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DEVELOPMENTAL EXPOSURE TO ORGANOPHOSPHATES TRIGGERS TRANSCRIPTIONAL CHANGES IN GENES ASSOCIATED WITH PARKINSON'S DISEASE IN VITRO AND IN VIVO

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Abstract

Epidemiologic studies support a connection between organophosphate pesticide exposures and subsequent risk of Parkinson's Disease (PD). We used differentiating, neuronotypic PC12 cells to compare organophosphates (chlorpyrifos, diazinon), an organochlorine (dieldrin) and a metal (Ni^{2+}) for their effects on the transcription of PD-related genes. Both of the organophosphates elicited significant changes in gene expression but with differing patterns: chlorpyrifos evoked both up- and downregulation whereas diazinon elicited overall reductions in expression. Dieldrin was without effect but Ni^{2+} produced a pattern resembling that of diazinon. We then exposed neonatal rats to chlorpyrifos or diazinon for the first four days after birth and examined the expression of PD-related genes in the brainstem and forebrain. Chlorpyrifos had no significant effect whereas diazinon produced significant increases and decreases in expression of the same PD genes that were targeted in vitro. Our results provide some of the first evidence for a mechanistic relationship between developmental organophosphate exposure and the genes known to confer PD risk in humans; but they also point to disparities between different organophosphates that reinforce the concept that their neurotoxic actions do not rest solely on their shared property as cholinesterase inhibitors. The parallel effects of diazinon and Ni^{2+} also show how otherwise unrelated developmental neurotoxicants can nevertheless produce similar outcomes by converging on common molecular pathways, further suggesting a need to examine metals such as Ni^{2+} as potential contributors to PD risk.

Keywords

Chlorpyrifos; Developmental neurotoxicity; Diazinon; Dieldrin; Gene transcription; Microarray; Nickel; Organophosphate insecticides; Organochlorine insecticides; Parkinson's disease

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INTRODUCTION

There is increasing evidence that many, if not most cases of Parkinson's disease (PD) involve a complex interaction of underlying genetic susceptibilities with environmental exposures to toxicants that lead to the loss of dopaminergic neurons of the nigrostriatal pathway. Occupational exposures to organophosphate insecticides are highly associated with increased risk of PD [13,18,21]. However, this class of insecticides is particularly notable for developmental neurotoxicity because the immature brain is far more susceptible to damage by organophosphates than is the adult [25,30,34]; indeed, early-life exposures that cause damage to PD-related brain structures may actually provide the key factor that leads to later emergence of the disorder [21]. Organophosphates are known to target dopamine neurons in the striatum [4,10,19,23,48] and to evoke oxidative stress, to which these neurons are especially susceptible [12,19,22,39,40].

Recent studies identified a number of genes that increase the risk of developing PD [1,6,8,9,14,15,24,55,56]. These findings raise the question of whether the same genes involved in familial PD risk are targeted by environmental factors associated with PD, and specifically whether this is triggered by early-life organophosphate exposure; this could readily provide a specific gene-environment interaction culminating in PD. In the present study, we explored this possibility for two commonly-used organophosphates, chlorpyrifos and diazinon, both of which have been well-characterized as developmental neurotoxicants [3,25,29,34,35]. Despite their similarities as cholinesterase inhibitors, chlorpyrifos and diazinon differ in their propensity to elicit oxidative stress [31,40,41] and therefore could have different effects on PD-related endpoints. For our evaluations, we used PC12 cells, a classical in vitro model for neuronal development [52] that has already been validated to reproduce the mechanisms and outcomes found after in vivo organophosphate exposures [26,27,38,39,41,42,46,47,51]. We evaluated gene expression patterns with cDNA microarrays, using a planned comparisons approach that focused on the relevant PD-related genes, rather than performing a blanket evaluation of the entire transcriptome [40,43,45–47]. We also contrasted the effects of the organophosphates to two otherwise unrelated neurotoxicants, an organochlorine (dieldrin) and a metal (divalent nickel). Dieldrin and nickel both cause oxidative stress targeting the dopaminergic phenotype [20,32,39,41,43] and organochlorines may also increase PD risk [13,53]. Finally, we contrasted the in vitro effects of chlorpyrifos and diazinon to those obtained in vivo in the developing rat brain after neonatal exposure, so as to determine the relative contributions of direct neurotoxicant effects on gene expression in isolated cells as compared to those that depend on more complex processes involving the intact brain.

