Dihydrobenzofuran Derivative Modulates Oxidative Stress-induced PC12 Cell Injury

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Abstract: The aim of this study was to investigate the effect of A-3922, a dihydrobenzofuran derivative, on hydroperoxide (H2O2)-induced PC12 cell injury. H2O2-induced PC12 cell injury was determined by lactate dehydrogenase (LDH) release. The concentration of lipid peroxide was determined from the amount of thiobarbituric acid reactive substances (TBARS) in PC12 cell homogenate. Following exposure to 150 μM H2O2 for 45 min (Condition I), the LDH activity increased to 181 ± 6 % of control activity (n = 11, P < 0.01). The LDH activity significantly decreased to 128 ± 6 % (n = 15, P < 0.01 vs Condition I) when PC12 cells were exposed to 150 μM H2O2 combined with 10 μM A-3922 (Condition II), and 161 ± 7 % (n = 15, P < 0.01 vs Condition I) when pre-treated with 10 μM A-3922 prior to the addition of 150 μM H2O2 (Condition III). Lipid peroxidation of PC12 cells induced by 25 mM 2,2'-azobis (2,4-dimethyl-valeronitrile), a lipophilic radical initiator, was inhibited by the addition of 100 μM A-3922. These results indicate that A-3922 exerts a protective effect against oxidative stress-induced PC12 cell injury, in part through the inhibition of lipid peroxidation in the cell membrane.

Key words: dihydrobenzofuran derivative, oxidative stress, PC12 cell, cell injury

Introduction

Oxidative stress is the cause of several neurodegenerative diseases, such as Alzheimer's disease (AD) 1) and Parkinson's disease (PD) 2-4), as well as pathological changes which occur in ischemia and reperfusion injury 5). Oxidative stress is the result of an imbalance in pro-oxidant/antioxidant homeostasis that leads to the generation of toxic reactive oxygen species (ROS). The ROS attack lipids and proteins, resulting in cell injury. Many attempts have been made to use natural 6-8) and synthetic 10) antioxidant molecules to protect against neuronal damage. A dihydrobenzofuran derivative, A-3922 is similar in structure to vitamin E, but has a dihydrobenzofuran structure instead of a chromane ring. It is thought that by substituting -OH with –NH2, dihydrobenzofuran has a greater antioxidant effect compared with vitamin E. Dihydrobenzofuran derivatives protect the brain and coronary artery from ischemia and reperfusion injury 11,12) and have antioxidant activities and an inhibitory effect

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on lipid peroxidation in several in vitro systems\textsuperscript{13-15}). They also exhibit antiangiogenic activities in the chorioallantoic membrane assay\textsuperscript{16}) and in the lipid hydroperoxide-induced rabbit corneal neovascularization model\textsuperscript{17}).

Hydroperoxide (H$_2$O$_2$), a major ROS, is known to cause lipid peroxidation and DNA damage in cells\textsuperscript{18}). It is possible that H$_2$O$_2$ is one of the causes of neurotoxicity in cerebral ischemia\textsuperscript{19}), and in the etiology of AD and PD\textsuperscript{20}). In vitro, H$_2$O$_2$ is able to induce injury in PC12 cells\textsuperscript{6,10,21}). In this study, we examined the effect of A-3922, a dihydrobenzofuran derivative, on H$_2$O$_2$-induced PC12 cell injury.

**Materials and Method**

**Chemicals**

A-3922, dihydrobenzofuran derivative was obtained from Nippon Soda Co., Ltd. (Odawara Japan). RPMI 1640 medium, horse serum, fetal bovine serum (FBS), penicillin and streptomycin were obtained from GIBCO (NY, USA). MTX "LDH" : lactate dehydrogenase assay kit was purchased from Kyokuto Pharmaceutical Industries, Ltd. (Tokyo, Japan). Hydroperoxide (H$_2$O$_2$), 2,2'-azovis (2,4-dimethyl-valeronitrile) (AMVN), thiobarbituric acid, phospholic acid and 1-butanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell culture**

