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Development of a simple measurement method for GluR2 protein expression as an index of neuronal vulnerability

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ABSTRACT

In vitro estimating strategies for potential neurotoxicity are required to screen multiple substances. In a previous study, we showed that exposure to low-concentrations of some chemicals, such as organotin, decreased the expression of GluR2 protein, which is a subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors, and led to neuronal vulnerability. This result suggested that GluR2 decreases as an index of neuronal cell sensitivity and vulnerability to various toxic insults. Accordingly, we developed a versatile method that is a large scale determination of GluR2 protein expression in the presence of environmental chemicals by means of AlphaLISA technology. Various analytical conditions were optimized, and then GluR2 protein amount was measured by the method using AlphaLISA. The GluR2 amounts were strongly correlated with that of measured by western blotting, which is currently used to determine GluR2 expression. An ideal standard curve could be written with the authentic GluR2 protein from 0 ng to 100 ng. Subsequently, twenty environmental chemicals were screened and nitenpyram was identified as a chemical which lead to decrease in GluR2 protein expression. This assay may provide a tool for detecting neurotoxic chemicals according to decreases in GluR2 protein expression.

1. Introduction

Mammals have been chronically exposed to environmental chemicals at low concentrations, and some environmental chemicals induce toxicity in individuals and in ecological systems. Thus, investigations of toxic environmental chemicals are necessary to prevent exposure. The central nervous system plays key roles in neuropsychiatric functions, such as behavior, learning, and memory, and comprises neuronal cells that recover poorly from damage. Life-long exposures to neurotoxic chemicals reportedly leads to altered behavior, mental retardation, and other neuronal disabilities, as well as diseases [1–3]. Moreover, during developmental stages, the immature blood–brain

Abbreviations: AMPA receptor, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; Glu, glutamate; HS, horse serum; MAP2, microtubule-associated protein 2; NAS, 1-naphthylacetylspermine; PBS, phosphate-buffered saline; TBT, tributyltin; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.

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barrier allows the passage of neurotoxic environmental chemicals, even at low concentrations [4,5]. Therefore, an index for neurotoxicity and a screening system for the index are required.

We previously showed that long-term exposure of rat cortical neurons to low concentrations of organotin decreases GluR2 protein expression, leading to increased neuronal susceptibility to glutamate stimulation compared with that in control neurons [6]. Subsequently, we showed that long-term lead exposure induces neuronal cell death in association with decreased GluR2 expression [7]. The GluR2 protein is a subunit of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, which is a glutamate receptor that mediates rapid excitatory synaptic transmissions in the central nervous system. GluR2 is critical for Ca^{2+} permeability of AMPA receptors. GluR2-containing AMPA receptors are impermeable to Ca^{2+} [8], whereas GluR2-lacking AMPA receptors are highly permeable to Ca^{2+} in a steady state, and the majority of functional AMPA receptors contain GluR2 [9]. GluR2 plays important roles in neuronal death, such as that associated with ischemia or Alzheimer's disease [10–12]. In addition, recent studies show that GluR2 is an essential regulator of the memory phenomena [13]. These studies suggest that decreases in GluR2 may be utilized as an index for conditions under which neuronal cells are sensitive and vulnerable to other stimulants. To investigate multiple neurotoxic chemicals for their effects on GluR2 expression, we need large-scale determinations of GluR2 protein expression in neuronal cells. However, western blotting for GluR2 expression is unsuitable for high throughput screening.

AlphaLISA® (developed by PerkinElmer, Inc.) is applied to high throughput screening methods with high reproducibility [14–16]. This analysis is a bead-based proximity immunoassay [17] that exploits oxygen channeling technology. AlphaLISA assays are performed with anti-analyte (a target protein) antibodies and two AlphaLISA beads, including streptavidin-coated alpha donor beads and IgG-coated alpha acceptor beads. AlphaLISA is an all-in-one-well assay that does not require transfer or wash steps, and allows assessments of analytes on a large scale [18].

