Induction of metabotropic glutamate receptor II in neuronal HT22 Cells as a neuroprotective effect of novel phenylenediamine derivatives

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Abstract
Phenylenediamine derivatives can function as a hydrogen donor and reportedly exert cytoprotective effects against oxidative stress possibly by acting as an antioxidant. However, our previous report suggested that there is no critical link between the antioxidant ability of these derivatives and prevention of death of neuronal HT22 cells and that some other mechanisms may be involved in protecting neuronal cells against oxidative stress. Here we found that these derivatives might inhibit the cell death pathway by inducing metabotropic glutamate receptor II (mGluRII). By microarray analysis, a phenylenediamine derivative was proved to induce the expression of the mRNA (Grm3) encoding one of the proteins of mGluRII in HT22 cells; and 2S-α-ethylglutamic acid, an antagonist of mGluRII, reduced the protective effects of the derivative. Our results suggest that these compounds protect neuronal cells against oxidative stress, at least in part, by inducing the mGluRII. These compounds may thus be useful tools as mGluRII inducers in neuronal cells.

Abbreviations
DMSO: Dimethyl Sulfoxide; DPPD: N, N’-diphenyl-p-phenylenediamine; ED50: Effective Dose of 50% inhibition; EGLU: (2S)-α-ethylglutamic acid; GSH: Reduced Glutathione; mGluR: Metabotropic Glutamate Receptor; LD50: Lethal Dose for 50% of the cells; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS: Reactive oxygen species; RT-PCR: Reverse Transcription-Polymerase Chain Reaction.

Introduction
The metabotropic glutamate receptor (mGluR) family greatly expands the potential cellular responses to glutamate within the nervous system [1]. Experiments on the use of selective mGluR agonists and antagonists have shown that these receptors play roles in synaptic plasticity, seizure activity, excitotoxicity, and neuroprotection [2-4]. mGluRII and III are coupled with the Gi and Go families of G-proteins, whereas mGluRI couples to Gq/11 [1]. mGluRI activates phospholipase C (PLC) to generate inositol 1,4,5-triphosphate (IP3) and diacylglycerol, which have multiple “second messenger” roles and finally protect neurons [1]. The activation of mGluRII protects against excitotoxicity and enhances the survival of cultured Purkinje cells, whereas group I antagonists cause retinal degeneration in developing rodents [5].

Phenylenediamine derivatives such as N, N’-diphenyl-p-phenylenediamine (DPPD) exert a cytoprotective effect in vitro against oxidative stress on various types of cells including neuronal cell lines PC12, HT22, and primary cortical neurons [6,7] and inhibit the differentiation of mouse preadipocytes, 3T3-L1 cells, into adipocytes possibly through induction of GDF-15/MIC-1 [8]. DPPD is supposed to protect neuronal cells against oxidative stress at least in part by acting as an antioxidant [7]. However, the neuroprotective effects that we found in a previous study [6] cannot be due to its antioxidant activities in light of the following 3 findings: 1) DPPD does not prevent H2O2-induced cell death; 2) DPPD does not prevent xanthione+xanthine oxidase-induced cell death; and 3) DPPD protects glutamate-treated cells at nM concentrations, which are about 1000 fold lower than those effective to decrease ROS levels [6]. These results suggest that a nM order of DPPD does not function as an antioxidant but that it may inhibit a certain event in the death pathway initiated by oxidative glutamate toxicity [6]. In the present study we addressed straightforwardly this issue; and based on several lines of experimental evidence, we concluded that these compounds at nM levels protected neuronal cells against oxidative stress, at least in part, by inducing the mGluRII.

Materials and methods
Compounds
Phenylenediamine derivatives shown in figure 1 were obtained from Ouchi Shinko Chemical Industrial (Tokyo, Japan). D10, D11, D12 and D13 are newly synthesized compounds for the present research. N-Methyl- and N, N-dimethyl-N’-phenyl-p-phenylenediamine (D10 and D11, respectively) were prepared by reductive alkylation of N-phenyl-p-phenylenediamine (PADA) and paraformaldehyde and following chromatographic separation. On the other hand, N-ethyl- and N,N-diethyl-derivative (D12 and D13, respectively) were prepared by direct alkylation of PADA with ethyl bromide. The compounds were dissolved in dimethyl sulfoxide (DMSO). The final concentration of...
DMSO in the culture medium was 0.1%. Other compounds, including (2S)-α-ethylglutamic acid (EGLU), were purchased from Sigma (St. Louis, MO, USA).

**HT22 culture and MTT assay**

HT22 cells were cultured as described previously [9-10]. The cells were maintained in 75-cm² flasks (Invitrogen, Carlsbad, CA) containing 10 mL of Dulbecco’s Modified Eagle medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (Invitrogen, Carlsbad, CA). For experiments the HT22 cells were seeded into 48-well plates at a density of 4 × 10⁴ cells/cm². After a 5-h incubation, the cells were then cultured further for 24 h. To evaluate cell survival of HT22 cells, we performed the MTT assay [9-10]. The data were presented as the mean ± SD (for in vitro experiments).

