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Chlorpyrifos developmental neurotoxicity: Interaction with glucocorticoids in PC12 cells

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ABSTRACT

Prenatal coexposures to glucocorticoids and organophosphate pesticides are widespread. Glucocorticoids are elevated by maternal stress and are commonly given in preterm labor; organophosphate exposures are virtually ubiquitous. We used PC12 cells undergoing neurodifferentiation in order to assess whether dexamethasone enhances the developmental neurotoxicity of chlorpyrifos, focusing on models relevant to human exposures. By themselves, each agent reduced the number of cells and the combined exposure elicited a correspondingly greater effect than with either agent alone. There was no general cytotoxicity, as cell growth was actually enhanced, and again, the combined treatment evoked greater cellular hypertrophy than with the individual compounds. The effects on neurodifferentiation were more complex. Chlorpyrifos alone had a promotional effect on neuritogenesis whereas dexamethasone impaired it; combined treatment showed an overall impairment greater than that seen with dexamethasone alone. The effect of chlorpyrifos on differentiation into specific neurotransmitter phenotypes was shifted by dexamethasone. Either agent alone promoted differentiation into the dopaminergic phenotype at the expense of the cholinergic phenotype. However, in dexamethasone-primed cells, chlorpyrifos actually enhanced cholinergic neurodifferentiation instead of suppressing this phenotype. Our results indicate that developmental exposure to glucocorticoids, either in the context of stress or the therapy of preterm labor, could enhance the developmental neurotoxicity of organophosphates and potentially of other neurotoxicants, as well as producing neurobehavioral outcomes distinct from those seen with either individual agent.

1. Introduction

One of the most critical issues in toxicology is to determine the factors that render specific subpopulations vulnerable to a given toxicant. The organophosphate pesticides provide a prime example. This class represents nearly 50% of worldwide insecticide use and exposure of the human population is virtually ubiquitous (Casida and Quistad, 2004); it is increasingly clear that individuals that carry genetic polymorphisms that reduce the activity of paraoxonase-1 are at greater risk of adverse effects (Costa et al., 2003; Povey, 2010). Nevertheless, there are other factors that govern susceptibility to organophosphate-induced neurotoxicity. In a number of previous studies, we explored the idea that prior exposures to chemicals from other classes that converge on some of the same neurotoxic endpoints as the organophosphates, could sensitize the brain to these pesticides (Aldridge et al., 2005; Meyer et al., 2005; Rhodes et al., 2004; Slotkin and Seidler, 2007); we focused on development because the major concern for the organophosphates is their propensity to elicit fetal and neonatal brain damage, including cell loss, impaired neurodifferentiation, deficient axonogenesis and synapse formation, and defective synaptic transmission, at exposures below the threshold for inhibition of cholinesterase, the mechanism that triggers systemic toxicity (Colborn, 2006; Rosas and Eskenazi, 2008; Slotkin, 2004). In particular, we evaluated the β-adrenergic agonist, terbutaline, a drug used "off-label" inappropriately, for long-term management of preterm labor (U.S. Food and Drug Administration, 2011). Preterm delivery is the largest contributor worldwide to cognitive loss, followed closely by the organophosphates as one of the most critical toxicologic factors (Bellinger, 2012). In evaluating whether the therapies of preterm labor might contribute to adverse neurodevelopmental outcomes, we found that prior exposure to terbutaline sensitized the developing brain to subsequent injury from chlorpyrifos, thus providing a proof-of-principle that early-life chemical exposures can create a subpopulation that is vulnerable to neurotoxicant injury (Aldridge et al., 2005; Meyer et al., 2005; Rhodes et al., 2004; Slotkin and Seidler, 2007).

In the current study, we expanded our investigations to the issue of whether glucocorticoids likewise enhance the neurodevelopmental effects of organophosphates. Glucocorticoid exposure is widespread. First, these agents are given as the consensus treatment for preterm

Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; NGF, nerve growth factor; TH, tyrosine hydroxylase.

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labor occurring between 24 and 34 weeks of gestation in order to prevent respiratory distress syndrome (Gilstrap et al., 1995); currently, one of every ten newborns in the U.S. has undergone this treatment (Matthews et al., 2002). Second, endogenous glucocorticoid release from stress can enhance the systemic toxicity of organophosphates or other toxicants (Aisa et al., 2009; Colomina et al., 1995; Pung et al., 2006; Shaikh et al., 2003). By themselves, excess glucocorticoids have an adverse impact on the numbers of neurons, structure and synaptic connectivity in the immature brain, ultimately contributing to a variety of neuroendocrine, behavioral and cardiovascular disorders (Cavalieri and Cohen, 2006; Drake et al., 2007; Meyer, 1985; Moritz et al., 2005; Pryce et al., 2011; Rokyta et al., 2008; Tegethoff et al., 2009). Here, we examined the interaction between dexamethasone and chlorpyrifos using PC12 cells, a well-established model of neurodifferentiation (Costa, 1998; Fujita et al., 1989; Teng and Greene, 1994) that has been used previously to evaluate the mechanisms of developmental neurotoxicity separately for glucocorticoids and organophosphates (Bagchi et al., 1995; Das and Barone, 1999; Ebert et al., 1997; Jameson et al., 2006a, b; Radio et al., 2008; Slotkin et al., 2007; Slotkin and Seidler, 2012; Song et al., 1998). We chose concentrations of each agent based on prior studies showing robust effects on differentiation without cytotoxicity (Jameson et al., 2006b; Qiao et al., 2001; Song et al., 1998): 100 nM dexamethasone and 10 or 50 μM chlorpyrifos. These concentrations are also appropriate for modeling human exposures. For dexamethasone, the typical levels in preterm infants after direct administration of a relatively low dose of 0.1–0.15 mg/kg is about 600 nM, or 60 nM in the fetus when the treatment is given to the mother (Charles et al., 1993; Osathanondh et al., 1977); as the recommended dose in preterm delivery is about three times higher (Gilstrap et al., 1995), our chosen concentration of 100 nM clearly occupies the therapeutic range. Similarly, our chosen chlorpyrifos concentrations lie near the upper end of levels measured in newborn infants in agricultural communities (Ostrea et al., 2002).

