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Evaluation of transcriptional activity of caspase-3 gene as a marker of acute neurotoxicity in rat cerebellar granular cells

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ABSTRACT

Caspase-3 is a key protein involved in the classical apoptosis mechanism in neurons, as in many other cells types. In the present research, we describe the transcriptional activity of caspase-3 gene as a marker of acute toxicity in a primary culture model of rat cerebellar granule neurons (CGNs). CGNs were incubated for 16 h in complete medium containing the chemicals at three concentrations (10, 100 μM and 1 mM). A total of 48 different compounds were tested. Gene transcriptional activity was determined by low-density array assays, and by single Taqman caspase-3 assays. Results from the PCR arrays showed that the caspase-3 gene was up-regulated when CGNs were exposed to neurotoxic chemicals. Significative correlations were found between the transcriptional activity of caspase-3 and the activity of some other genes related to apoptosis, cell-cycle and ROS detoxification. In our experiments, acute exposure of CGNs to well-documented pro-apoptotic xenobiotics modulated significantly caspase-3 gene expression, whereas chemicals not related to apoptosis did not modify caspase-3 gene expression. In conclusion, acute exposure of CGNs to neurotoxic compounds modulates the transcriptional activity of genes involved in the classical apoptotic pathway, oxidative stress and cell-cycle control. Transcriptional activity of caspase-3 correlates significantly with these changes and it could be a good indicator of acute neurotoxicity.

1. Introduction

The apoptotic death of neuronal cells is a very complex process that can be activated by many different endogenous factors and signals from the cell environment. Neuronal loss due to apoptotic processes is a common event involved in the progression of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) or amyotrophic lateral sclerosis (ALS). Thus, it is of great importance to be able to detect the potential neurotoxicity of new chemicals or pharmaceutical intended for human use that can cause the aberrant activation of the apoptosis program. Mitochondrial alteration and DNA damage may constitute key hallmarks of the toxic xenobiotics that activate the apoptotic process in neuronal cells, where apoptosis can be stimulated by different tissue-specific factors. Neuronal apoptosis has been related to excitotoxicity processes (extrinsic pathway), mitochondrial or DNA damage (intrinsic pathway), cell-cycle activation or aberrant activity of molecules like cdk5. The classical apoptosis process involves the generation of a permeability transition pore complex (PTPC) that allows the release of proteins such as cytochrome c, apoptosis inducing factor (AIF), endonuclease G, and the second mitochondria-derived activator of caspase, Smac, also known as DIABLO (Xiang et al., 1996; Uberti et al., 1998; Sakhi et al., 1996). Once cytochrome-c is released into the cytoplasm, it triggers the formation of the apopotosome: a complex composed of cytocrome-c, Apaf-1, and dATP (Xiang et al., 1996; Uberti et al., 1998; Sakhi et al., 1996; Smith et al., 2006). This complex contributes to the activation of the initiator procaspase-9. The caspase cascade is amplified in successive steps by the activation of executioner caspases such as caspase-3, caspase-6, and caspase-7 (Kruman et al., 2004). After that the executioner caspases are activated it is not possible to stop the apoptosis process.

Over the last 50 years, research has been conducted worldwide to evaluate the potential use of *in vitro* cell systems for predicting acute toxic effects *in vivo*. Many studies have shown good correla-

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tion between *in vitro* basal cytotoxicity data and *in vivo* LD50 values and/or human lethal and toxic blood concentrations. Acute systemic toxicity *in vivo* can result from toxicity at the cellular level, i.e., cytotoxicity, which in turn results from interference with structures and/or properties essential for cell survival, proliferation and/or function. These effects can involve, for example, the integrity of membranes and the cytoskeleton, metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division. The toxicity types that can be tested readily in cell cultures are the: basal cytotoxicity that involves one or more of the above-mentioned structures or processes, when all of the cell types studied show similar sensitivities; organ-specific (cell-specific) cytotoxicity that occurs where some types of differentiated cells are more sensitive to the effects of a particular toxicant than others, as a result of biotransformation, binding to specific receptors, or uptake by specific mechanisms (Ekwall, 1983). The late type of cytotoxicity can cause severe damage, for example, to highly sensitive cells that form the human nervous system, and that hardly can be reversed.

In previously published researches of our group, we showed that well-known neuronal apoptosis inducers, like colchicine, cause a significant increase in the activity of caspase-3 gene in rat cerebellar granule neurons (CGNs) (Jorda et al., 2003; Yeste-Velasco et al., 2008). The low-density real-time PCR analysis also showed that caused a significant up-regulation not only in caspase-3 gene, but also in cell-cycle-related molecules and ROS detoxification enzymes. In the present research, we intended to evaluate the usefulness of transcriptional gene activity analysis of key molecules involved in apoptosis, like caspase-3, as an automatisable and reliable method to assess the acute neurotoxicity of xenobiotics in primary cultures of CGNs. This research was developed within the A-Cute-Tox project, and the chemicals tested were selected by an international expert group under the supervision of the European Chemicals Bureau and the European Centre for Validation of Alternative Methods.

