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OXIDATIVE STRESS FROM DIVERSE DEVELOPMENTAL NEUROTOXICANTS: ANTIOXIDANTS PROTECT AGAINST LIPID PEROXIDATION WITHOUT PREVENTING CELL LOSS

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Abstract

Oxidative stress has been hypothesized to provide a mechanism by which apparently unrelated chemicals can nevertheless produce similar developmental neurotoxic outcomes. We used differentiating PC12 cells to compare the effects of agents from four different classes and then to evaluate antioxidant amelioration: fipronil, perfluorooctanesulfonamide (PFOSA), dieldrin and chlorpyrifos. The rank order for lipid peroxidation corresponded to the ability to evoke cell loss: fipronil > PFOSA > dieldrin > chlorpyrifos. The same sequence was found for an index of cell enlargement (protein/DNA ratio) but the effects on neurite outgrowth (membrane/total protein) diverged, with fipronil producing a decrease and PFOSA an increase. Cotreatment with antioxidants reduced (ascorbate) or eliminated (Vitamin E) lipid peroxidation caused by each of the agents but failed to protect against cell loss, with the sole exception of chlorpyrifos, for which we earlier showed partial protection by Vitamin E; addition of higher NGF concentrations protected neither against oxidative stress nor cell loss. Despite the failure to prevent cell loss, ascorbate protected the cells from the effects of PFOSA on neuritic outgrowth; NGF, and to a lesser extent, ascorbate, offset the effects of fipronil on both cell enlargement and neuritogenesis. At the same time, the ameliorant treatments also worsened some of the other toxicant effects. Our results point out the problems in concluding that, just because a neurotoxicant produces oxidative stress, antioxidant therapy will be effective in preventing damage. Instead, additional mechanisms for each agent may provide alternative routes to neurotoxicity, or may be additive or synergistic with oxidative stress.

Keywords

Ascorbic acid (Vitamin C); Chlorpyrifos; Dieldrin; Fipronil; Oxidative Stress; PC12 cells; Perfluorooctanesulfonamide; Vitamin E

INTRODUCTION

Thousands of chemicals in active production are thought to be developmental neurotoxicants, and because only a small percentage have been evaluated [3,11], widespread exposures of the

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human population are likely to contribute to what has been termed a “silent pandemic” of neurodevelopmental disorders [11]. Despite underlying differences in the originating mechanisms of action of various neurotoxicants, they often produce convergent outcomes, while at the same time, there are sometimes unexpected disparities between related chemicals from the same class [2,30,42,44,⁴⁵,⁴⁹⁻⁵¹,⁵⁵,57,60,68,69]. One likely route by which different agents produce similar neurotoxic outcomes is through their shared ability to produce oxidative stress [10,13,32,33]. Compared to other tissues, the brain is especially vulnerable to oxidant damage because its membrane lipid composition is enriched in oxidizable polyunsaturated fatty acids [13] and because of its high oxygen consumption. This is exacerbated in the *developing* brain, which faces the increased metabolic demand required for growth, and yet has lower antioxidant reserves [13] and a reduced complement of glia, the cells that protect neurons from oxidative damage [61]. Indeed, many developmental neurotoxicants elicit oxidative stress in the immature central nervous system [15,21,23,31,34,41,62].

