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Organophosphorus pesticides induce cytokine release from differentiated human THP1 cells

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BJ Proskocil, AC Grodzki, AD Fryer, PJ Lein, and DB Jacoby designed the research study.
BJ Proskocil and AC Grodzki analyzed the data.
BJ Proskocil, AC Grodzki, AD Fryer, and PJ Lein wrote the paper.

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RUNNING TITLE: Organophosphorus pesticides stimulate macrophages

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ABSTRACT

Epidemiologic studies link organophosphorus pesticides (OPs) to increased incidence of asthma. In guinea pigs, OP-induced airway hyperreactivity requires macrophages and TNFα. Here, we determine whether OPs interact directly with macrophages to alter cytokine expression or release. Human THP1 cells differentiated into macrophages were exposed to parathion, chlorpyrifos, diazinon, or their oxon, phosphate or phosphorothioate metabolites for 24 h, in the absence or presence of reagents that block cholinergic receptors. TNFα, IL-1β, PDGF and TGFβ mRNA and protein were quantified by qPCR and ELISA, respectively. OP effects on NF-κB, acetylcholinesterase, and intracellular calcium were also measured. Parent OPs and their oxon metabolites upregulated cytokine mRNA and stimulated cytokine release. TNFα release, which was the most robust response, was triggered by parent but not oxon compounds. Cytokine expression was also increased by diethyl dithiophosphate, but not diethyl thiophosphate or diethyl phosphate metabolites. Parent OPs, but not oxon metabolites, activated NF-κB. Parent and oxon metabolites decreased acetylcholinesterase activity, but comparable acetylcholinesterase inhibition by eserine did not mimic OP effects on cytokines. Consistent with non-cholinergic mechanisms of OP effects on macrophages, pharmacologic antagonism of muscarinic or nicotinic receptors did not prevent OP-induced cytokine expression or release. These data indicate that phosphorothioate OP compounds directly stimulate macrophages to release TNFα, potentially via activation of NF-κB, and suggest that therapies that target NF-κB may prevent OP-induced airway hyperreactivity.

KEY WORDS: acetylcholinesterase, calcium, chlorpyrifos, diazinon, macrophages, NF-κB, parathion, phosphorothioates, TNFα

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INTRODUCTION

Organophosphorus pesticides (OPs) are extensively used to control insects in not only agricultural but also suburban and urban settings, and thus human exposure is widespread (1). Acute OP toxicity is mediated by inhibition of acetylcholinesterase (AChE), resulting in overstimulation of nicotinic and muscarinic receptors that triggers cholinergic crisis associated with peripheral and central respiratory paralysis. However, most human exposures involve considerably lower OP concentrations that do not cause cholinergic crisis. Exposure occurs via inhalation, absorption through the skin and eyes, or ingestion (1). For example, in agricultural areas, OPs are tracked into homes where concentrations in house dust correlate with OP metabolites in urine of children in that home (2). Ingestion of OP-contaminated foods is another important source of exposure as indicated by studies in Seattle in which OP metabolites were detected in urine of 99% of children but were no longer detectable when children diets were switched to organic foods (3). In young children, urinary concentrations of OP metabolite correlate with a significant decrease in pulmonary function and increase in other symptoms consistent with asthma (4). In adults, occupational exposure to OPs is associated with wheeze, respiratory dysfunction, and asthma (5), which can persist even after the exposure. Thus, there is widespread human exposure to OPs, and multiple studies have identified an association between OP exposure and chronic respiratory symptoms including asthma.

In the lung, parasympathetic postganglionic nerves release acetylcholine to activate M3 muscarinic receptors on airway smooth muscle to cause bronchoconstriction. Acetylcholine also activates M2 muscarinic receptors on prejunctional parasympathetic nerves to inhibit further release of acetylcholine, thus limiting bronchoconstriction (6). Loss of neuronal M2 receptor function increases acetylcholine release, potentiating vagally-induced bronchoconstriction, and this is associated with asthma (7). We have shown that chlorpyrifos, diazinon and parathion each potentiate bronchoconstriction by inhibiting neuronal M2 receptor function (8-10) independent of AChE inhibition (8, 9). These observations suggest

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that environmental concentrations of OPs that do not cause significant AChE inhibition may, nonetheless, be sufficient to trigger airway hyperreactivity.

OP-induced loss of M2 function and potentiation of bronchoconstriction occur independent of direct effects of OPs on muscarinic receptors in airway nerves (11), suggesting that OPs alter neuronal M2 function downstream of direct effects on non-neuronal cells in the airways. There is increasing recognition that OPs are immunomodulators (5). For example, subacute doses of chlorpyrifos and diazinon increase TNFα and interleukin-6 production in macrophages derived from multiple sources, including lungs (12). Macrophages release cytokines and growth factors known to modulate M2 muscarinic receptor function and/or expression (13-15). We demonstrated that inhibition of macrophages with clodronate or inhibition of TNFα with etanercept each independently protected neuronal M2 receptors and prevented airway hyperreactivity in guinea pigs following exposure to parathion (10). In that same study, we showed that when guinea pig alveolar macrophages were isolated from parathion-treated animals, TNFα and IL-1β mRNA expression was significantly increased, and when alveolar macrophages were isolated from naïve guinea pigs and treated with parathion, IL-1β mRNA expression and TNFα protein release were increased. These previously published data suggest a model in which OPs stimulate macrophages to increase cytokine expression that consequently inhibit neuronal M2 receptor activity to cause airway hyperreactivity. Here, we further tested this hypothesis by determining whether these OPs and their metabolites directly stimulate macrophages to release cytokines and growth factors known to modulate M2 muscarinic receptor expression and/or function. We also investigated the mechanism(s) by which this may occur. Some of these results have been previously reported in the form of abstracts (16-19).

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MATERIALS AND METHODS

Materials: Parathion, chlorpyrifos, diazinon, paraoxon, chlorpyrifos oxon, diazoxon were purchased from Chem Service (West Chester, PA, USA). Diethyl phosphate (DEP) was obtained from Acros Organics (Gell, Belgium). *O*,*O*-diethyl thiophosphate potassium salt (DETP), *O*,*O*-diethyl dithiophosphate (DEDTP), atropine, mecamylamine, eserine, and DMSO were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

THP1 cells. THP1 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Gibco, Waltham, MA, USA) containing 100 I.U. penicillin and 100 μg/ml streptomycin, 10% FBS (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). THP1 cells were differentiated into macrophage-like cells using 25 ng/ml PMA (Sigma-Aldrich) for 48 h (20).

Quantitative polymerase chain reaction (qPCR). THP1 RNA was reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA, USA). cDNA was amplified using QuantiTect SYBR Green (Qiagen, Valencia, CA, USA) on the Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Specific primers were synthesized (Table 1; Integrated DNA Technologies, Coralville, IA, USA), and PCR products were quantified on the 7500 Fast Real-Time PCR System (Applied Biosystems). Relative concentration of mRNA was calculated using a serially diluted sample (21) and normalized to 18S rRNA.