MATERIALS AND METHODS

Cell cultures

Because of the clonal instability of the PC12 cell line [11], the experiments were performed on cells that had undergone fewer than five passages. As described previously [28,51], PC12 cells (American Type Culture Collection, 1721.1-CRL, 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% inactivated horse serum (Sigma Chemical Co., St. Louis, MO), 5% inactivated fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen). Incubations were carried out with 7.5% CO₂ at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation [16,39,52] twenty-four hours after seeding, the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (Invitrogen). Along with the nerve growth factor, we added 30 µM of each of the test agents: chlorpyrifos (Chem Service, West Chester, PA), diazinon (Chem Service), dieldrin (Chem Service) or NiCl₂ (Sigma). The concentration was

chosen from earlier studies that demonstrated adverse effects on differentiation of PC12 cells without outright cytotoxicity [17,26,39,46]. Because of the limited water solubility of the three insecticides, these agents were dissolved in dimethylsulfoxide (final concentration 0.1%), which was also added to the control cultures and to cultures containing NiCl₂; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation [26,28,51]. Cultures were examined 24 and 72 hr after commencing exposure, with 5–8 independent cultures evaluated for each treatment at each time point. Each culture was run on a separate array. We used two time points so as to be able to evaluate changes in gene expression regardless of whether the mRNA for a given gene has a rapid turnover (and hence can rise rapidly) or a slower turnover that would require a longer period to show corresponding increases or decreases. For chlorpyrifos, we evaluated the effects both on undifferentiated cells (without addition of nerve growth factor) and during differentiation, whereas for the other agents, we studied the effects only during differentiation.

Animal treatments

No additional animal studies were conducted for this work. Instead, we mined gene expression data from microarrays used in earlier studies of the effects of chlorpyrifos and diazinon in neonatal rats [40]; here, we focused on the PD-related genes, which were not evaluated heretofore. Details of the animal treatments have been published [40] and therefore will be described only briefly. Neonatal rats received daily injections of chlorpyrifos or diazinon in dimethylsulfoxide vehicle on postnatal days 1–4, whereas control animals received equivalent injections of the vehicle. For both agents, we utilized doses below the threshold for growth retardation and the first signs of systemic toxicity [5,36,54]: 1 mg/kg for chlorpyrifos and 1 or 2 mg/kg for diazinon. The chlorpyrifos treatment and the higher dose of diazinon elicit less than 20% cholinesterase inhibition, well below the 70% threshold necessary for symptoms of cholinergic hyperstimulation [7], whereas the lower dose of diazinon produces no measurable inhibition [33,34,49,50,54]. On postnatal day 5 (24 hr after the last dose), one male pup was selected from each of five litters in each treatment group and separate determinations were made for the forebrain and brainstem from each animal. The two regions were derived by sectioning rostral to the thalamus, so that the area designated as “brainstem” also contains the midbrain (including the substantia nigra), the locus of the dopamine cell bodies that are affected in PD, whereas the “forebrain” contains the corpus striatum, the region to which those neurons project.