In the current study, we explored whether the shared property of oxidative stress accounts for the developmental neurotoxicant profiles of otherwise unrelated agents, and further, whether antioxidant therapies can thus provide protection against toxicants from different classes. We focused on four representative compounds: a phenylpyrazole pesticide (fipronil), a perfluoroalkyl compound (perfluorooctanesulfonamide, PFOSA), an organochlorine pesticide (dieldrin) and an organophosphate pesticide (chlorpyrifos, CPF). Each of these produces oxidative stress, cell loss and altered neurodifferentiation when examined in standard test systems, such as PC12 cells [26,46,48] and each is environmentally important in its own right. Organophosphates represent 50% of world-wide insecticide use [5] and the adverse effects of these agents on brain development, particularly those of CPF [7,8,35,36,42,43], have led to increasing restrictions on their application in the U.S. and other countries. Some of the usages vacated by the organophosphates are being filled by agents that act through different mechanisms, notably fipronil, a phenylpyrazole insecticide that noncompetitively antagonizes GABA_A receptors [6,14] as well as glutamate-activated chloride channels [16,70]; fipronil is widely applied in agriculture, termite/fire ant control and in pet ectoparasite treatment [20, 65]. Dieldrin produces fetal neural damage [66], also interacts with GABA_A channels [4] and elicits oxidative stress and apoptosis in developing neuronal cells [24,25]. Perfluoroalkyls are widely used as surfactants and also are formed as breakdown products of larger polymers in industrial use; they accumulate in the environment due to their chemical stability and general lack of biodegradation [27-29], and also produce developmental neurotoxicity both in vivo [22,27-29] and in vitro [46].

For our evaluations, we used PC12 cells, a well-defined neurodevelopmental model [63] that readily recapitulates key events in neuronal cell development and thus has served to evaluate the effects of a wide variety of neurotoxicants. With the introduction of nerve growth factor (NGF), PC12 cells undergo neurodifferentiation, acquiring distinct neurotransmitter phenotypes, neurotransmitter receptors, neuritic projections and electrical excitability [9,59, 63]. Using the PC12 model, we have already characterized the neurotoxicant profiles of fipronil, PFOSA, dieldrin and CPF, along with their ability to elicit oxidative stress [26,46, 48]. Here, we compared effects on indices of cell number, cell size, neurite outgrowth and lipid peroxidation with each agent alone, and with amelioration treatments involving the antioxidants, ascorbate and Vitamin E. We also assessed the effects of adding a higher concentration of NGF, which supports the survival and differentiation of PC12 cells, to evaluate the possibility that the toxicants compromise its trophic effect. Our evaluations focused on biochemical parameters that define cell number and size. Each neural cell contains only a single nucleus [67], so that the DNA content in the culture dish reflects the total number of cells [59]. Indices of growth were provided by measurements of protein subfractions related to cell size and membrane surface area [17,64]. The total protein/DNA ratio rises with cell enlargement. With the onset of differentiation, the development of neuritic projections

necessitates a rise in the relative contribution of membrane proteins, so that the increase in the membrane/total protein ratio gives an indication of augmented membrane “complexity”. For oxidative stress, we measured the concentration malondialdehyde (MDA) to indicate formation of lipid peroxides.

MATERIALS AND METHODS

Cell cultures

Because of the clonal instability of the PC12 cell line [9], the experiments were performed on cells that had undergone fewer than five passages. As described previously [40,59], PC12 cells (kindly provided by Dr. Patrick Casey, Duke University Medical Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), and 50 µg/ml penicillin streptomycin (Invitrogen, Carlsbad, CA). Incubations were carried out with 5% CO₂ at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation [18,48,63] twenty-four hours after seeding, the medium was changed to include 50 ng/ml of 2.5 S murine nerve growth factor (Promega, Madison, WI). Along with the nerve growth factor, we added each of the test agents in a final concentration of either 30 or 50 µM: fipronil (Chem Service, West Chester, PA), PFOSA (Battelle, Columbus, OH), dieldrin (Chem Service) or CPF (Chem Service). Ameliorants were added simultaneously: 10 µM sodium ascorbate (Sigma), 10 µM Vitamin E (α-tocopherol, Sigma) or NGF at 3× the normal concentration. The concentrations of the neurotoxicants were chosen from earlier studies that demonstrated adverse effects on differentiation of PC12 cells [19,26,38,46,48,56]; for the antioxidants, the 10 µM concentration was based on successful prevention of Ag⁺-induced cell loss by ascorbate [37] and partial protection against CPF by Vitamin E [47]. Because of their limited water solubility, the toxicants were dissolved in 100% dimethylsulfoxide (0.1% final concentration after dilution in the culture medium), and Vitamin E was dissolved in 95% ethanol (0.05% final concentration), and controls for those experiments contained the corresponding vehicle. These vehicle have no effect on PC12 cell growth or differentiation [38,40,59]. The medium was changed every 48 hr, including replacement of all test chemicals, and studies were conducted after six days in culture. Cells were examined with a microscope to verify neurite formation, indicative of neurodifferentiation.