ELISA. TNFα and IL-1β protein were measured in conditioned media on the VersaMax plate-reader (450 nm; Molecular Devices, Sunnyvale, CA, USA). Detection limits are 15.6 pg/ml for TNFα and 3.9 pg/ml for IL-1β (R&D Systems, Minneapolis, MN, USA). Protein concentration was calculated from the slope of a standard curve.

NF-κB activation. THP1-XBlue cells (InvivoGen, San Diego, CA, USA) were maintained in THP1 media supplemented with 100 μg/ml normocin and 200 μg/ml zeocin (InvivoGen). Differentiated THP1-

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XBlue cells (25 ng/ml PMA for 48 h) were exposed to OPs for 24 h and secreted embryonic alkaline phosphatase (SEAP) activity was quantified using Quanti-Blue reagent (InvivoGen) on a SpectraMax spectrophotometer (630 nm; Molecular Devices) as a measure of NF-κB activation.

Acetylcholinesterase assay. AChE activity was determined using the standard Ellman assay (22) with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ASChI) as substrate, and 100 μM tetraisopropyl pyrophosphoramide to inhibit pseudocholinesterase. AChE activity was normalized to protein concentration (BCA assay; Pierce, Rockford, IL, USA).

Data analysis. All data were analyzed by Shapiro-Wilk and D'Agostino & Pearson normality tests. mRNA expression and protein concentration in exposed cultures were graphed as fold-change over controls in each experiment to demonstrate the magnitude of the effect and to account for changes in baseline expression. Data were then analyzed by Kruskal-Wallis (nonparametric one-way ANOVA) and corrected by Dunn's Multiple Comparison Test (Prism 7, GraphPad, La Jolla, CA, USA). NF-κB activity was analyzed by one-way ANOVA on log-transformed data for parathion, chlorpyrifos, chlorpyrifos oxon and diazinon using Tukey's multiple comparison test and for paraoxon and diazoxon using the Kruskal-Wallis and post hoc Dunn's Multiple Comparison Test. AChE activity was analyzed by one-way ANOVA with post hoc Tukey's Multiple Comparison Test. Statistical probability of $p \leq 0.05$ was considered significant. Data are represented as mean ± standard error of the mean (SEM).

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RESULTS

Parent OPs increase TNFα, IL-1β, PDGF, and TGFβ mRNA expression. The parent OPs parathion (Figure 1A, D, G, J; black bars), chlorpyrifos (Figure 1B, E, H, K; black bars), and diazinon (Figure 1C, F, I, L; black bars) concentration-dependently, and significantly, increased TNFα (Figure 1A-C), IL-1β (Figure 1D-F), PDGF (Figure 1G-I), and TGFβ (Figure 1J-L) mRNA expression in differentiated THP1 cells after 24 h of exposure. Diazinon induced the largest increase in cytokine and growth factor mRNA expression (for example, parathion induced a 17-fold increase in IL-1β, while diazinon caused a 143-fold increase in IL-1β).

In contrast, while some concentrations of the oxon metabolites increased cytokine and growth factor mRNA expression (see gray bars for paraoxon in Figure 1A, D, G, J; chlorpyrifos oxon in Figure 1B, E, H, K; and diazoxon in Figure 1C, F, I, L), with the exception of paraoxon effects on TNFα mRNA expression (Figure 1A), the effects did not exhibit classic monotonic concentration-effect relationships. Moreover, the magnitude of the increase in mRNA levels observed in THP1 cells exposed to the oxon metabolites were less than those observed in THP1 cells exposed to the corresponding parent compound.

Parent OPs increase TNFα protein expression in conditioned media. Conditioned media was collected from THP1 cells 24 h after exposure to parathion, chlorpyrifos, or diazinon or their oxon metabolites. Of the proteins examined, only TNFα protein was significantly increased in conditioned media by all three OPs (Figure 1M-O and Supplemental Figure E1). Parathion, chlorpyrifos and diazinon increased TNFα protein at concentrations $\geq$ 30 μM (Figure 1M-O, respectively, black bars). Similar to observations of OP effects on mRNA levels, the largest increase in TNFα protein was seen in diazinon-exposed cells (42-fold increase; Figure 1O). None of the oxon metabolites significantly increased TNFα protein in conditioned media (Figure 1M-O, respectively, gray bars).

IL-1β protein was significantly increased in conditioned media only by diazinon at 100 μM (Supplemental Figure E1C). PDGF protein was significantly decreased by paraoxon at 0.001 μM and 0.1

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μM and chlorpyrifos oxon at 100 μM (Supplemental Figure E1D-E). TGFβ protein was undetectable in media from control or OP-exposed THP1 cells (data not shown).

Parent OPs influence cytokine expression and release at concentrations that do not cause cellular toxicity. A 24 h exposure to parathion, chlorpyrifos, diazinon (Supplemental Figure E2, black bars) or their respective oxon metabolites paraoxon, chlorpyrifos oxon, diazoxon (Supplemental Figure E2, gray bars) over the same concentration range used to assess OP effects on cytokine expression and release had no effect on THP1 mitochondrial function as measured by an MTT assay (Supplemental Figure E2A-C) and membrane integrity as measured by a lactate dehydrogenase (LDH) cytotoxicity assay (Supplemental Figure E2D-F). A live/dead cell assay indicated that some concentrations of the OPs and their oxons had a small, but significant, effect on THP1 cell viability (Supplemental Figure E2G-J). The percentage of living cells after exposure to 30 μM parathion (99.2% ± 1.2), 100 μM parathion (96.2% ± 2.2), 1 μM paraoxon (96.9% ± 1.3), 100 μM paraoxon (90.6% ± 2.8), and 100 μM diazoxon (95.0% ± 1.0) was significantly decreased compared to vehicle treated cells (Supplemental Figure E2G, I). None of the concentrations of chlorpyrifos, chlorpyrifos oxon, nor diazinon decreased cellular viability (Supplemental Figure E2H, I).

A phosphorothioate OP metabolite similarly increases cytokine expression in THP1 cells. Parathion, chlorpyrifos and diazinon are all classified as phosphorothioate OPs. The parent compounds have negligible AChE inhibiting activity, but rather must be oxidized by exchanging a sulfur for an oxygen, to form the oxon, which potently inhibits AChE (23). Oxidation can occur in the environment (24) or via cytochrome P450-mediated metabolism in biological organisms (25). OPs can also be hydrolyzed to form metabolites with no AChE activity, including diethyl phosphate (DEP), diethyl thiophosphate (DETP), or diethyl dithiophosphate (DEDTP; Figure 2A). These metabolites, which are not specific to any one OP, are often measured in urine as biomarkers of general OP exposure (26).