Microarray determinations

Our earlier studies detailed all the techniques for mRNA isolation, preparation of cDNA, conversion to cRNA incorporating cyanine-3 (reference RNA) or cyanine-5 (sample RNA), verification of RNA purity and quality, hybridization to the microarrays, washing and scanning [40,45,46]. These all involve commercial kits and standardized procedures, and since the current studies were done identically, the techniques will not be described here. The mRNA used for the reference standard was created by pooling aliquots from each of the samples in the study so as to ensure measurable levels of all genes expressed over the background. Array normalizations and error detection were also carried out by standard procedures described previously [40,45,46]. We used Agilent Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA), type G4131A for the studies of chlorpyrifos in undifferentiated and differentiating cells and for the *in vivo* studies, whereas type G4131F was used for the studies of diazinon, dieldrin and Ni²⁺ in differentiating cells. The two chips contain exactly the same gene sequences but the latter has a lower detection threshold; however, all the genes reported here passed the quality control filters with both arrays.

The genes selected for examination were compiled from the published literature identifying the genes associated with PD [1,6,8,9,14,15,24,55,56], along with the Parkinson's Disease

Mutation Database maintained by The Parkinson's Institute and Clinical Center, Sunnyvale, CA

(http://www.thepi.org/index.php?submenu=BasicResearch&src=gendocs&ref=MutationIntro&category=Basic_Research).

For many of the genes, the arrays contain multiple probes and/or replicates of the same probe in different locations on the chip, and these were used to verify the reliability of values and the validity of the measures on the chip. In these cases, to avoid artificially inflating the number of genes utilized for statistical analysis, we limited each gene to a single set of values, selecting those obtained for the probe showing the smallest intragroup variance. The other values for that gene were used only to corroborate direction and magnitude of change. We also validated the readings on the arrays through the use of duplicate arrays for one sample selected from each treatment group [40,45].

Statistical procedures

Because of the requirement to normalize the data across arrays and within each gene, the absolute values for a given gene are meaningless, so only the relative differences between treatments can be compared. Accordingly, results are presented as means and standard errors of the percentage change from control values to allow for visual comparison of the effects across families of genes. However, statistical comparisons were based on the actual ratios (log-transformed, since the data are in the form of ratios) rather than the percent change.

For the in vitro studies, our design involved planned comparisons of four agents at two time points, as well as the effects of one agent (chlorpyrifos) in undifferentiated vs. differentiating states. It was therefore important to protect against the increased probability of type 1 errors engendered by repeated testing of the same data base. Accordingly, before looking for effects on individual genes, we performed a global ANOVA incorporating all the variables in a single comparison: treatment, time, and all genes involved in the planned comparisons. Lower-order ANOVAs on subdivisions of the data set were then carried out as permitted by the interactions of treatment with the other variables; however, where there were no interactions, we report only main treatment effects without further subdivision. Differences for individual treatments were evaluated with Fisher's Protected Least Significant Difference test. Similarly, for the in vivo evaluations, there were factors of treatment, brain region and gene, requiring an initial multivariate ANOVA with lower-order tests as required by treatment interactions with the other variables. For all tests, treatment effects were considered significant at $p < 0.05$ (two-tailed, since we were interested in both increases and decreases in gene expression).

In addition to these parametric tests of the direction and magnitude of changes in gene expression, we evaluated the incidence of significant differences as compared to the predicted false positive rate, using Fisher's Exact Test; we used a one-tailed criterion of $p < 0.05$, since finding a significant decrease in the incidence of detected differences relative to the false positive rate would be biologically implausible and statistically meaningless. At a criterion of $p < 0.05$, only one gene out of every 20 tested can be expected to show a difference at random.

RESULTS

The microarrays detected 21 PD-related genes that passed the quality-control filters and that were expressed in both the cell cultures and brain samples (Table 1). Several additional PD genes were either not annotated on the microarray or failed the quality control filters: *ccdc62*, *gigyl2* (*park11*), *hladrb5*, *htral1* (*park13*), *omi*.

In vitro effects

Exposure of undifferentiated or differentiating PC12 cells to chlorpyrifos elicited time-dependent changes in gene expression (treatment $\times$ gene $\times$ time, $p < 0.0002$) that were not dependent on the differentiation state of the cells (no treatment $\times$ state interaction). Initially, after 24h of exposure, there were no significant overall changes (Figure 1A) but by 72h there were gene-specific effects (treatment $\times$ gene, $p < 0.0001$, Figure 1B). Two genes were robustly upregulated (*acmsd* and *snca*), whereas four others showed significant decrements (*atp13a*, *bst1*, *gba3*, *lamp3*). The overall incidence of significant gene expression changes, 6 out of 22 genes, was significantly greater than the expected false positive rate of 1 gene ($p < 0.05$, Fisher's Exact Test).

