5-HT RECEPTOR SUBTYPES ACTIVATIONS MODULATE NEURONAL CELL DEATH INDUCED BY OXIDATIVE STRESS IN PRIMARILY CULTURED CEREBRAL CORTICAL NEURONS.

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Introduction

Oxygen-derived free radical generation has been implicated in the etiology of some neurodegenerative diseases and in neuronal death after acute injury such as ischemia-reperfusion or trauma1). In particular, superoxide anion ($O_2^{\cdot-}$), which has limited toxic effects in itself, can react with nitric oxide (NO) to form peroxynitrite anions, which are highly cytotoxic, or dismutate into hydrogen peroxide (H_2O_2), a reaction that is accelerated by superoxide dismutase. Generated reactive oxygen species (ROS) have been implicated as potential modulators of apoptosis.[3] Under pathological situations such as ischemia-reperfusion, various cell types including neurons produce large amounts of H_2O_2. Because of its high membrane permeability, H_2O_2 can be cytotoxic not only for the producing cell but also for neighboring cells. Therefore, *in vitro* H_2O_2 toxicity has become a well-established model for neurodegenerative disease. Exposure of cultured cortical neurons to H_2O_2 could induce neuronal death that proceeds via an apoptotic cell suicide pathway. The major of H_2O_2-induced neurotoxicity is mediated by the formation of hydroxyl radicals, which might be involved in the delayed accumulation of extracellular glutamate and NMDA receptor activation, followed by massive Ca^{2+} influx, which contribute to the activation of molecular mechanisms involved in apoptosis.[1]

5-HT is an important CNS neurotransmitter and the distribution of neurons containing 5-HT is very wide-spread. The cells occur in *raphe nuclei*, which project via the medial forebrain bundle, to many parts of cortex, hippocampus, basal ganglia, limbic system and hypothalamus.[5] 5-HT_3 receptor is a ligand gated cation channel that increases intracellular cation ions such as Na^{2+}, Ca^{2+}, K^+ by its activation. By previous reports, the action of $5HT_3$-receptor antagonist has been shown to prevent glutamate-mediated excitotoxcity via non-competitive antagonism of NMDA receptors. 5-HT_{1A} receptors are predominantly inhibitory in their effects and have been shown to protect cultured neurons from excitotoxic as well as from apoptotic damage.[4] We studied the effects of 5-HT receptor subtypes (5-HT_3, 5-HT_{1A}) ligands on H_2O_2-induced neuronal cell death.

Methods and Materials

Primary cultures of rat cerebral cortical neurons were prepared from rat embryos cerebral hemispheres (embryonic day 15). The dissociated cells were seeded on poly-lysine coated multi well plate ($2X10^6$/ml) and cultured in dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 5mM KCl. Twenty-four hours after seeding, the culture medium was changed to DMEM containing 5% fetal bovine serum and 15mM KCl. Experiments were performed when cultures were 7 days old.

For experiments, cerebral cortical neurons were washed to remove DMEM and placed in an Mg^{2+} and glucose-free incubation buffer. And they exposed to H_2O_2 (100 μM) for 20min, (the agonists and antagonists were applied 15min prior to H_2O_2) and further incubated for 15 hr in serum-free DMEM.

The MTT assay was performed to measure cell viability. The microfluorescence assay of 2, 7-dichlorofluorescin, the fluorescent product of 2, 7-dichlorofluorescin diacetate (DCF-DA), was used to monitor the generation of ROS and nitric oxide. For measurement of apoptosis, Hoechst 33342, which is a chromatin dye staining all nuclei, was used.

Results and Discussion

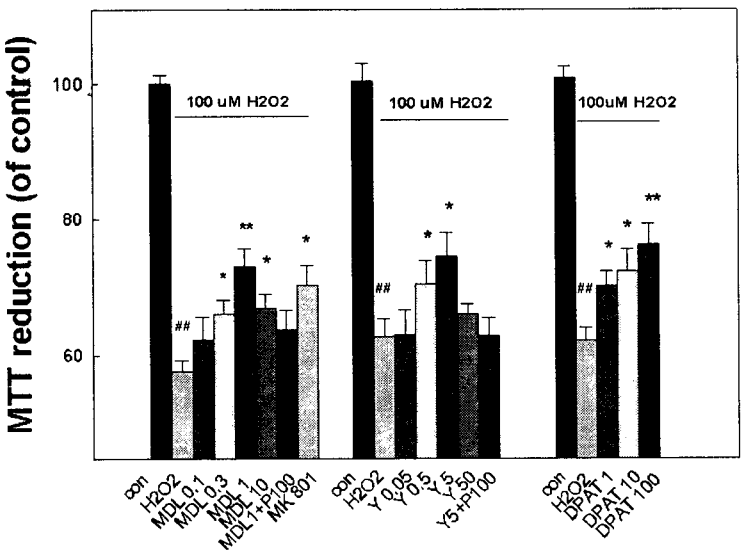

Fig.1. Inhibitory effects of MDL 72222, Y25130 and DPAT on H_2O_2-induced decrease of MTT reduction in cerebral cortical neurons. After washing and equilibration of 20 min with incubation buffer, cells were incubated with the same buffer containing 100 μM H_2O_2 for 20 min, and further incubated H_2O_2- and serum-free DMEM for 15 h (post-incubation) at 37 °C. The compounds were pretreated 20 min prior to the H_2O_2 treatment and added during the H_2O_2 exposure period and post-

incubation period. At the end of the incubation, cells were processed for MTT assay. Values represent mean ± SEM. ## $p<0.01$ compared to control. **$p<0.01$ compared to 100 μM H_2O_2.