DNA and protein ratios

Cells were harvested, washed, and the DNA and protein fractions isolated and analyzed as described previously [38,40,59]. To prepare the cell membrane fraction, the homogenates were sedimented at 40,000 × g for 10 min and the pellet was washed and resedimented. Aliquots of the final resuspension were then assayed for membrane protein. We then calculated the total protein/DNA ratio as an index of cell size and the membrane/total protein ratio as an index of neuritic outgrowth.

Oxidative stress

We evaluated the degree of lipid peroxidation by assessing the concentration of MDA through its colorimetric reaction with thiobarbituric acid, using a modification [39] of published procedures [12]. To give the MDA concentration per cell, values were calculated relative to the amount of DNA.

Data analysis

All studies were performed in multiple batches of cells, with several independent cultures for each treatment in each batch. Results are presented as mean ± SE, with treatment comparisons carried out by analysis of variance (ANOVA, data log-transformed whenever the variance was

heterogeneous) followed by Fisher's protected least significant difference test for post hoc comparisons of individual treatments. In the initial test, we evaluated two ANOVA factors (treatment, cell batch) and 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 $p < 0.05$.

RESULTS

Fipronil

By themselves, antioxidant treatments evoked a significant reduction in lipid peroxidation, whereas excess NGF had no effect on the concentration of MDA (Fig. 1A). In agreement with earlier findings [26], fipronil elicited robust oxidative stress, increasing MDA levels more than three-fold at 30 μ M and six-fold at 50 μ M. Although ascorbic acid was ineffective in preventing the oxidative stress caused by fipronil, addition of Vitamin E reduced the MDA concentration to normal levels. A three-fold increase of NGF had no effect on the oxidative stress caused by fipronil.

Just as reported in a previous study [26], fipronil evoked a significant loss of cells, as indicated by a decrease in the DNA content of the cultures (Fig. 1B). None of the ameliorant treatments had any impact on this effect; the reductions in DNA were the same regardless of inclusion of ascorbate, Vitamin E or excess NGF.

Ascorbate and elevated NGF concentrations evoked slight, but significant increases in cell size, as indicated by a rise in the total protein/DNA ratio (Fig. 1C). Fipronil evoked a large increase, in keeping with earlier findings [26]. This indicates that the cell loss did not simply represent general cytotoxicity, which would have compromised both cell number and growth. Neither of the antioxidants prevented this effect; however, higher NGF concentrations partially attenuated the increase.

Fipronil reduced the membrane/total protein ratio, indicative of suppressed neurite formation (Fig. 1D), again, recapitulating an earlier report [26]. Ascorbate had equivocal effects on the fipronil response. At the lower fipronil concentration, ascorbate produced a partial protection that was not statistically significant from the fipronil group but was also not significant from the control; at the higher fipronil concentration, a protective effect was no longer apparent. Vitamin E also failed to prevent the fipronil-induced decline in the ratio. However, higher NGF was again protective, in this case completely restoring the membrane/total protein ratio to normal.

PFOSA

In the PFOSA studies, the antioxidants by themselves again produced a significant reduction in MDA levels (Fig. 2A). PFOSA at either 30 or 50 μ M elicited oxidative stress, as indicated by an increase in MDA, just as reported earlier [46], although to a lesser extent than seen with fipronil (note different scales for Fig. 2A vs. 1A). At the lower PFOSA concentration, addition of ascorbate reduced the MDA level to normal whereas Vitamin E decreased it all the way down to the level obtained with Vitamin E alone. At the higher PFOSA concentration, ascorbate was partially effective in reducing lipid peroxidation and Vitamin E more so, again dropping the MDA concentration below that seen in controls. NGF had no salutary effect on lipid peroxidation caused by PFOSA.