In this study, we developed a novel *in vitro* assay to screen multiple compounds for their effects on GluR2 expression by means of AlphaLISA technology (Fig. 1). Subsequently, twenty potentially neurotoxic chemicals were screened, and then nitenpyram was identified as a novel chemical which lead to decrease in GluR2 protein expression.

2. Materials and methods

2.1. Materials

Eagle's minimal essential salt medium (Eagle's MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Nichirei Biosciences (Tokyo, Japan). Horse serum (HS) was purchased from Gibco (Life Technologies, Carlsbad, CA, USA). Acifluorfen, bithionol, bromofenofos, carbofuran, *cis*-permethirin, D-(+)-glucose, 4,4′-dichlorobenzophenone, dertamethrin, diclofop, dimethyl sulfoxide (DMSO), fensulfothion, fenthion, methiocarb, $NaHCO_3$, nitenpyram, nitrixynil, ortho phenyl phenol, oxyclozanid, phenylmethylsulfonyl fluoride (PMSF), procloraz, rafoxianid, Sodium dodecyl sulfate (SDS), tribromsalan and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) were purchased from Wako (Tokyo, Japan). Allethrin, arabinosylcytosine, anti-β-actin antibody (AC-15) and 1-naphthylacetylspermine (NAS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pentobarbital was purchased from Kyoritsu (Tokyo, Japan). Captafol and dichlorvos were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Tris–HCl, nonidet P-40, ethylenediaminetetraacetic acid (EDTA), mercaptoethanol, and protease inhibitor cocktail were purchased from Nacalai Tesque (Kyoto, Japan). pGEX-6P and PreScission protease were purchased from GE healthcare (Piscataway, NJ). BL-21-CodonPlus (DE3) RIPL was purchased from Agilent Technologies (Santa Clara, CA). Mouse anti-GluR2 monoclonal antibody (MAB397) was purchased from Millipore (Billerica, MA, USA). Rabbit anti-GluR2 pAb (BS3658) was purchased from Bioworld Technology, Inc. (St. Louis, MN, USA). ChromaLink™ Biotin (DMF Soluble) was purchased from solulink (San Diego, CA, USA). Zeba Desalt Spin Columns (0.5 mL) and Pierce™ BCA Protein Assay Kits were purchased from Thermo Fisher Scientific K.K. (Rockford, IL, USA). Bromoxynil was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

AlphaLISA streptavidin donor micro-beads (676002S), anti-rabbit IgG (Fc specific) AlphaLISA acceptor beads (AL104C), AlphaLISA immunoassay buffer (AL000C), and 1/2 AreaPlate-96 plates were purchased from PerkinElmer, Inc. (Waltham, MA02451, USA).

2.2. Cell culture

The present study was approved by the animal ethics committee of Hiroshima University. The following procedures were performed under sterile conditions. Primary cultures were obtained from the cerebral cortex of fetal rats (at 18 days of gestation) as described previously [19]. Fetuses were taken from pregnant Slc; Wistar/ST rats under pentobarbital anesthesia. Subsequently, parts of the cerebral cortex were dissected using a razor blade, and cells were dissociated by gentle pipetting and plated on culture plates (4×10^6 cells/cm^2). Cultures were incubated in Eagle's MEM supplemented with 10% fetal calf serum, L-glutamine (2 mM), D-(+)-glucose (11 mM), $NaHCO_3$ (24 mM), and HEPES (10 mM). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Cultures were incubated in MEM containing 10% FCS (1–7 days) or 10% HS (8–11 days) and the medium was exchanged every 2–3 days. Arabinosylcytosine (10 μM) was added to inhibit the proliferation of non-neuronal cells at 6 days *in vitro* (DIV). Cultures were used for experiments at 11 DIV. This protocol has been shown to produce cultures containing approximately 90% neurons, as indicated by immunostaining for the neuron marker, microtubule-associated protein 2 (MAP2).