We determined cell viability by MTT assay that measures mitochondrial function of cells. MTT reduction rate decreased to 57-62 % by 100 uM H_2O_2. MK 801, NMDA receptor antagonist, suppressed the H_2O_2-induced neuronal cell death indicating H_2O_2-induced neuronal cell death is related to the NMDA receptor activation by excessively released glutamate. Tropanyl-3, 5-dichlorobenzoate (MDL 72222) and N-(1-Azabicyclo[2.2.2] oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide hydrochloride (Y 25130), 5-HT_3 antagonists, significantly blocked the H_2O_2-induced decrease of MTT reduction in dose-dependent manners. When the neurons were treated with MDL 72222, MTT reduction was recovered by 15.26 % in the concentration of 1μM compared to H_2O_2 only. Y 25130 suppressed H_2O_2-induced decrease of MTT reduction by 11.72 % and DPAT, $5HT_{1A}$ agonist, by 13.96%. The treatment of 5-HT_3 antagonists in the presence of the receptor agonist, 1-phnylbiguanide hydrochloride, did not show neuroprotective effect indicating that the effect was mediated via 5-HT_3 receptor blockade. 5-HT_3 receptor is coupled to the activation of cation channels. Thus, It might be concluded that the receptor antagonists inhibited H_2O_2-induced cell death via blocking the cation channels.

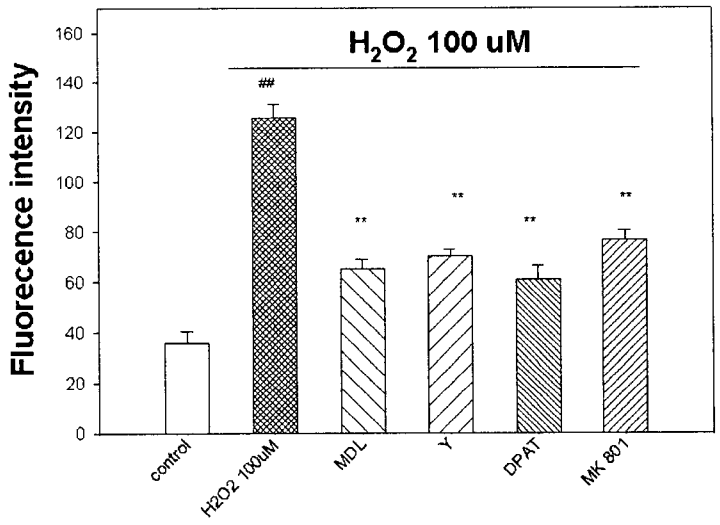

Fig.2. Inhibitory effects of 5-HT receptor ligands and MK 801 on H_2O_2-induced ROS generation in cerebral cortical neurons. Values represent mean ± SEM of relative fluorescence intensity. ##$p<0.01$ compared to control. **$P<0.01$ compared to 100 uM H_2O_2.

H_2O_2 is associated with accelerated formation of ROS and nitric oxide. In this experiment, these intracellular ROS generation was measured using DCF-DA, a fluorescent dye. H_2O_2 (100 μM)-treated cells showed bright cell body with increased fluorescence intensity indicating significantly increased ROS generation, compared to non-treated control cells. The H_2O_2-induced fluorescence intensity increase was blocked by MDL 72222, Y25130 and DPAT (Fig.2).

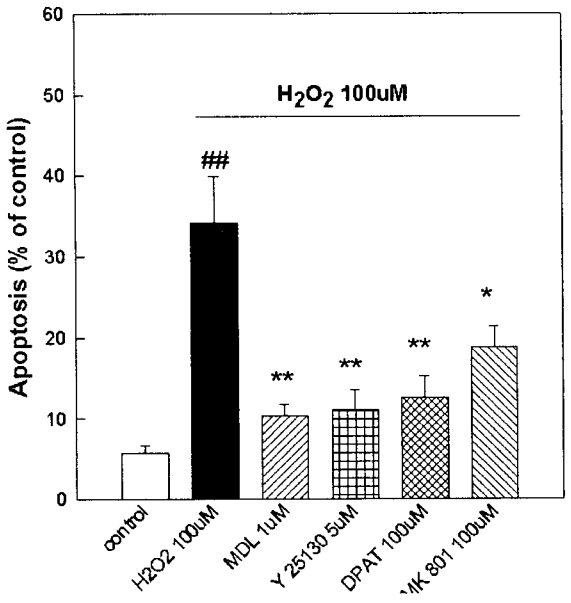

Fig.3. Inhibitory effects of MDL 72222, Y25130 and DPAT on apoptosis of cultured cerebral cortical neurons as measured by Hoechst 33342 staining. Apoptotic cells were counted from 5 to 6 fields per well. ##$p<0.01$ compared to control. *$p<0.05$, **$p<0.01$ compared to 100 μM H_2O_2.

Many studies have been reported that H_2O_2 and O_2^- play important roles in apoptosis.[2] The present study confirmed that H_2O_2-induced neuronal cell death in cultured cortical neurons is apoptosis, not necrosis, evidenced by Hoechst 33342 staining. MDL 72222, Y25130 and DPAT decreased the apoptotic cell death induced by 100 μM H_2O_2 (Fig. 3). The production of ROS may also be associated with H_2O_2-induced apoptosis. In vitro H_2O_2 causes oxidative cellular damage in neurons and induces superoxide anion and nitric oxide production, which are known as potential inducers of apoptosis.[6] In conclusion, $5HT_3$ receptor antagonists and $5HT_{1A}$ receptor agonist inhibited H_2O_2-induced neuronal apoptosis via respective receptor blockade and activation, and indirect inhibition of glutamate release, $[Ca^{2+}]^i$ elevation and ROS generation. Further study is necessary to clarify the precise mechanism of neuroprotective effects of 5-HT receptor subtypes ligands.

References

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