2. Materials and methods

2.1. Materials

The chemicals used in this study are shown in Table 1, and all of them were provided by Sigma Chemical Co. (St. Louis, MO, USA). Cell culture media and fetal calf serum (FCS) was from GIBCO (Life Technologies, Paisley, UK). Total RNA isolation NucleoSpin® RNAII kit was purchased from Macherey–Nagel (Macherey–Nagel GmbH & Co. KG, Düren, Germany). High-Capacity cDNA Archive Kit, TaqMan® Universal PCR Master Mix, No AmpErase® UNG, TaqMan® Gene Expression Assays and low-density arrays were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). PCR reactions were developed in a Perkin Elmer 2400 Thermal Cycler and 7900HT system (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR data were quantified using the SDS 2.2 software package (Applied Biosystems). Other chemical reagents were of analytical quality and purchased from Scharlab (Barcelona, Spain).

Table 1
Compounds tested.

(−)-Epinephrine bitartrate	Chloral hydrate	Methanol
(±)-Verapamil hydrochloride	cis-Diammineplatinum (II) dichloride	Nicotine
17α-Ethynylestradiol	Colchicine	Ochratoxin A
2,4-Dichlorophenoxyacetic acid	Cyclosporine A	Orphenadrine hydrochloride
5-Fluorouracil	Dichlorvos	Parathion
2-Propanol	Diethylene glycol	Phenobarbital
Acetonitrile	Digoxin	Physostigmine
Acrylaldehyde	Dimethylformamide	Pyrene
Amiodarone hydrochloride	Diquat dibromide	Rifampicine
Amitriptyline hydrochloride	Ethanol	Sodium chloride
Arsenic trioxide	Ethylene glycol	Sodium fluoride
Atropine	Glufosinate-ammonium	Sodium selenate
Butylhydroperoxide	Hexachlorobenzene	Strychnine
Cadmium (II) chloride	Lindane	Tetracycline hydrochloride
Caffeine	Lithium sulphate	Thallium sulphate
Carbamazepine	Malathion	Warfarin

2.2. Granule cell culture and drug treatment

Primary cultures of CGNs were prepared from 7-day-old Sprague Dawley rat pups, following the method of Verdaguer et al. (2004). Briefly, cerebella were freed of meninges, minced, trypsinised and treated with DNAse. Cells were dissociated by repeated pipetting, and separated from non-dissociated tissue by sedimentation. Cell density was adjusted to 8×10^5 cells/mL and cells were seeded on poly-L-lysine coated six-well plates. Cultures were grown in Eagle's basal medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/mL gentamicin and 25 mM of KCl. Cytosine arabinoside (10 μM) was added 16–18 h after plating, in order to inhibit the growth of non-neuronal cells. Cultures prepared by this method were enriched more than 95% in granule neurons. After 7 days *in vitro* (DIV), CGNs were incubated for 16 h in complete medium containing each chemical, at concentrations of 10, 100 and 1000 μM. In a first set of experiments, CGNs were exposed to colchicine, nicotine, malathion, phenobarbital, caffeine and atropine (at the mentioned concentrations, for 16 h) and the gene transcriptional activity was determined by real-time low-density array assays. In a second set of experiments, CGNs were exposed to all the compounds listed in Table 1, at concentrations of 10, 100 and 1000 μM, for 16 h. Caspase-3 transcriptional activity was determined by Real-time TaqMan® Gene Expression Assay.

2.3. Determination of gene transcriptional activity by low-density array assays

Isolation of total RNA was performed after 16 h of incubation with the chemical to be tested. Total RNA isolation was carried out according with the guidelines of the NucleoSpin® RNA II kit. Briefly, cell culture medium was removed and cells rinsed with PBS and lysed directly in the culture dish by adding lysis buffer containing β mercaptoethanol. For each preparation, one NucleoSpin® RNA II column was placed in a 2 mL centrifuge tube and the lysate loaded. After centrifugation, 30 s at 8.000*g*, the column was desalted, loaded with 95 μL of DNAse I reaction mixture. After 15 min of digestion at room temperature, the RNA was eluted from the column and recovered in 50 μL H_2O (RNAse-free). RNA content in the samples was measured at 260 nm and purity of the samples was determined by the A260/280 ratio. RNA samples were finally stored at −80 °C until reverse transcription was performed (less than 48 h later).

First strand cDNA was reversely transcribed from 25 μL of total RNA, at 0.2 μg/μL, by using a High-Capacity cDNA Archive Kit. Reaction mix was prepared according with the manufacturer's guidelines and the reaction was performed in a Perkin Elmer 2400 Thermal Cycler, according with the following program: Step 1 at 65 °C for 5 min; Step 2 at 50 °C for 120 min. The cDNA samples were used for TaqMan low-density arrays analysis.

We designed the configuration of a low-density array in a 32 assays format. This format includes a mandatory control designed by the manufacturer as a housekeeping gene: ribosomal 18S RNA. We