Like chlorpyrifos, exposure of differentiating PC12 cells to diazinon also produced significant time-dependent changes (treatment $\times$ time, $p < 0.02$) in the expression of PD-related genes (Figure 2A). Again, there were no significant effects after 24h of exposure but overall reductions emerged by 72h, reflecting a main treatment effect ($p < 0.004$) without distinction among the various genes (no treatment $\times$ gene interaction). In contrast, the organochlorine, dieldrin, failed to produce any significant changes in PD-related gene expression (Figure 2B). Exposure to Ni^{2+} produced a pattern similar to that of diazinon, with an overall significant decrement without distinction among the various genes (Figure 2C); however, the effect of Ni^{2+} did not display time-dependence, reflecting a more immediate onset of effects than with diazinon, so that effects at 24h and 72h were indistinguishable from each other (no treatment $\times$ time interaction).

In vivo effects

Treatment of neonatal rats with chlorpyrifos for the first four days after birth did not evoke significant changes in PD-related gene expression in the forebrain or brainstem assessed on postnatal day 5 (Figure 3A). However, diazinon elicited significant, gene-specific effects at either dose (treatment $\times$ gene interaction, $p < 0.0001$). At 1 mg/kg (Figure 3B), diazinon exposure evoked significant changes in the expression of 9 out of the 21 genes ($p < 0.005$ vs. the expected false positive rate of 1 gene), with upregulation of *acmsd* and *lamp3*, and downregulation of *atp13a*, *gak*, *hip1r*, *park2*, *park7*, *pink1* and *pla2g6*. At the 2 mg/kg dose (Figure 3C), we again saw changes in 9 out of the 21 genes, with increases for *acmsd* and *lamp3*, and decreases for *atp13a*, *bst1*, *gak*, *hip14*, *park2*, *park7* and *pla2g6*. Effects on the forebrain and brainstem were equivalent overall (no treatment $\times$ region interaction).

DISCUSSION

The results shown here provide direct evidence that early-life exposure to organophosphates targets the expression of the same family of genes that are related to the emergence of PD in humans. There are two important ramifications of these findings. First, they reinforce a mechanistic connection between such exposures and the subsequent risk of PD, thus providing a biological basis for epidemiologic findings [8,13,18,21]. Second, the convergence of organophosphate effects on the same genes that confirm PD risk supports the idea that there is a specific subpopulation (i.e. those with gene variants that confer risk) that may be particularly vulnerable to the effects of organophosphates or other environmental toxicants, leading ultimately to PD or other neurodegenerative disorders.

Our findings point to both direct and indirect mechanisms by which organophosphates converge on PD-related genes. The studies in vitro with the PC12 model revealed time-dependent effects on gene expression for both chlorpyrifos and diazinon but with distinctly different profiles. Chlorpyrifos produced a mixed pattern of up- and down-regulation whereas diazinon uniformly reduced expression of PD-related genes. Importantly, this

indicates that the effects are unrelated to cholinesterase inhibition, the shared property of all organophosphates. This conclusion was further reinforced by the divergent *in vivo* effects of chlorpyrifos and diazinon *in vivo*, even though we matched the doses to produce precisely the same, low degree of cholinesterase inhibition [49,50]. Indeed, there are prominent disparities between chlorpyrifos and diazinon in their abilities to elicit oxidative stress or excitotoxicity that are likely to contribute to fundamental differences in their effects on PD-related gene expression [31,37,41]. In turn, this suggests that exposure assessments in humans may similarly reveal selective relationships of exposure to different organophosphate pesticides and the risk of PD. The *in vitro* studies also indicated a relative lack of dependence of the effects of chlorpyrifos on the differentiation state of the cells, in direct contrast to effects on other gene families more closely related to neurodifferentiation *per se* [37,41–43,46,47]. This suggests that the impact on PD-related genes is not likely to be restricted to a specific developmental window.