PFOSA evoked a significant decrease in DNA content (Fig. 2B), although again the effect was less than with fipronil (note different scales for Fig. 2B vs. 1B). None of the ameliorant treatments prevented the loss of cells caused by PFOSA. Like fipronil, PFOSA evoked cell

enlargement, as evidenced by a rise in the total protein/DNA ratio (Fig. 2C); in this case, the ameliorants actually enhanced the effect of PFOSA, producing uniformly greater increases in the ratio for the groups receiving the combination treatments. Unlike fipronil, PFOSA evoked an *increase* in the membrane/total protein ratio (Fig. 2D), in agreement with earlier findings [46]; for this endpoint, ascorbate provided protection but neither Vitamin E nor excess NGF were effective.

Dieldrin

Just as seen earlier [48], dieldrin evoked oxidative stress (Fig. 3A); cotreatment with ascorbate reduced the MDA concentration back to normal whereas Vitamin E reduced it to even lower values. Dieldrin reduced DNA levels, an effect which was unaltered by any of the ameliorant treatments (Fig. 3B). Similarly, dieldrin evoked a significant increase in the index of cell size, without any protective effect provided by ascorbate, Vitamin E or NGF (Fig. 3C). Dieldrin did not evoke a sufficient change in the membrane/total protein ratio to achieve statistical significance, so we were unable to assess the effects of the ameliorants on this index (data not shown).

Chlorpyrifos

As with the other agents and in agreement with our earlier work [39,47,48], CPF evoked oxidative stress (Fig. 4A) but to a smaller extent than the other agents. Ascorbate provided sufficient protection to reduce the MDA to control levels, whereas NGF did not; although not evaluated here, we showed previously that Vitamin E is even more effective against CPF-induced oxidative stress [47], just as seen in the current study for the other toxicants. Neither ascorbate nor excess NGF provided protection against the loss of cells caused by CPF, and indeed, ascorbate worsened the effect at the lower CPF concentration (Fig. 4B); in contrast, we showed earlier that Vitamin E provides partial protection [47]. The increase in cell size caused by CPF was not reduced by ascorbate or higher NGF and in fact, they exacerbated the effect (Fig. 4C). As with dieldrin, the effect of CPF on membrane/total protein was insufficient to allow for testing of ameliorant effects (data not shown).

DISCUSSION

Although all four agents studied here produced lipid peroxidation and cell loss, our results point to diversity in some of the outcomes and in the ability of antioxidants to protect the cells from the neurotoxicants. The degree of oxidative stress was highly predictive of the rank order for eliciting reductions in cell number, namely, fipronil > PFOSA > dieldrin > CPF. Nevertheless, antioxidant cotreatments, although effective in reducing (ascorbate) or totally eliminating (Vitamin E) the evidence of oxidative stress, were largely ineffective in preventing the decline in DNA. In contrast, we showed earlier that ascorbate protects PC12 cells almost completely from both the oxidative stress *and* cell loss evoked by Ag⁺ [37], and that Vitamin E provides partial protection against the lower concentration of CPF [47]. It is therefore clear that oxidative stress, by itself, does not account for the net adverse outcomes, albeit that it might still provide one of the contributors. Indeed, in earlier work, we showed distinct disparities in the transcriptional responses of antioxidant and excitatory pathways to challenges with oxidative neurotoxicants from different classes [52]. The conclusion is inescapable that the mechanisms contributing to these divergent responses are likely to be more important than the mere presence of oxidative stress itself. Accordingly, antioxidant therapy is unlikely to provide a panacea against neurotoxicant effects, a conclusion that is in agreement with earlier findings for CPF, indicating that protection necessitated antagonizing multiple contributory mechanisms, rather than any single pathway [47]. In turn, this also effectively rules out the possibility that, even though the ameliorants protected against lipid peroxidation, they might not have protected proteins and DNA from oxidative damage, a distinction that would be

unlikely in any circumstance, since lipid peroxidation typically is a more sensitive endpoint. Future examination of the specific mechanisms (other than oxidative stress) that contribute to cell loss should resolve many of these issues; these may include adverse effects on cell replication, promotion of apoptosis, or necrosis [26,47,48,52,54,56,57].