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A significant difference between the molecular structure of parent OPs, which were observed to increase TNFα release, and their oxon metabolites, which had no effect on TNFα release, is the phosphorothioate linkage (P=S) in the parent compounds vs. a phosphate group (P=O) in the oxon metabolites. Therefore, we tested the effect on cytokine expression and TNFα release of phosphorothioate vs. phosphate OP metabolites (Figure 2A). Neither DEP, a non-phosphorothioate, nor DETP, a phosphorothioate, increased TNFα or IL-1β mRNA expression or protein release (Figure 2B-E). These compounds also did not increase PDGF or TGFβ mRNA expression (Supplemental Figure E3A-B). In contrast, DEDTP, a phosphorothioate with two sulfurs, induced a large, significant increase in TNFα and IL-1β mRNA (Figure 2B-C) and a small but still significant, increase in PDGF mRNA expression (Supplemental Figure E3A). DEDTP did not increase TGFβ mRNA expression (Supplemental Figure E3B). Although not significant, DEDTP increased TNFα (Figure 2D) and IL-1β (Figure 2E) protein release.

Parent OPs increase NF-κB activity. We next tested whether OPs activate NF-κB using the NF-κB-reporter cell line THP1-XBlue. After a 24 h exposure, parent OPs parathion, chlorpyrifos, and diazinon each significantly increased NF-κB activation (Figure 3A-C respectively, black bars), while their oxon metabolites did not (Figure 3A-C respectively, gray bars). Parathion showed the largest increase, with peak activation of NF-κB observed at 10 μM (Figure 3A), while the effect of diazinon was minimal (Figure 3C).

OP effects on cytokine expression and release are not mediated by cholinesterase inhibition. Many of the neurotoxic effects of OPs are mediated by AChE inhibition, therefore, AChE activity was measured at the highest concentrations of the OP parent compounds and oxon metabolites tested in the cytokine expression and release studies. At 100 μM, parathion, paraoxon, chlorpyrifos, chlorpyrifos oxon, diazinon, and diazoxon each significantly inhibited AChE activity by approximately 75% compared to AChE activity in vehicle control THP1 cells (Figure 4A). To determine whether AChE inhibition mediated the effects of OPs on cytokine expression, we tested eserine at 100 μM, a concentration that inhibited AChE to a level comparable to that observed in the OP-exposed THP1 cells (Figure 4A). Unlike

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diazinon at 100 μM, eserine at 100 μM did not increase cellular levels of TNFα or IL-1β mRNA (Figure 4B and D) or media levels of TNFα or IL-1β protein (Figure 4C and E).

Pharmacologically antagonizing muscarinic or nicotinic receptors did not prevent diazinon effects on cytokine expression and release. To determine whether muscarinic or nicotinic cholinergic receptors mediate OP-induced cytokine expression, THP1 cells were pretreated with either 100 μM atropine or 100 μM mecamylamine for 1 h prior to addition of 100 μM diazinon for 24 h to block muscarinic or nicotinic receptors, respectively. The IC50 for atropine is in the nanomolar range (27) while the IC50 for mecamylamine is in the low micromolar range (28). Thus at 100 μM, atropine sufficiently blocks muscarinic receptors and mecamylamine blocks nicotinic receptors on macrophages (29, 30). Neither atropine nor mecamylamine blocked upregulation of TNFα or IL-1β mRNA or increased release of TNFα or IL-1β protein into conditioned medium in THP1 cells exposed to diazinon (Figure 5). In the absence of diazinon, neither atropine nor mecamylamine had any effect on cytokine mRNA or protein levels.

OPs and their oxon metabolites do not increase intracellular calcium in THP1 cells. To investigate whether Ca^{2+} mediates OP-induced cytokine expression in macrophages, we measured intracellular calcium levels in THP1 cells loaded with the Fluo4 Ca^{2+} indicator dye immediately prior to acute exposure to OPs (3-100 μM) or their oxon metabolites (0.1-100 μM). None of the three parent OPs or their oxon metabolites increased intracellular Ca^{2+} significantly above baseline levels within 10 min after administration (Supplemental Figure E4). In contrast, ionomycin, added as a positive control following the 10 min exposure to OPs, significantly increased intracellular calcium levels.

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DISCUSSION

Exposure to OPs is linked to increased incidence of asthma and asthma exacerbations and to respiratory dysfunction (5). However, the mechanisms that mediate OP-induced asthma and airway hyperreactivity are not well understood. In guinea pigs, we have shown that OPs cause airway hyperreactivity (8-10) via neuronal M2 muscarinic receptor dysfunction. M2 receptors normally limit ACh release (8-10), and loss of their function leads to increased ACh release and increased bronchoconstriction. Loss of M2 receptor function is reported in some asthma patients (7). We have previously shown that OP-induced M2 dysfunction is mediated by macrophages and TNFα, and that OPs increase TNFα and IL-1β expression in guinea pig alveolar macrophages (10). Here we extend these previous findings with the striking observation that the parent forms of parathion, chlorpyrifos, diazinon, and the diethyl dithiophosphate metabolite, but not the oxon metabolites, directly stimulate macrophages, potentially via NF-κB, to increase release of TNFα. This is significant because TNFα has been reported to downregulate neuronal M2 receptors and increase airway reactivity (10). These data suggest that OPs cause airway hyperreactivity via non-cholinergic mechanisms of immunomodulation, identifying a potential pathway to target for therapeutic interventions to prevent OP-induced airway dysfunction.

TNFα, IL-1β, PDGF, and TGFβ have all been shown to modulate M2 muscarinic receptor expression and/or function (13-15), and because of this, we focused on these cytokines and growth factors for this study. Here we show that although mRNA is increased for all these inflammatory cytokines by OPs, only TNFα protein release into conditioned medium is increased in differentiated THP1 cells by all three OPs. These data confirm a prior study showing enhanced TNFα protein release from isolated guinea pig alveolar macrophages treated *ex vivo* with parathion (10). The link between macrophages and TNFα is important because blocking TNFα *in vivo* prevented OP-induced airway hyperreactivity and protected neuronal M2 muscarinic receptor function in guinea pigs 24 h after OP exposure; whereas blocking IL-1β had no effect on acute OP-induced hyperreactivity (10). We have previously demonstrated that OP-

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induced airway hyperreactivity and M2 dysfunction can persist up to at least 7 days (9), so it may be possible that IL-1β, PDGF, and TGFβ have a role in chronic effects of OP exposure, but this possibility has not been tested. OPs may stimulate macrophages to release other cytokines and factors not investigated here, and future experiments using multiplex analysis may provide insights as to other factors released by OP-stimulated macrophages that may influence lung function.

