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Metabolomic changes induced by nicotine in adult zebrafish skeletal muscle

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ABSTRACT

Acute exposure to nicotinic agonists induces myotoxicity in zebrafish embryos. The main goal of this work was to evaluate the potential myotoxicity of nicotine acetylcholine receptor agonists on adult zebrafish muscle tissue by using nicotine as a model compound. Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) datasets were processed with different chemometric tools based on the selection of Regions of Interest (ROI) and Multivariate Curve-Resolution (ROI-MCR procedure) Alternating Least Squares (ALS) for the analysis of different exposure experiments. Analysis of Variance Simultaneous Component Analysis (ASCA) of changes on metabolite peak profile areas showed significant nicotine concentration and exposure time-dependent changes, clearly differentiating between exposed and non-exposed samples and between short (2 h) and long exposure times (6 h or 24 h). Most of the changes observed in the concentrations of different metabolites are probably secondary to the observed hyperlocomotion, as they have been also observed in humans after strenuous muscular exercise. The absence of myotoxicity might be related with the reduced calcium permeability of adult muscle-type nicotinic acetylcholine receptors (nAChRs).

1. Introduction

Electronic cigarettes, which use liquid nicotine, are increasingly popular. Liquid nicotine used in electronic cigarette devices is highly concentrated (6–72 mg/mL), unreliably packaged, and poorly regulated (Gill et al., 2015; Noble et al., 2016). More than 8200 liquid nicotine exposures among children < 6 years old were reported to US poison control centers from January 2012 through April 2017 (Govindarajan et al., 2018). These exposures are concerning because a small volume of concentrated nicotine solution could easily deliver the estimated lethal dose of 6.5–13.0 mg/kg body weight to a young child, 6 and at least 2 young children have already died of liquid nicotine. Thus, e-liquids containing liquid nicotine pose a significant risk to public health, particularly to children, who may be drawn to their bright colors and fragrant flavorings like cherry, chocolate and bubble gum (Gill et al., 2015; Noble et al., 2016).

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels composed of five subunits arranged symmetrically around a central ion-channel pore. Whereas neuronal-type nAChRs are mainly found throughout the peripheral nervous system (PNS) and central system (CNS), muscle-type nAChR is found in vertebrate skeletal muscle, where they mediate neuromuscular transmission at the neuromuscular junctions (NMJs) (Kalamida et al., 2007). Overactivation of these receptors during development with nAChRs agonists, as nicotine, results in severe effects in both neuronal and muscle tissues (Menelaou et al., 2015; Welsh et al., 2009). At the muscle tissue level, developmental exposure to 15–30 μM nicotine induces myotoxicity, with muscle fibers degeneration after the prolonged activation of muscle-type nAChRs (Welsh et al., 2009). Receptor overactivation and the concomitant dramatic rise in muscle calcium could cause this myopathy via excitotoxic mechanisms (Engel et al., 1982, 2003; Gomez et al., 2002). As the permeability to calcium of the fetal and adult muscle-type nAChR is different, further studies should be addressed to analyze the potential myotoxicity of nicotinic agonist in adult fish.

Metabolomics is an emerging technique to evaluate physiological responses associated to drug action, toxic effects, and metabolic disorders (Peng et al., 2015). Many analytical techniques have been used for the detection of different metabolites and other related substances. Nuclear magnetic resonance (NMR) was an early popular approach. This methodology was recently used for determining changes in the metabolomic profile in the brain of mice exposed to nicotine (Li et al., 2014). However, NMR is limited by slow processing speed and poor sensitivity leading the detection of metabolites primarily present in high abundance (Want et al., 2005). Recently, because of its high

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selectivity, sensitivity and enhanced identification capabilities in aquatic organisms, liquid chromatography coupled to mass spectrometers (LC-MS) has showed great potential in the field of metabolomics. Concerning MS instrumentation, high-resolution mass spectrometers (HRMS) are the most powerful analysers due to their ability to improve accurate mass determination (Chen et al., 2016; Gómez-Canela et al., 2016; Knolhoff and Croley, 2016; Su et al., 2016). Zebrafish (*Danio rerio*) is a vertebrate model increasingly used in neurotoxicological research (Faria et al., 2015; Gómez-Canela et al., 2017b; Prats et al., 2017). By using this animal model is it possible to determine the effects of a neurotoxicant at different levels of organization, from molecular to behavioural. The behavioural effects of the exposure of adult zebrafish to 50 μM nicotine for 2 h, 6 h and 24 h have been previously analysed by using the open field test (OFT) paradigm (Gómez-Canela et al., 2017b) showing hyperlocomotion, defined as abnormally fast swimming for an extended period of time (6 h and 24 h after exposure). Moreover, highly mobility duration was significantly higher in animals exposed to nicotine for 24 h. At this time, a positive thigmotaxis, defined as the preference for staying in close proximity to the edge of the arena, was also found.

