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A decrease of intracellular ATP is compensated by increased respiration and acidification at sub-lethal parathion concentrations in murine embryonic neuronal cells: Measurements in metabolic cell-culture chips

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ABSTRACT

We present a label-free *in vitro* method for testing the toxic potentials of chemical substances using primary neuronal cells. The cells were prepared from 16-day-old NMRI mouse embryos and cultured on silicon chips (www.bionas.de) under the influence of different parathion concentrations with sensors for respiration (Clark-type oxygen electrodes), acidification (pH-ISFETs) and cell adhesion (interdigitated electrode structures, IDES). After 12 days *in vitro*, the sensor readouts were simultaneously recorded for 350 min in the presence of parathion applying a serial 1:3 dilution. The parathion-dependent data was fitted by logistic functions. IC_{50} values of approximately 105 μM, 65 μM, and 54 μM were found for respiration, acidification, and adhesion, respectively. An IC_{50} value of approximately 36 μM was determined from the intracellular ATP-levels of cells, which were detected by an ATP-luminescence assay using microwell plates. While the intracellular ATP level and cell adhesion showed no deviation from a simple logistic decay, increases of approximately 29% in the respiration and 15% in the acidification rates above the control values were found at low parathion concentrations, indicating hormesis. These increases could be fitted by a modified logistic function. We believe that the label-free, continuous, multi-parametric monitoring of cell-metabolic processes may have applications in systems-biology and biomedical research, as well as in environmental monitoring. The parallel characterization of IC_{50} values and hormetic effects may provide new insights into the metabolic mechanisms of toxic challenges to the cell.

1. Introduction

It is known that many xenobiotic substances, for instance insecticides, which enter into food from the environment, are harmful to human health, especially for children. Toxicological investigations of existing and new substances are regulated in the test guidelines of the Organization for Economic Co-Operation and Development (OECD) and the European Union (OECD, 1996). Furthermore, EU and US guidelines require the characterization of substances according to their neurotoxic effects on human development (Kaufmann, 2003) and their overall neurotoxic effects (Grandjean and Landrigan, 2006). In Europe, the new chemical policy on the Registration, Evaluation and Authorization of Chemicals (REACH) came into force in 2007. If conventional, validated and regulatory-accepted methods had been used, a large number of animals would have needed to be sacrificed during the course of the necessary investigations. Even though alternative methods do exist (http://alttox.org/ttrc/validation-ra/validated-ra-methods.html), only a few cell-based methods, such as the Limulus Amoebocyte Lysate (LAL) test (Blechova and Pivodova, 2001), are routinely used.

Cell-based *in vitro* systems emerging from Micro Total Analysis Systems (μTAS) (Daridon et al., 2001) or lab-on-chip systems (El-Ali et al., 2006; Fuhr et al., 1997; Wolf et al., 2006) are commonly used for cell monitoring, cell sorting or as micro-bioreactors. They can also be employed to reduce animal testing in the fields of environmental monitoring, medical diagnostics or drug development. Microsystems with integrated sensors (Bousse et al.,

Abbreviations: AChE, acetylcholine-esterase; CM, culture medium; CM+, culture medium with parathion; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium with 4.5 g/l glucose, without Na-pyruvate, without L-glutamine; ES, equine serum; FBS, fetal bovine serum; GABA, gamma amino butyric acid; IC_{50}, half-maximal (50%)-inhibitory concentration; IDES, interdigitating electrode structures; ISFET, ion-selective field-effect transistor; MC, metabolic chip SC 1000; MM, measuring medium; MM+, measuring medium with parathion; MMT, measuring medium with Triton X 100; MEPC, murine embryonic primary frontal cortex cells; PBS, phosphate buffered saline without Ca^{2+} and Mg^{2+}; PDL, poly-D-lysine; P/S, penicillin/streptomycin; SD, standard deviation; SM, seeding medium; SM+, seeding medium with parathion.

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1994; Bousse and Parce, 1994) can be used for the non-invasive *in vitro* measurement of a number of cell-physiological parameters. Commercial systems allow for high-throughput cell-based assays, as well as superior electroactive tissue diagnostics (see e.g. www.alphaomega-eng.com, www.multichannelsystems.com, www.plexon.com).