**Oligonucleotide microarray analysis**

Total RNA was isolated from vehicle (DMSO)- or D11 (10 µM)-treated HT22 cells by using TRIzol Reagent (Invitrogen, Carlsbad, CA) [11-13]. cDNA was synthesized by using the Superscript II system (Invitrogen) with a T7-Oligo(dT) primer. Biotin-labeled cRNA was prepared by in vitro transcription and fragmented by incubation at 94°C for 35 minutes in 40 mmol/L Tris acetate buffer (pH 8.1) containing 100 mM/L potassium acetate and 30 mM/L magnesium acetate. Fragmented cRNA was hybridized at 45°C for 16 hours to a GeneChip Mouse Mouse Gene 430 2.0 Array (Affymetrix, Santa Clara, CA), which contains over 39000 transcripts. Probe arrays were washed and stained using a Fluidics Station 450 and scanned with a GeneChip Scanner 300. Affymetrix GeneChip Operating Software (GCOS v1.4) was used for analysis.

**RT-PCR**

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously (9-10, 33-34) using the following primers: for β-actin (287 bp), 5'- ATC CGT AAA GAC CTC TAT GC-3' (forward) and 5'- AAC GCA GCT CAG TAA CAG TC-3' (reverse); and for Grm3 (534bp), 5'- CAAAGTCTACAGAATGCA-3' (forward) and 5'- CTGTCACCAATGCTCAGCTCT-3' (reverse).

**Results and discussion**

**Protective effects by DPPD derivatives**

In HT22 cells and immature cortical neurons, which do not function normally NMDA receptors, high concentrations of glutamate induce a novel type of neuronal death mediated by depletion of reduced glutathione (GSH), termed “oxidative glutamate toxicity” [14,15]. Although these 2 types of neuronal death by glutamate, i.e., excitotoxicity and oxidative glutamate toxicity, are distinct from each other, oxidative stress is involved in both types [14,15], which is why several antioxidant molecules are reported to protect neurons against both types of toxicity [16,17]. Thus, one possible strategy for the development of neuroprotective drugs is to search for low-molecular-weight compounds that can regulate the redox state [18-22]. For the present study, we prepared new lines of DPPD derivatives from D10 to D13 (Figure 1); because DPPD is too hydrophobic and also is reported to be toxic as an allergen and carcinogen possibly through activation of cytochrome P450 family genes (Cyp2c55 and Cyp3a41a) significantly [23]. Glutamate receptor metabotropic 3 (grm3) was identified as the gene most induced by this compound, and thus we examined the expression of this gene by RT-PCR (Figure 3A). We observed that Grm3 mRNA was clearly induced by D11 (100 nM), although the levels of β-actin were the same, suggesting that mRNA expression of this gene was really induced by D11 in HT22 cells.

**Pharmacological analysis**

Next, we studied the protective effect by DPPD in HT22 cells. D10 is less lipophilic than D1, D13 or DPPD. D11 and D13 had a very low ED50 (70.68 and 17.48 nM, respectively) and had a broad safety zone. These results suggest that the induction of grm3 mRNA expression of this gene was really induced by D11 in HT22 cells.

**Discussion**

By the present study, we found that D11, a newly synthesized phenylenediamine derivative, protected HT22 cells against oxidative...
HT22 cells were seeded into 24-well plates at a density of 4 × 10^4 cells/cm^2. After a 5-hr incubation, various concentrations of the compounds were added. One hour later, the cells were treated for 20 h with 5 mM glutamate and then subjected to the MTT assay. The values, which represent the percentage of the control MTT activity, are means ± S.D.s (n=4). The experiments presented here were repeated at least 3 times with 4 samples for each determination.

**Figure 2.** Effects by the compounds on oxidative glutamate toxicity. HT22 cells were incubated in the absence or presence of 100 nM D11 or vehicle (DMSO) for 24 h. RT-PCR was performed following 3 mechanisms, 1) antioxidant activities [7,8], 2) activation of arylhydrocarbon receptors [23,24], and 3) induction of mGluRII. These genes were selected for their significant up-regulation (p < 0.0003) in response to D11. HT22 cells were incubated in the absence or presence of 100 nM D11 or vehicle for 24 h in normal medium. Total RNA was isolated and subjected to microarray analysis. Fold change (D11/vehicle), GeneBank names, gene descriptions, and gene symbols are indicated.

**Table 1.** Effective dose of 50% inhibition (ED_{50}) of oxidative glutamate toxicity, lethal dose for 50% of the cells (LD_{50}) and safety zone (=LD_{50}/ED_{50}) of each compound.

| Abbreviation | ED_{50}[nM] | LD_{50}[nM] | LD_{50}/ED_{50} |
|--------------|------------|------------|----------------|
| DPPD         | 7.50       | 17544.30   | 2338.67        |
| D10          | 145.96     | 9813.54    | 67.21          |
| D11          | 70.68      | 4050.45    | 57.37          |
| D12          | 73.75      | 7420.60    | 100.61         |
| D13          | 17.48      | 3371.59    | 1928.49        |

stress at least possibly through induction of the mGluRII gene. This possibility is supported by the findings that 1) D11 induced expression of the mGluRII gene; and 2) EGLU, a mGluRII antagonist, partially abolished the protective effect afforded by D11. Because HT-22 cells lack ionotropic glutamate receptors and do not constitutively express mGluRII, our results suggest a new type of neuroprotective effect of phenylenediamine derivatives. We propose that diphenylphenylene diamine derivatives can protect neuronal cells possibly through the following 3 mechanisms, 1) antioxidant activities [7,8], 2) activation of arylhydrocarbon receptors [23,24], and 3) induction of mGluRII. Mechanisms 1 and 2 seem to require μM levels of compounds, whereas only mechanism 3 is operative at the nM level. Thus, we can assume that the major pathway contributing to the inhibition of cell death by oxidative glutamate toxicity is the induction of mGluRII. Because these compounds might induce mGluRII in neurons in vivo, they might become effective, novel therapeutics for neuroprotection against neurodegeneration.

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