The aim of this study has been to evaluate the potential myotoxicity of nicotinic agonists by analyzing changes in the metabolic profiles of zebrafish skeletal muscle after exposure to two concentrations of nicotine, 20 μM and 50 μM during 2 h, 6 h and 24 h, by using an Orbitrap analyzer. Orbitrap operates in ultra-high resolution (100,000 at *full width at half maximum*) with exact mass, with a mass accuracy higher than 2 ppm in full scan mode and acquires a mass range between *m/z* 50 and 6000 (Makarov and Scigelova, 2010; Perry et al., 2008). The large amount of data gathered cannot be easily assessed unless chemometric tools are employed to assist in data interpretation. Regions of Interest (ROI) (Gorrochategui et al., 2015) and Multivariate Curve-Resolution-Alternating Least Squares (MCR-ALS) (Peré-Trepat et al., 2005; Tauler, 1995) were employed as general approaches for proper investigation and resolution of complex and extensive LC-MS data sets (in full spectral scan mode). In addition, Analysis of Variance Simultaneous Component Analysis (ASCA) (Jansen et al., 2005; Smilde et al., 2005) was applied to MCR-ALS resolved peak profile areas to investigate what metabolites were more influenced by nicotine levels and exposure time. To our knowledge, this is the first time that changes in metabolites induced by the exposure of nicotine are described by using LC-HRMS.

2. Experimental

2.1. Chemicals and materials

Pure standard metabolites used for the analytical method development (amino acids, sugars, nucleotides, nucleosides and others) as well as nicotine HPLC grade standard (≥ 99%) used for the zebrafish exposures were supplied from Sigma-Aldrich (St. Louis, USA). Stock individual standard solutions (1000 ng μL^{-1}) were prepared dissolving accurate amounts of pure standards in ultra-pure water (HPLC grade). A standard mixture sample of these compounds was prepared at 10 ng μL^{-1} concentration level also in HPLC water. Acetonitrile and HPLC grade water were purchased from Merck (Darmstadt, Germany). Wild-type zebrafish were obtained from Piscicultura Superior (Barcelona, Spain) and maintained in fish water (reverse-osmosis purified water containing 90 μg mL^{-1} of Instant Ocean®, 0.58 mM $CaSO_4 \cdot 2H_2O$) at 28 ± 1 °C under a 12:12 light:dark photoperiod in the Institute of Environmental Assessment and Water Research (IDAEA-CSIC) facilities under standard conditions. All procedures were approved by the Institutional Animal Care and Use Committee at the Research and Development Centre of the Spanish Research Council (CID-CSIC) and conducted in accordance with institutional guidelines under a license from the local government (agreement number 9027).

2.2. Acute toxicity of nicotine

In order to select the nicotine concentrations, acute toxicity was assessed following the Organization for Economic Co-operation and Development (OECD) guideline (Organization for Economic Cooperation and Development (OECD), 1992). Exposure experiments were carried out in Pyrex® beakers, each containing 1 L of Instant Ocean (Aquarium systems, Sarrebourg, France) at 28 °C and ten male adult animals. Live/dead animals were counted after 24-h by gently prodding and observing movement of appendages. Initially, different nicotine concentrations were tested starting with 10, 50, 100 and 150 μM. In a second test, the range of concentrations was bounded between 60 and 90 μM and finally, a last trial was performed between 60 and 70 μM. Controls (fish water only) showed no measurable toxicity. Following this, the test procedure was further optimized by adjusting concentrations to enable a better estimation of LC_{50}. Concentrations where 100%, 50% and 0% of the animals died were repeated two or three times to ensure reliable results. Lethal median concentration effects and its 95% confidence interval (CI) were estimated by fitting immobility concentration responses to the Hill regression model (Eq. (1)).

$$I(C_i) = \frac{1}{1 + \left(\frac{C_i}{LC_{50}}\right)^{-Hill}} \quad (1)$$

where, I(Ci) is the proportion of immobile animals at concentration Ci; Ci is the concentration of the respective compound (i); LC_{50} is the median lethal concentration to the 50% of population and Hill is the shape constant. The 24 h-LC_{50} for nicotine was 65 μM.

The maximum tolerated concentration (MTC) has been defined as the concentration for which no lethality was observed above that seen in vehicle treated siblings (Berghmans et al., 2008). MTC is the preferred concentration for detecting effects minimizing the confounding effect of systemic toxicity (Faria et al., 2018). MTC for nicotine was 50 μM, and this concentration was the highest concentration tested in this study.

2.3. Experimental design

Sexually mature male zebrafish were placed in 4 L aerated glass tanks with fish water at 28 ± 1 °C at a rate of 4 animals L^{-1}. Two concentrations of nicotine were tested in this study, 20 μM and 50 μM, dissolved in fish water. Vehicle controls were carried out in parallel under the same conditions. Five fish were recovered from each tank at 2, 6 and 24 h (with a total of 15 specimens in each tank). Figure SI1 shows the schema of the experimental design used for the nicotine exposure in adults of zebrafish. Animals from each experimental group were anesthetized in ice. Skeletal muscle was obtained from the caudal region, introduced in microcentrifuge tubes, immediately frozen in liquid nitrogen and stored at − 80 °C until extraction. Samples were spiked with 5 ng μL^{-1} of IS (L-methionine sulfone) that was used as extraction and analytical control. Three hundred microliters of a MeOH:H_2O (90:10) mixture was added of each 100 mg of zebrafish muscle dissected. Then, samples were homogenized in an ultrasonic homogenizer (BRANSON Sonifier® 150) during 5 min. After this step, samples were shaken during 20 min in a vibrating plate and then were centrifuged for 10 min at 13,000 rpm to 4 °C. Samples were kept on ice throughout the entire procedure. The supernatant was filtered with 0.20 μM PTFE filters (DISMIC®-13JP, ADVANTEC®) and then kept in amber chromatographic vials at − 80 °C (to avoid any possible degradation) until LC-HRMS analysis.

2.4. LC-HRMS

Metabolites were measured using liquid chromatography coupled to high-resolution mass spectrometry (LC-Orbitrap-MS). An Orbitrap/