Pheochromocytoma 12 cells (PC12 cells) derived from a rat cell line, were obtained from Human Science Resources Bank (Osaka, Japan). PC12 cells were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$. The cells were grown in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin in 75T flasks (Corning-Nippi, NY, USA). To determine H$_2$O$_2$-induced cell injury, the cells (3 x 10$^4$ cell/mL) were incubated in 96-well collagen coated plates (NCO 3903, Corning, NY, USA) for 24 h before being incubated for 45 min in fresh medium containing various concentration of H$_2$O$_2$. The medium was then replaced with fresh phenol-red-free medium and incubated for 24 h (Table 1: Condition I). To test the effect of A-3922, PC12 cells (3 x 10$^4$ cell/mL) were incubated in a 96-well collagen coated plate (NCO 3903, Corning) for 24 h and then incubated for 45 min in fresh medium containing various concentration of A-3922 and 150 μM H$_2$O$_2$. The medium was then replaced with fresh phenol-red-free medium and incubated for 24 h (Table 1: Condition II). To test the effect of pretreating PC12 cells with A-3922, cells (3 x 10$^4$ cell/mL) were incubated in a 96-well collagen coated plate (NCO 3903, Corning) for 24 h and then incubated for 45 min with fresh medium containing various concentrations of A-3922. The culture medium was then replaced with fresh medium containing 150 μM H$_2$O$_2$ and incubated for a further 45 min before being incubated for 24 h in fresh phenol-red-free medium (Table 1: Condition III).

**Analysis of Cellular injury**

Cell injury was assessed by measuring the release of LDH into the culture medium. After 24 h incubation in phenol-red-free medium, 10 μL of medium was collected and transferred to a 96-well plate (Costar\textsuperscript{®}, Corning). LDH activity was determined using the LDH assay kit and by measuring absorbance at 570 nm. LDH activity in each sample was expressed as percent of activity in control supernatants (without H$_2$O$_2$ or A-3922 exposure).
**A-3922 Modulates PC12 Cell Injury**

Table 1. Effect of 10 μM A-3922 pretreatment on 150 μM H2O2-induced cell injury

<table>
<thead>
<tr>
<th>Culture Condition: pretreatment</th>
<th>Medium Change</th>
<th>Medium Change</th>
<th>measurement of LDH</th>
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<tbody>
<tr>
<td>Duration of exposure</td>
<td></td>
<td></td>
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<tr>
<td>45 min</td>
<td>45 min</td>
<td>24 hours</td>
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<table>
<thead>
<tr>
<th>Condition</th>
<th>% of control</th>
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<tbody>
<tr>
<td>I</td>
<td>181 ± 6 **</td>
</tr>
<tr>
<td>II</td>
<td>128 ± 6 **</td>
</tr>
<tr>
<td>III</td>
<td>161 ± 7 **</td>
</tr>
</tbody>
</table>

Data are mean ± SE, Condition I: n = 11, Condition II: n = 15, Condition III: n = 15, **P < 0.01 vs Condition I

**Measurements of thiobarbituric acid reactive substances (TBARS) in PC12 cell homogenate**

10 μL of 25 mM 2,2′-azovis (2,4-dimethyl-valeronitrile) (AMVN), a lipophilic radical initiator, was added to 180 μL of 5% PC12 cells homogenate and the reaction volume made up to 200 μL with dH2O. The homogenates were incubated for 60 min at 37°C after which the supernatant was analyzed for TBA reactive substances using the thiobarbituric acid (TBA) assay. To determine basal levels of TBARS in PC12 cells, 10 μL of distilled water was added to the PC12 cell homogenate and incubated at 37°C for 60 min. The supernatant was then analyzed using the TBA assay. Various concentrations of A-3922 were added simultaneously with the initiator and the PC12 cell homogenates were assayed to determine the extent of inhibition.

Lipid peroxide concentration was determined by measuring the level of TBARS using a modification of the methods described by Ohkawa et al. The reaction mixture contained 0.2 mL of sample, 1.2 mL of 1% phosphoric acid, and 0.4 mL of 0.6% thiobarbituric acid. Each reaction mixture was incubated at 100°C for 45 min, then chilled for 15 min in an ice bath prior to the addition with mixing of 1.5 mL L-butanol. The mixtures were centrifuged at 1500 x g for 10 min, and the absorbance at 535 nm of each supernatant was measured using an Ultrospec 3300 Pro spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Statistical analysis**

Data was expressed as mean ± SE. Statistical significance was assessed by Non-repeated measures ANOVA and Dunnett’s test. P values less than 0.01 were considered significant.

**Result**

**H2O2-induced cell injury**

H2O2-induced cell injury was determined by the measurement of LDH activity in the medium. Following the addition of 100, 150, 300, 500, and 1000 μM H2O2, LDH activity increased to 116±3% (n=5, P<0.01), 169±4% (n=5, P<0.01), 277±3% (n=5, P<0.01), 324±17% (n=5, P<0.01), and 359±6% (n=5, P<0.01) of control activity, respectively (Fig. 1).

**Effect of A-3922 on H2O2-induced PC12 cell injury**

Exposure to 150 μM H2O2 for 45 min significantly increased LDH activity in medium
to 169±4% of control activity (n=9, P<0.01). The addition of 1, 5, 10, 30 and 50 µM A-3922 with 150 µM H₂O₂, significantly decreased LDH activity to 139±5% (n=9, P<0.01), 127±7% (n=9, P<0.01), 112±2% (n=9, P<0.01), 105±2% (n=9, P<0.01) and 103±2% (n=9, P<0.01) of control activity (Fig. 2).