OP-induced cytokine expression is not the result of macrophage cytotoxicity. THP1 mitochondrial function and plasma membrane integrity were unaffected by exposure to parent OPs or their oxon metabolites. High concentrations of parathion, paraoxon, and diazoxon caused a small, but significant, decrease in THP1 viability as determined using a live/dead cell assay. Similarly, 100 µM chlorpyrifos has been reported to cause minimal cell death in the human monocyte cell line U937 (31), and neither 100 µM chlorpyrifos nor 100 µM diazinon caused significant cell death in human peripheral blood monocytes (32). Collectively, these data show that OPs at concentrations < 100 µM are not overtly cytotoxic to monocytic cells. Importantly, the small decrease in THP1 cell viability observed with high doses of parathion, paraoxon, and diazoxon in this study did not correlate with increased cytokine expression observed in THP1 cells exposed to the parent OPs.

Little is known about the deposition of OPs in human lungs. We could find no published studies measuring OPs or their metabolites in induced sputum, bronchoalveolar lavage, or lung biopsies. Many OPs are lipophilic and can be stored in adipose tissue for days to weeks (33). For example, following oral administration of the OP malathion to rats, the initial highest concentrations were found in blood and muscle, but malathion was stored in adipose tissue (33). The OP fenthion is initially taken up by adipose tissue and does not cause cholinergic symptoms until 24-48 h after exposure (34). Lung surfactant is 90% lipids and may be a reservoir for OPs in the lung. When parathion or paraoxon were infused directly into guinea pig lungs, both chemicals were nearly all retained (35). Although OPs are predominantly metabolized by liver cytochrome P450s, guinea pig lungs express some of the same hepatic cytochrome

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P450s (36) and can locally metabolize OPs (35). Thus, OPs can be retained and metabolized by the lung, where they would come into contact with lung macrophages.

The ability of parent OPs to stimulate differentiated THP1 cells is independent of their ability to inhibit AChE, adding to a growing list of research showing OPs targets molecules other than AChE to cause toxicity. Inhibiting AChE is a property shared by both parent compounds and oxon metabolites, however the oxon forms can be 100-fold more potent (23). Despite this, the oxon metabolites did not stimulate an increase in cytokine expression or release. Additionally, eserine, at a concentration that caused AChE inhibition comparable to that observed in THP1 cells exposed to OPs, did not mimic the effects of OP parent compounds on cytokine expression and release in differentiated THP1 cells. Macrophages express functional nicotinic and muscarinic receptors (37, 38), and OPs are capable of modulating both (39, 40). However, the ability of parent OPs to stimulate differentiated THP1 cells was unaffected by pharmacologic antagonism of nicotinic or muscarinic receptors. Using a calcium indicator dye, we additionally excluded increased calcium influx as a messenger contributing to OP-induced cytokine transcription and release. This is consistent with data collected from guinea pig alveolar macrophages, in which parathion did not induce a calcium influx nor did it potentiate a calcium influx in response to FMLP (Proskocil B, unpublished data), a potent activator of macrophages. Collectively, these data show that the mechanism(s) underlying OP-induced increases in cytokine expression and TNFα release in macrophages does not involve canonical mechanisms of OP toxicity involving AChE inhibition, modulation of cholinergic receptor activity, or changes in intracellular calcium levels.

A striking finding of this study is that the parent OPs, but not their oxon metabolites, stimulated differentiated THP1 cells to release TNFα (Table 2). This confirms previous observations that parathion modestly increased IL-1β mRNA and significantly increased TNFα protein release from cultured guinea pig alveolar macrophages, while paraoxon had no effect (10). Parent OPs are phosphorothioates while their oxon metabolites are phosphodiesters (see chemical structures; Figure 2). We tested whether the

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presence of a sulfur group, which distinguishes the former from the latter, is critical to activation of THP1 cells. Neither diethyl phosphate (DEP; a phosphodiester without a sulfur group) nor diethyl phosphorothioate (DETP; a phosphorothioate with one sulfur group) increased cytokine mRNA expression or protein release from THP1 cells. However, diethyl dithiophosphate (DEDTP; a phosphorothioate with two sulfur groups) significantly increased TNFα, IL-1β, and PDGF mRNA and increased TNFα and IL-1β protein by 75- and 36-fold respectively, although the differences were not significant. Thus, having a phosphorothioate bond and additional sulfur groups appears to increase the potential to stimulate macrophages and may explain why parent OPs stimulate macrophages, while oxon forms do not. DEDTP in urine is widely used as a biomarker of OP exposure but is thought to have little biological activity. Our data suggests that DEDTP directly affects macrophage function. In support of this conclusion, Medina Buelvas, *et al.* (41) reported an increase in alternatively activated (M2) macrophages in lymph nodes of mice treated with intraperitoneal DEDTP for 8 days. These data are important because many OP studies have focused on the oxon metabolites in the context of neurotoxicity. Our data demonstrate that parent OPs and DEDTP may also have important biological effects that are unique from those associated with the neurotoxic oxon metabolites.

The mechanism by which the parent OPs and DEDTP alter cytokine synthesis and release in macrophages remains to be determined. Mac-1 (CD11b/CD18) is a heparin-binding integrin receptor expressed by macrophages and it binds phosphorothioate oligonucleotides (42). A study by Hosoi *et al.* (43), demonstrated that phosphorothioate oligonucleotides were 200 times more potent than phosphodiesters oligonucleotides (of the same size) in inhibiting Mac-1 mediated DNA-dependent kinase activity in a fibroblast cell line. This may be one mechanism by which phosphorothioates, like the parent OPs and DEDTP, interact with macrophages. In support of this possibility, only the parent OPs triggered NF-κB activation in a THP1 NF-κB reporter cell line. We did not determine whether parent OPs activated AP-1 to increase TGF-β and PDGF mRNA expression or whether this increase in growth factors was the result of downstream signaling after NF-κB activation and/or TNFα release. Addressing these questions, as well

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as identifying the NF-κB and AP-1 binding sites in the promoter regions of cytokine genes upregulated by OPs are important questions for future studies. An extensive literature supports a critical role for NF-κB in transducing diverse environmental stimuli to upregulate cytokine expression in inflammatory cells (44). While it has yet to be determined whether blocking NF-κB activation prevents OP effects on cytokine expression and release in macrophages, our data are consistent with this hypothesis.

A limitation to this research is that we did not confirm whether OPs increase cytokine expression in human primary alveolar macrophages. We have previously shown that OPs increase TNFα and IL-1β expression in guinea pig alveolar macrophages isolated from bronchoalveolar lavage. While useful information might be obtained using alveolar macrophages collected from healthy humans; bronchoalveolar lavages are not routinely performed on healthy individuals. Human alveolar macrophages obtained from individuals with pulmonary disease would likely be more activated and a heterogeneous population compared to THP1 cells. In guinea pigs, we have previously shown that if guinea pigs are sensitized to ovalbumin, parathion-induced airway hyperreactivity is significantly enhanced compared to non-sensitized animals (45). Although macrophages were not investigated in that study, we would infer that human alveolar macrophages isolated from asthmatics or atopic individuals may have an enhanced response to OP exposure, which may potentiate airway reactivity. Macrophages differentiated from human peripheral blood monocytes could also be used to investigate OP-induced increase in cytokine expression. THP1 cells are spontaneously immortalized monocytes derived from blood from a child with acute monocytic leukemia. THP1 cells, differentiated THP1 cells, human monocytes, and human macrophages derived from peripheral monocytes have some differences and some similarities in gene expression profiles in response to stimulants (46, 47).

