol decreased the expressions of these two enzymes and the glutamate-cysteine ligase catalytic subunit. These enzymes are involved in detoxifying ROS and also replenishing NADPH. Therefore, although still speculative, serotonin 5-HT2B antagonism may be crucial in enhancing the expression of these enzymes.

In conclusion, this report is the first to show the importance of serotonin 5-HT2B-receptor antagonism in exerting an inhibitory effect against H_2O_2 injury. However, the precise machinery/mechanism involved requires further investigation. Therefore, our future studies will aim to delineate the protein(s) underlying this process.

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Conflict of interests

The authors have no conflict of interest to declare.

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