Cellular metabolic parameters such as respiration and acidification have been detected, e.g. by Clark-type electrodes (Ceriotti et al., 2007; Thedinga et al., 2007) and ion-sensitive field-effect transistors (ISFETs) (Baumann et al., 1999; Lehmann et al., 2001; Poghossian et al., 2009), which are integrated in the microsensor chips. To characterize cell adhesion, the electric impedance of cells is detected with interdigitated electrode structures (IDES; Ehret et al., 1997; Ceriotti et al., 2007). IDES measurements may provide information on cell number, adhesion status, morphology and viability (Koester et al., 2010). High content screening systems are already commercially available (www.roche-applied-science.com). The Bionas Analysis System 2500 (Bionas GmbH, Warnemünde, Germany, www.bionas.de) used in our investigations allows for the *in vitro*, non-invasive measurement of the three metabolic parameters of respiration, acidification and cell adhesion, using six SC 1000 metabolic chips (MCs; Bionas GmbH) in parallel, and spanning time-frames ranging from minutes to days (Ehret et al., 2001; Thedinga et al., 2007).

We developed an *in vitro* test strategy for measuring the cell-metabolic effects of environmental toxins with MCs. We used the organophosphate parathion as an exemplary test substance in combination with murine embryonic, primary frontal cortex cells (MEPCs) because of their sensitivity to organophosphates.

Parathion, better known as E605, is a potent insecticide that is toxic to humans (Lotti, 2000). Parathion and its metabolites are known to induce acute and systemic effects in different mammalian tissues. Systemic effects, such as blood pressure and acetylcholine-esterase (AChE) activity alterations, are caused by parathion interfering with mitochondrial ATP formation, as tested in rats and mice. Holtz and Westermann (1959) found an LD_{50} value of 7.2 mg/kg for mice. LD_{50} values of 5–25 mg/kg and 2–30 mg/kg (orally applied substance per body mass) are given for mice and rats, respectively, on Extoxnet (http://extoxnet.orst.edu/pips/parathio.htm).

Dose-range finding experiments were conducted in cell-culture plates using the phenol-red indicated pH change of the culture medium (CM) as a criterion for the metabolic activities of the cells.

Following dose-range finding experiments, we performed an intracellular ATP assay. These experiments allowed us to limit the MC experiments for determining the IC_{50} values of cellular respiration, acidification and adhesion for 6 (5+ control) different concentrations. While the intracellular ATP-level and cell adhesion showed no deviation from a simple logistic decay, increases in the respiration and cellular acidification rates were found at low parathion concentrations, indicating a stimulating effect for these parameters.

The concept of the stimulating effects of low doses of potentially toxic agents on the metabolic activity had already been described by Philippus Theophrastus Aureolus Bombast von Hohenheim, known as Paracelsus (1493–1541). Nowadays, these effects are discussed as "hormesis" (Calabrese, 2008; Schabenberger et al., 1999; Schulz, 1887; Stebbing, 1982; Van Ewijk and Hoekstra, 1993).

2. Materials and methods

2.1. Solutions

The poly-D-lysine (PDL) precoating solution for the MCs consisted of 5 mg PDL lyophilisate (Sigma–Aldrich, Schnelldorf, Germany) diluted in 50 ml phosphate buffered saline (PBS). Laminin solution for the MCs consisted of 0.5 mg/ml laminin (Roche Applied Science, Penzberg, Germany). The cell-seeding medium (SM) contained 77% Dulbecco's modified Eagle's medium (DMEM) with phenol red, 10% fetal bovine serum (FBS), 10% equine serum (ES), 100 U/ml penicillin and 100 μg/ml streptomycin and 1% L-glutamine (200 mM) (all purchased from Biochrom AG, Berlin, Germany) as well as 1% methanol (used as solubilizer for parathion).

The CM contained 87% DMEM with phenol red, 10% ES, 100 U/ml penicillin and 100 μg/ml streptomycin and 1% L-glutamine (200 mM) and 1% methanol. CM was used in the cell cultures for the ATP assay and in the MCs. For the MC measurements with the Bionas 2500, measuring medium (MM), i.e. unbuffered CM without $NaHCO_3$ was used.