If all the effects of organophosphates on gene expression represented direct actions at the cellular level, then we would expect to see the same pattern after exposure *in vivo*. However, for chlorpyrifos, we failed to see any significant changes in gene expression on postnatal day 5, 24 hours after the last dose of a four-day exposure regimen. In contrast, diazinon did produce significant effects but these were not all in the downward direction as would have been predicted solely from the *in vitro* findings. The apparent dichotomies between *in vitro* and *in vivo* findings have an important caveat in that we examined only a single time point for the *in vivo* exposures. In the case of chlorpyrifos, it is certainly conceivable that effects emerge later than at the 24 hour posttreatment time point, especially given that the effects *in vitro* were not immediate; indeed, this chlorpyrifos exposure paradigm is known to produce lasting hyperexcitability of striatal dopamine neurons [2,48]. This is reinforced by the findings for diazinon *in vivo*, which evoked both up- and down-regulation of PD-related genes, whereas the direct effects *in vitro* showed consistent reductions. Thus, the values at 24 hours posttreatment *in vivo* could represent rebound increases for some of the same genes that showed initial suppression as a direct effect of diazinon. Comparing these complex time-course relationships between the *in vitro* and *in vivo* models is further complicated by the fact that the cultures were exposed continuously, whereas the animals received episodic exposures (daily treatment). Notwithstanding these detailed differences, the two main conclusions are that the PD-related genes are indeed targeted by diazinon *in vivo* and that, reinforcing the *in vitro* findings, there are distinct differences between chlorpyrifos and diazinon that are likely to be of biologic significance in their impact on PD. Nevertheless, the dichotomy between the effects of chlorpyrifos *in vitro* and *in vivo* should provide a cautionary note to proposals that cell culture systems can entirely replace animal studies to detect developmental neurotoxicity.

Our *in vitro* studies also point to potentially important conclusions about other classes of toxicants. Although organochlorines have also been implicated in PD [13,53], dieldrin did not evoke any of the changes in gene expression seen with the organophosphates. This implies that PD risk associated with organochlorines will likely not share the same gene-environment relationship seen for the organophosphates; specifically, we would predict a weaker relationship for the effects of organochlorine exposures in subpopulations containing the at-risk PD genes, than would be seen for comparable organophosphate exposures. Further, the fact that Ni^{2+} elicited a pattern of PD-related gene expression resembling that of diazinon points to the need to explore whether this toxicant increases the risk of PD in humans. Indeed, we have previously noted convergent effects of diazinon and Ni^{2+} on gene pathways mediating several different components of neurotoxicity [41–44,47], again indicating how otherwise unrelated neurotoxicants can nevertheless converge on common final pathways to produce similar outcomes. In this case, the resemblance between diazinon and Ni^{2+} , combined with the dichotomy between diazinon and chlorpyrifos, reinforce the

fact that organophosphates exert toxicant actions through mechanisms other than their shared property as cholinesterase inhibitors.

Our results provide some of the first mechanistic connections for gene-environment interactions that connect organophosphate exposure to subpopulations that are vulnerable to developing PD. The approach taken here points to how transcriptional profiling that combines in vitro and in vivo comparisons, can contribute to deconvoluting complex gene-environment interactions related to specific disease outcomes.

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Abbreviations

ANOVA	analysis of variance
PD	Parkinson's disease

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Highlights

- Organophosphate pesticides target expression of genes related to Parkinson's Disease
- Effects were seen in vivo in neonatal rat brain regions, and in PC12 cells
- These results provide a mechanistic link for the association of pesticide exposure with PD

Figure 1.