**Effect of A-3922 pretreatment on H₂O₂-induced cell injury**

Table 1 shows the duration of 10 µM A-3922 and/or 150 µM H₂O₂ exposure and the LDH activity after 24 h incubation. Pretreatment of cells with A-3922 (Condition III) significantly decreased LDH activity in the medium to 161±7% of control activity (n=15, P<0.01).

**Effect of A-3922 on AMVN-induced lipid peroxidation in PC12 cell homogenate**

The basal level of TBARS in the PC12 cell homogenate was 8.7±2.3 nmol MDA/mg pro-
Fig. 3. Effect of A-3922 on AMVN-induced lipid peroxidation in PC12 homogenate.
The ordinate shows the percentage of control : TBARS levels in PC12 cell homogenate without
AMVN. The abscissa shows the concentration of A-3922. Data show the average ± SE for four
independent experiments.

When 25 mM AMVN was added to the PC12 cell homogenate, the TBARS
concentration significantly increased to 177 ± 1.0 nmol MDA/mg protein (203 ± 12%, n = 6, p
< 0.01). Addition of 100 μM A-3922, decreased AMVN-induced TBARS concentration to
14.1 ± 1.6 nmol MDA/mg protein (162 ± 18%, n = 6) (Fig. 3). Data shown in brackets is
TBARS levels expressed as % of control.

Discussion

In this study, H2O2-induced PC12 cell injury was prevented by the dihydrobenzofuran
derivative, A-3922.

The addition of H2O2 to PC12 cells initiates the Fenton reaction in which H2O2 reacts
with iron salts (Fe2+ and other organic molecules, and generates hydroxyl radicals (OH').
Hydroxyl radicals attack endoplasmic reticulum and mitochondrial membrane lipid, undergo-
ing a single electron reduction and oxygenation to produce lipid peroxide, initiating a dam-
aging chain reaction in the membrane. As a result of membrane damage, LDH is released
into the medium. Therefore, an increase in LDH release is an indicator of PC12 cell
injury and probably cell death. H2O2 is often used to investigate the mechanism of ROS-
induced cell death. H2O2-treated PC12 cells show apoptotic changes including activation of
caspase-3. H2O2 may modify the molecules attached and/or integrated in the membrane,
which triggers apoptotic signals and cell death. Mosley et al suggested that neuroinflamma-
tory processes play a significant role in the pathogenesis of PD. Mononuclear phagocyte
inflammatory responses damage the blood brain barrier, increase oxidative stress and release
neurotoxic factors. The neuronal injury signals enhance oxidative stress, generate protein
adducts, lipid peroxidation and DNA adducts, and lead to further neuronal damage
and neuronal death. There is abundant biochemical and histological evidence for oxidative
stress in PD which includes increased levels of carbonyl and nitrotyrosin protein modifica-
tions, lipid peroxidation, DNA damage, and reduction of glutathione and ferritin. Addition-
ally, glutathione (GSH) content in the substantia nigra pars compacta of PD patients is
decreased by 40-50%, but not in other regions of the brain, nor in age-matched controls or
in patients with other diseases affecting dopaminergic neurons. The depletion of GSH
may render cells more sensitive to the toxic effect of oxidative stress. Therefore, anti-inflammatory or anti-oxidant therapies might provide protection against neuroinflammation and consequent neurodegeneration in PD. In this study, H2O2-induced injury in PC12 cells pretreated with 10 μM A-3922 (Condition III) was decreased by 89%, and AMVN-induced lipid peroxidation of PC12 cells decreased to 87% when the cells were pretreated with 10 μM A-3922. This suggests, A-3922 may act like a vitamin E, as a chain-breaking antioxidant in the membranes. Additionally, A-3922 was a more effective antioxidant at a concentration of 1 μM (62% inhibition) than vitamin E (44% inhibition) in levofloxacin and blue LED light exposure induced retinal lipid peroxidation.

Currently, a radical scavenger, pramipexole is used in the treatment of PD. It is a dopamine D2/D3 receptor agonist that exerts a protective effect against oxidative stress-induced PC12 cell death in part through inhibition of JNK and p38 MAP kinase. The mechanism(s) by which A-3922 has a protective effect against H2O2-induced PC12 cell injury are unclear. However, the pramipexole-like antioxidant activity of A-3922 makes it a candidate for inclusion in a combination drug treatment to protect against neuroinflammation and neurodegeneration in PD.

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