In conclusion, our data suggest a novel mechanism by which OPs induce airway hyperreactivity through stimulation of alveolar macrophages by parent OPs to increase NF-κB activation resulting in TNFα protein release. Although the oxon forms of these pesticides have been long considered to be mediators of

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OP toxicity, our data convincingly demonstrate that parent OP compounds more potently and consistently stimulate macrophages. We further postulate that the phosphorothioate linkage in parent OPs may be important in determining how parent OPs interact with macrophages and perhaps other cells.

Specialized macrophages are located throughout the body and may be similarly affected by OPs. Parent OPs increase IL-6 and TNFα mRNA in cultured microglia (48), a macrophage-like glia cell in the brain, and exposure to OPs has been linked to increased incidence of Parkinson's disease (49) and autism (50). Collectively, this data adds to a growing list of research that show parent OPs have biological effects, especially on cells in the macrophage family, and that these effects are independent of AChE inhibition. Future experiments determining the mechanism by which parent OPs and other phosphorothioates interact with macrophages to induce cytokine expression will provide promising targets for development of therapeutics to prevent deleterious effects of OP exposure on airway function.

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Table 1. Primers

18S rRNA	5’	GTAACCCGTTGAACCCCATT
	3’	CCATCCAATCGGTAGTAGCG
Human TNFα	5’	TCAGCCTCTTCTCCTTCCTG
	3’	TCAGCTTGAGGGTTTGCTAC
Human IL-1β	5’	AAGCTGATGGCCCTAAACAG
	3’	CAGGTCATTCTCCTGGAAGG
Human PDGF	5’	CAGTCAGATCCACAGCATCC
	3’	TCTCGTAAATGACCGTCCTG
Human TGFβ	5’	CAACAATTCCTGGCGATACC
	3’	GTAGTGAACCCGTTGATGTCC

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Table 2. Summary of data

Increased		**PTH** μM	PX μM	**CPF** μM	CPFO μM	**DZN** μM	DZO μM	DEDTP μM	DETP μM	DEP μM
TNFα	mRNA	30, 100	30, 100	30, 100	0.001 and 0.1 30, 100	30, 100	0.001	30 and 300	ns	ns
	Protein	30, 100	ns	30, 100	ns	100	ns	300*	ns	ns
IL-1β	mRNA	30, 100	ns	10, 30, 100	0.001 and 100	10, 30, 100	0.001	30 and 300	ns	ns
	protein	ns	ns	ns	ns	100	ns	300*	ns	ns
PDGF	mRNA	10, 30, 100	0.001, 0.01, 0.1	1, 10, 30, 100	0.001, 0.01, 0.1	10, 20, 100	0.001	30 and 300	ns	ns
	protein	ns	ns	ns	ns	ns	ns			
TGFβ	mRNA	10, 30, 100	100	30, 100	0.001, 0.1, 100	30, 100	ns	ns	ns	ns
	protein	nd	nd	nd	nd	nd	nd			
NF-κB activation		3, 10, 30, 100	ns	30	ns	10	ns			

Shown is a summary of data for parathion (PTH), paraoxon (PX), chlorpyrifos (CPF), chlorpyrifos oxon (CPFO), diazinon (DZN), and diazoxon (DZO) as well as *O*,*O*-diethyl dithiophosphate (DEDTP), *O*,*O*-diethyl thiophosphate potassium salt (DETP), and diethyl phosphate (DEP) on cytokine mRNA and protein release in THP1 cells. Parent compounds are shown in bold; all concentrations are in μM. Data for parathion/oxon, chlorpyrifos/oxon and diazinon/oxon are from Figure 1 and E1. Protein levels for TGF-β were all less than the lower limit of the standard curve (lowest concentration 31.3 ng/ml). Data for NF-κB activation are from Figure 3. Data for DEDTP, DEP, and DETP are from Figure 2 and E3. *DEDTP at 300 μM caused a large increase in TNF*α* and IL-1β but it was not statistically significant. Abbreviations used: 'ns' indicates no significant increase; 'nd' not detected, i.e. below standard curve; 'and' indicates no dose response.

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FIGURE LEGENDS

Figure 1. OPs upregulate cytokine and growth factor mRNA expression and TNFα release in THP1 cells. Differentiated THP1 cells were treated with parathion or paraoxon (A, D, G, J, M), chlorpyrifos or chlorpyrifos oxon (B, E, H, K, N), or diazinon or diazoxon (C, F, I, L, O) for 24 h. Cellular levels of mRNA specific for TNFα (A-C), IL-1β (D-F), PDGF (G-I), and TGFβ (J-L) were quantified by real time PCR and normalized to 18S rRNA. Conditioned media was collected from THP1 cells and quantified by ELISA to quantify the amount of TNFα protein released by the cells into the media (M-O). The effect of OPs on mRNA expression and protein release was expressed as a fold change over mRNA expression or protein release, respectively, in vehicle-treated cells (0.1% DMSO) within each experiment. Data are presented as the mean + SEM (each exposure was performed in triplicate wells; n = 4-10 separate experiments for each exposure). *Significantly different from vehicle control at $p \leq 0.05$.

Figure 2. Influence of phosphorothioate vs. phosphate OP metabolites on TNFα and IL-1β mRNA expression and protein release in THP1 cells. Differentiated THP1 cells were treated with 100 µM chlorpyrifos (CPF) or the OP metabolites diethyl dithiophosphate (DEDTP; 10–300 µM), diethyl thiophosphate potassium salt (DETP; 10–300 µM), or diethyl phosphate (DEP; 10–300 µM) for 24 h. (A) Schematic of OP metabolism. X is the chemical structure that specifically identifies each OP. The effects of CPF vs. OP metabolites on TNFα and IL-1β mRNA (B-C) and protein (D-E) levels in THP1 cells and conditioned media, respectively. The effect on cytokine expression was expressed as a fold change over expression in vehicle controls (0.1% DMSO) within each experiment. Data are presented as the mean + SEM (each exposure was performed in triplicate wells; n = 4 separate experiments). *Significantly different from vehicle control at $p \leq 0.05$.