To measure parathion effects, a dilution series of a parathion stock solution of 34.33 mM parathion in methanol (originally purchased as 10 mg/ml in methanol; LGC Standards GmbH, Wesel, Germany) in SM, CM and MM was used, respectively. The 34.33 mM parathion stock solution was diluted 1:100 in SM, CM and MM, respectively. The resulting concentration (343.3 μM), was subsequently diluted in 1:3 steps starting at 343.3 μM and ending at 1.41 μM for the ATP assays and 4.24 μM for metabolic measurements, respectively. Media containing parathion are designated as SM+, CM+ and MM+ below. The measurements of respiration, acidification and adhesion were conducted in MM or MM+. The cell lysis medium with Triton X 100 (MMT) consisted of 1% Triton X 100 and 99% MM without $NaHCO_3$.

2.2. Cell culture

Pregnant NMRI-mice were obtained from Charles River Laboratory (Sulzfeld, Germany). The mice were allowed to adapt to housing conditions for 8–12 h with conventional rodent chow and water provided *ad libitum*. On gestational day 16, the mice were sacrificed by cervical dislocation without chemical compounds being applied. For our experiments, the uteri were dissected under sterile conditions and the embryos decapitated before cell preparation (for details see Koester et al., 2010). We chose this method to avoid any pharmacological influence on the embryonic cells. All these procedures were approved by the local Animal Care Committee and are in accordance with the European Council Directive of 24 November 1986 (86/609/EEC).

First, experiments were conducted to find the optimal cell-seeding number. For this, 48-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany; max. trough volume up to 1.8 ml; culturing surface 100.4 mm^2) were used because of their geometry, which is similar to that of the MCs (max. trough volume 560 μl, culturing surface: 75.4 mm^2. The MCs were pretreated by cleaning with 5% Contrad solution (Decon Laboratories Inc. PA, USA), disinfected with 70% ethanol, and subsequently rinsed with PBS under sterile conditions.

The following steps were applied to pretreated MCs, as well as to the 48 well plates. Each MC or well was precoated with 150 μl PDL-precoating solution and stored overnight at room temperature. After aspiration of the precoating solution, 150 μl of a laminin solution (0.5 mg/ml diluted 1:20 in DMEM) was transferred into the MCs or wells for 2 h and then aspirated before cell seeding. A maintenance volume of 400 μl was used for wells and MCs. The cell concentration was adjusted using a Neubauer cell-counting chamber. To find the optimal cell-seeding number for cell spreading and neuronal network formation, a serial 1:2 dilution with six steps was prepared starting at a cell concentration of 4.0×10^6 cells/ml. The best results were obtained at a cell concentration of 2.5×10^5 cells/ml after 12 DIV (*days in vitro*). The CM was depleted after less than two DIV at concentrations above 2.5×10^5 cells/ml and much cell debris was found. At concentrations lower than 2.5×10^5 cells/ml, the cells were not able to survive 12 DIV.

For parathion experiments, the cell concentration was adjusted to 5.0×10^5 cells/ml. The cells were seeded out in 200 μl SM onto pre-coated wells or MCs. Thereafter, 200 μl SM for controls and SM+ for the dilution series were added to each well or MC. The cells were cultured at 37 °C, 95% humidity and 10% carbon dioxide for 12 days (Fig. 1). One day after cell seeding, SM and SM+ were completely replaced by CM and CM+, respectively. The cell cultures were maintained by completely renewing the media every second day. The MC measurements were conducted after 12 DIV.

2.3. Dose-range finding

For dose-range finding experiments we used the medium-diluted phenol red as indicator for the metabolic activities of the cells. The experiments were conducted three times in 48-well plates using a serial 1:10 dilution from 0.01 to 1000 μM. This range generously spans the broad LD_{50} value range of rats that had been estimated in Extoxnet (http://extoxnet.orst.edu/pips/parathio.htm) using the molecular weight of parathion and assuming an animal body density of 1 kg/l and 17.2–85.2 μM and 6.9–103.0 μM, respectively (see Section 4). It was expanded towards lower concentrations because *in vitro* experiments are known to be usually more sensitive, which might be explained by a higher number of substance molecules per cell and the lack of detoxification mechanisms in the *in vitro* systems. For dose-range finding experiments, we used the medium-diluted phenol red as indicator of the metabolic activities of the cells. The dose-range finding experiments were designed to reduce the number of MCs required for the IC_{50} determinations.