Notwithstanding the general failure of antioxidants to provide blanket protection against cell loss, we did obtain partial amelioration of other actions for some of the agents. Although ascorbate was less effective than Vitamin E in preventing oxidative stress caused by PFOSA, it was more protective against the promotion of neuritic outgrowth. Similarly, excess NGF, which had no impact on lipid peroxidation, offset the cell enlargement and suppression of neurite formation evoked by fipronil, with the latter effect shared to a lesser extent by ascorbate but not by Vitamin E, the most effective antioxidant. Obviously, these ameliorant actions clearly are unrelated to oxidative stress per se and it would be valuable to explore the other cellular mechanisms by which the actions of ascorbate and Vitamin E diverge. In contrast, none of the strategies prevented any of the adverse effects of dieldrin other than the reduction in lipid peroxidation evoked by the antioxidants. Equally important, we also observed situations in which the supposedly protective treatments actually *worsened* the outcome. All the ameliorants exacerbated the cell enlargement caused by PFOSA and the cell loss caused by CPF was exacerbated by addition of ascorbate. Indeed, we showed earlier that ascorbate worsens the developmental neurotoxicity of nicotine, another prooxidant molecule, in the fetal monkey brain in vivo [58]. Our results thus point out the problems in concluding that, just because a neurotoxicant produces oxidative stress, antioxidant therapy will be effective in preventing damage. Instead, additional mechanisms for each agent may provide important alternative routes to neurotoxicity, or may be additive or synergistic with oxidative stress. Indeed, there is significant evidence that shared effects on cell signaling cascades and neurotrophic factors other than NGF, that control cell replication, differentiation and apoptosis are potentially more relevant targets [1,50,51,53-57]. Accordingly, treatments to ameliorate developmental neurotoxicity are likely to require administration of multiple agents, rather than any single approach.

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Abbreviations

ANOVA	analysis of variance
CPF	chlorpyrifos
MDA	malondialdehyde
NGF	nerve growth factor
PFOSA	perfluorooctanesulfonamide

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Figure 1.

Effects of fipronil (FIP) and amelioration by 10 μ M ascorbate (Asc), 10 μ M Vitamin E (VitE) or excess NGF, on indices of lipid peroxidation (A), cell number (B), cell size (C) and neurite outgrowth (D). Data represent mean $\pm$ SE of the number of determinations shown in parentheses. ANOVA appears at the top of each panel and asterisks denote values that differ significantly from the control group.

Figure 2.

Effects of perfluorooctanesulfonamide (PFOSA) and amelioration by 10 μ M ascorbate (Asc), 10 μ M Vitamin E (VitE) or excess NGF, on indices of lipid peroxidation (A), cell number (B), cell size (C) and neurite outgrowth (D). Data represent mean $\pm$ SE of the number of determinations shown in parentheses. ANOVA appears at the top of each panel and asterisks denote values that differ significantly from the control group. Note the different scales in (A) and (B) as compared to Figure 1.

Figure 3. Effects of dieldrin (DLD) and amelioration by 10 μ M ascorbate (Asc), 10 μ M Vitamin E (VitE) or excess NGF, on indices of lipid peroxidation (A), cell number (B) and cell size (C). Data represent mean $\pm$ SE of the number of determinations shown in parentheses. ANOVA appears at the top of each panel and asterisks denote values that differ significantly from the control group. Note the different scales in (A) and (B) as compared to Figure 1.

Figure 4.

Effects of chlorpyrifos (CPF) and amelioration by 10 μ M ascorbate (Asc), 10 μ M Vitamin E (VitE) or excess NGF, on indices of lipid peroxidation (A), cell number (B) and cell size (C). Data represent mean $\pm$ SE of the number of determinations shown in parentheses. ANOVA appears at the top of each panel and asterisks denote values that differ significantly from the control group. Note the different scales in (A) and (B) as compared to Figure 1.