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Figure 3. Parent OP compounds, but not oxon metabolites, activate NF-κB in THP1 cells. Differentiated THP1-XBlue cells were exposed to parathion or paraoxon (A), chlorpyrifos or chlorpyrifos oxon (B), or diazinon or diazoxon (C) for 24 h. SEAP released into the culture medium was quantified as a measure of NF-κB activation. Values from OP-exposed cells were normalized to controls (0.1% DMSO) and expressed as a percent change from vehicle controls. Data presented as the mean + SEM (n = 8-20 wells per group in 4 different experiments). *Significantly different from vehicle control at $p \leq 0.05$.

Figure 4. OP effects on cytokines in THP1 cells are not mediated by acetylcholinesterase (AChE) inhibition. Differentiated THP1 cells were treated for 24 h with either vehicle (0.2% DMSO), 100 μM of the cholinesterase inhibitor, eserine, or 100 μM of the OPs parathion (PT), paraoxon (PX), chlorpyrifos (CPF), chlorpyrifos oxon (CPFO), diazinon (DZN), or diazoxon (DZO). (A) AChE activity is expressed as activity per min per mg of protein. Data presented as whisker box plots. The horizontal line in each box represents the mean; the lower and upper box limits, the 25th and 75th percentile, respectively; whiskers, the 1–99th percentile (n=4 wells per group in 4 different experiments). Concentrations of DZN and eserine that cause comparable inhibition of AChE activity differentially influence RNA (B, D) and protein (C, E) levels of TNFα (B, C) and IL-1β (D, E) in THP1 cells. The effect of DZN and eserine on mRNA and protein expression is expressed as a fold-change over expression in vehicle control cells (0.2% DMSO) within each experiment. Data are represented as mean + SEM (each exposure was performed in triplicate wells; n = 5 separate experiments for each exposure). *Significantly different from vehicle control at $p \leq 0.05$.

Figure 5. Pharmacologic antagonism of muscarinic or nicotinic acetylcholine receptors has no effect on diazinon-induced cytokine expression in THP1 cells. Differentiated THP1 cells were pre-treated for 1 h with 100 μM atropine (Atr) to block muscarinic receptors or 100 μM mecamylamine (Meca) to block nicotinic receptors prior to the addition of 100 μM diazinon (DZN) or vehicle (veh; 0.1% DMSO) for 24

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h. Cellular levels of TNFα (A) and IL-1β (C) mRNA were quantified by real time PCR and normalized to 18S mRNA. Levels of TNFα (B) and IL-1β (D) protein in conditioned media were quantified by ELISA. The effect of DZN on mRNA and protein expression was expressed as a fold change over expression in vehicle control cells not exposed to atropine or mecamylamine (white bar) within each experiment. Data are presented as the mean + SEM (each exposure was performed in triplicate wells; $n = 4\text{-}5$ separate experiments for each exposure). *Significantly different from vehicle control (white bar) at $p \leq 0.05$.