2. Materials and methods

2.1. Cell cultures

Because of the clonal instability of the PC12 cell line (Fujita et al., 1989), the experiments were performed on cells that had undergone fewer than five passages. As described previously (Qiao et al., 2003; Song et al., 1998), PC12 cells (American Type Culture Collection CRL-1721, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum (Sigma Chemical Co., St. Louis, MO), 5% fetal bovine serum (Sigma), and 50 μg/ml penicillin streptomycin (Invitrogen). Incubations were carried out with 5% CO_2 at 37 °C, standard conditions for PC12 cells. To initiate neurodifferentiation (Jameson et al., 2006a; Slotkin et al., 2007; Teng and Greene, 1994), the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (NGF; Promega Corporation, Madison, WI); each culture was examined under a microscope to verify the outgrowth of neurites after NGF treatment.

There were three different exposure paradigms, each using final concentrations of 100 nM dexamethasone (Sigma) and 10 or 50 μM chlorpyrifos (Chem Service, West Chester, PA). For studies of simultaneous exposure during neurodifferentiation, both agents were introduced concurrently with NGF, beginning 24 h after the initial plating, and cells were cultured for an additional 6 days. To determine whether priming of the cells with dexamethasone had a different effect, we first added the dexamethasone simultaneously with NGF but delayed the chlorpyrifos exposure to begin 24 h later; determinations were made 6 days after starting dexamethasone (i.e. 24 h pretreatment with dexamethasone followed by 5 days of co-treatment with dexamethasone and chlorpyrifos). In a third set of studies, undifferentiated cells (without NGF) were exposed to dexamethasone starting 24 h after initial plating; after 24 h of exposure, chlorpyrifos was then introduced along with NGF and continued for 5 days of co-exposure with the dexamethasone. For all three exposure protocols, the medium was changed every 48 h with the continued inclusion of NGF, dexamethasone and chlorpyrifos. Because of its limited water solubility, chlorpyrifos was dissolved in dimethylsulfoxide (Sigma; final concentration 0.1%), which was also added to all the samples regardless of treatment; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation (Qiao et al., 2001; Song et al., 1998).

2.2. Assays

Cells were harvested, washed, and the DNA and protein fractions were isolated and analyzed as described previously (Slotkin et al., 2007). Measurements of DNA, total protein and membrane protein were used as biomarkers for cell number, cell growth, and neurite growth (Qiao et al., 2003; Song et al., 1998). Neuronotypic cells contain a single nucleus, so that the DNA content per dish provides a measure of cell number (Winick and Noble, 1965). Since the DNA per cell is constant, cell growth entails an obligatory increase in the total protein per cell (protein/DNA ratio) as well as membrane protein per cell (membrane protein/DNA ratio). If cell growth represents simply an increase in the perikaryal area, then the ratio of membrane to total protein would fall in parallel with the decline in the surface-to-volume ratio (volume increases with the cube of the perikaryal radius, whereas surface area increases with the square of the radius); however, when neurites are formed as a consequence of neurodifferentiation, this produces a specific *rise* in the ratio. Each of these biomarkers has been validated in prior studies by direct measurement of cell number (Powers et al., 2010; Roy et al., 2005), perikaryal area (Roy et al., 2005) and neurite formation (Das and Barone, 1999; Howard et al., 2005; Song et al., 1998).

To assess neurodifferentiation into dopamine and acetylcholine phenotypes, we assayed the activities of tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively (Jameson et al., 2006a, b). TH activity was measured using [^{14}C]tyrosine as a substrate and trapping the evolved $^{14}CO_2$ after decarboxylation coupled to L-aromatic amino acid decarboxylase. Each assay contained 55 μM [1-^{14}C]L-tyrosine (Moravek Biochemicals, Brea, CA; specific activity, 51 mCi/mmol, diluted to 3.3 mCi/mmol with unlabeled tyrosine) as substrate and activity was calculated as pmol synthesized per hour per μg DNA (i.e. activity per cell). ChAT assays utilized a substrate of 50 μM [^{14}C]acetyl-coenzyme A (specific activity 60 mCi/mmol; PerkinElmer Life Sciences, Waltham, MA). Labeled acetylcholine was extracted and activity was calculated on the same basis as for TH.

2.3. Data analysis

All studies were performed using 2–3 separate batches of cells, with multiple independent cultures for each treatment in each batch ($n = 8–12$); each batch of cells comprised a separately prepared, frozen and thawed passage. Results are presented as mean ± SE, with treatment comparisons carried out by multivariate analysis of variance (ANOVA; data log-transformed when variance was heterogeneous) followed by Fisher's Protected Least Significant Difference Test for post-hoc comparisons of individual treatments. Each treatment paradigm involved an initial three-factor ANOVA: factor 1 = with vs. without dexamethasone; factor 2 = chlorpyrifos concentration (0, 10 μM, 50 μM); factor 3 = cell batch. In each case, we found that the treatment effects were the same across the different batches of cells, although the absolute values differed from batch to batch. Accordingly, we normalized the results across batches prior to combining them for presentation. Significance was assumed at $p < 0.05$.