Effects of chlorpyrifos exposure on expression of PD-related genes in undifferentiated and differentiating PC12 cells. Multivariate ANOVA (factors of treatment, differentiation state, time, gene) indicated a treatment $\times$ time $\times$ gene interaction ($p < 0.0002$). Accordingly, values were separated into the 24h (A) and 72h (B) time points and reexamined for lower-order treatment effects, which appear at the top of each panel. There was no significant effect at 24h but the treatment $\times$ gene interaction was maintained at 72h; for the latter, asterisks shown below each gene denote a significant main treatment effect. Note that we did not conduct separate evaluations for differentiation state because of the absence of a treatment $\times$ state interaction. NS = not significant.

Figure 2. Effects of (A) diazinon, (B) dieldrin, or (C) Ni²⁺ exposure on expression of PD-related genes in differentiating PC12 cells. Multivariate ANOVA (factors of treatment, time, gene) across all treatments indicated a main treatment effect ($p < 0.0002$) as well as an interaction of treatment $\times$ time ($p < 0.02$). Accordingly, values were separated into the three different exposures and reexamined for treatment effects, which appear at the top of each panel. For diazinon (A), there was a treatment $\times$ time interaction, so a lower-order test was conducted that identified a significant overall decrement at 72h. For Ni²⁺ (C), there was a main treatment effect with no interactions, so only the overall decrement is shown. Note that we did not conduct separate evaluations for each gene because of the absence of a treatment $\times$ gene interaction. NS = not significant.

Figure 3. Effects of in vivo exposure to (A) chlorpyrifos 1 mg/kg, (B) diazinon 1 mg/kg or (C) diazinon 2 mg/kg, on expression of PD-related genes in neonatal rat brainstem and forebrain. Each agent was given daily on postnatal days 1–4 and samples were obtained on postnatal day 5. Multivariate ANOVA (treatment, region, gene) indicated a significant treatment $\times$ gene interaction ($p < 0.0002$), so values were separated for the individual exposures and reexamined for treatment effects. Although chlorpyrifos had no overall effect, either dose of diazinon produced a significant treatment $\times$ gene interaction (ANOVA shown at the top of each panel), necessitating lower-order tests for each gene; asterisks indicate values that are significantly different from the control. Note that we did not perform tests separately for each region because of the absence of a treatment $\times$ region interaction. NS = not significant.

Table 1

Parkinson-Related Genes Detected on the Microarrays

Gene	Protein encoded	Genbank Accession Number
<i>acmsd</i>	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	NM_134372
<i>atp13a (park9)</i>	ATPase type 13A	XM_214310
<i>bst1</i>	bone marrow stromal cell antigen 1	NM_030848
<i>fbxo7</i>	F-box only protein 7	NM_001012222
<i>gak</i>	cyclin G associated kinase	NM_031030
<i>gba2</i>	glucosidase beta 2	XM_216490
<i>gba3</i>	glucosidase, beta, acid 3 (cytosolic)	XM_223486
<i>hip1r</i>	huntingtin interacting protein 1 related	AB005052
<i>lamp3</i>	lysosomal-associated membrane protein 3	NM_001012015
<i>lrk2 (park8)</i>	leucine-rich repeat kinase 2	AA818238
<i>mapt</i>	microtubule-associated protein tau	NM_017212
<i>mccc1</i>	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	NM_001009653
<i>park2</i>	parkin	NM_020093
<i>djl (park7)</i>	fertility protein SP22	NM_057143
<i>pink1 (park6)</i>	PTEN induced kinase 1	XM_216565
<i>pla2g6</i>	phospholipase A2, group VI	NM_001005560
<i>snca (park1)</i>	α -synuclein	NM_019169
<i>sncaip</i>	α -synuclein interacting protein (synphilin)	XM_225768
<i>stk39</i>	serine/threonine kinase 39	NM_019362
<i>syt11</i>	synaptotagmin 11	NM_031667
<i>uchl1 (park5)</i>	ubiquitin carboxy-terminal hydrolase L1	NM_017237