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Figure 1

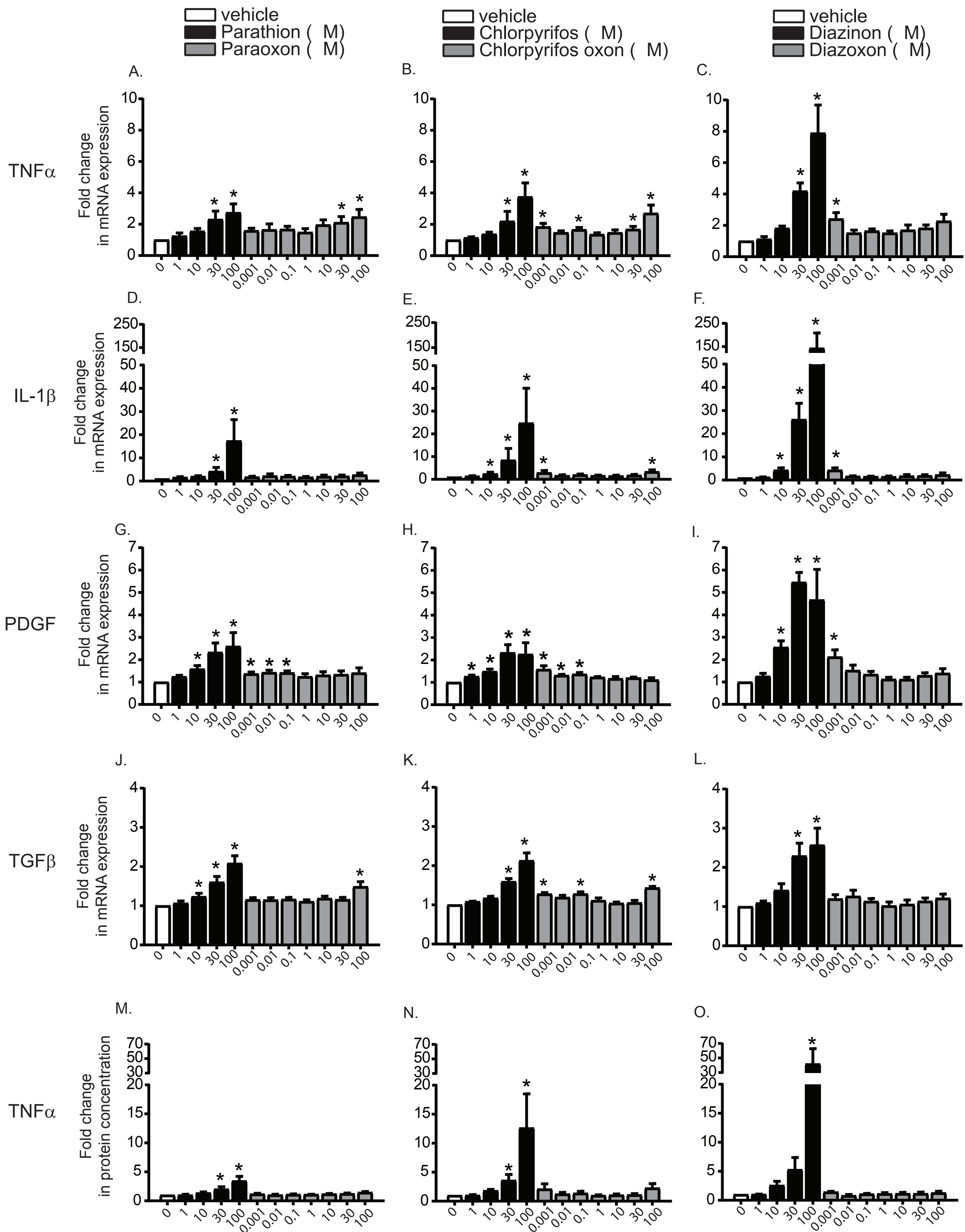

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Figure 2

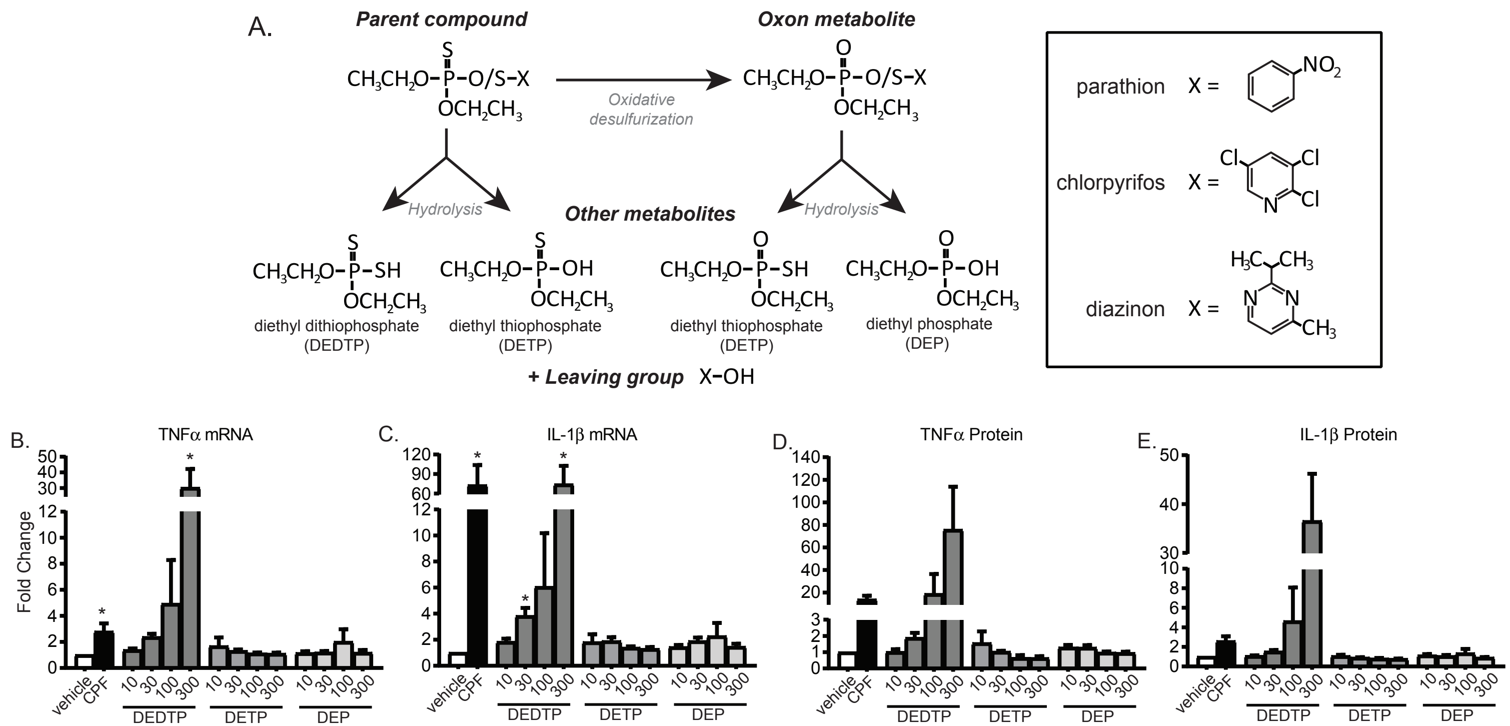

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Figure 3

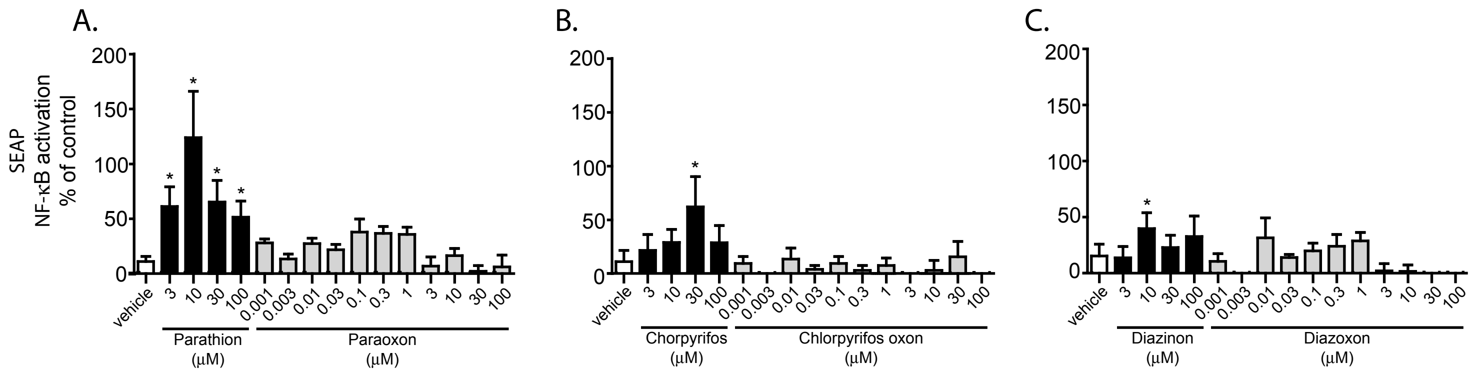

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Figure 4

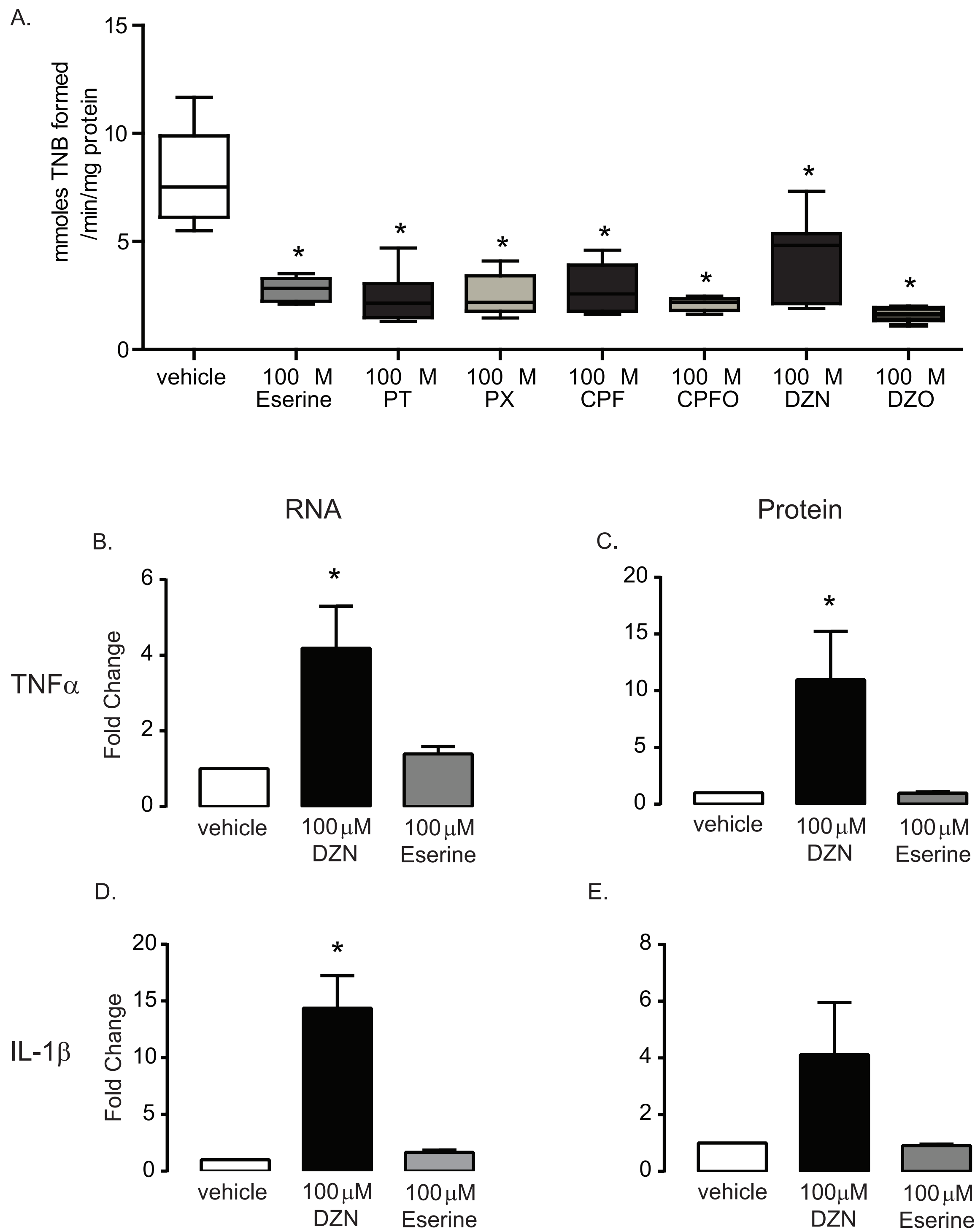

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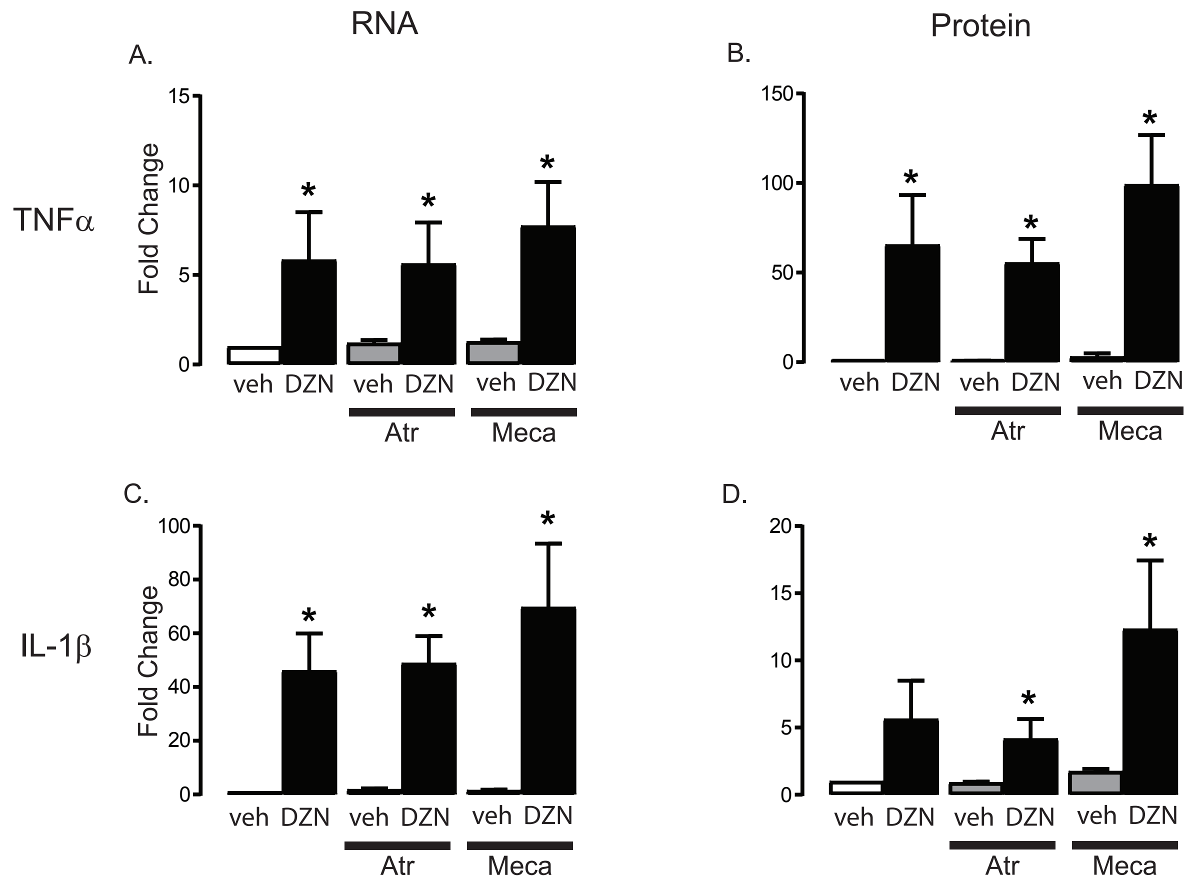
Figure 5



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ONLINE DATA SUPPLEMENT

Organophosphorus pesticides induce cytokine release from differentiated human THP1 cells

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SUPPLEMENTAL METHODS

Organophosphorus pesticides (OPs). Parathion (*O*,*O*-diethyl-*O*-p-nitrophenyl phosphorothioate), paraoxon (diethyl-p-nitrophenylphosphate), chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2yl phosphorothioate), chlorpyrifos oxon (diethyl-3,5,6-trichloro-2-pyridinyl phosphate), diazinon (*O*,*O*-diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate), and diazoxon (diethyl (6-methyl-2-propan-2-ylpyrimidin-4-yl) phosphate) were purchased from Chem Service (West Chester, PA, USA). The OP metabolites DETP (*O*,*O*-diethyl thiophosphate potassium salt) and DEDTP (*O*,*O*-diethyl dithiophosphate) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and DEP (diethyl phosphate) was purchased from Acros Organics (Geel, Belgium). OPs, their oxon metabolites and inactive metabolites were initially diluted or resuspended in DMSO (Sigma-Aldrich) and then further diluted in THP1 media.

THP1 cells. THP1 cells (TIB-202, ATCC, Manassas, VA, USA) were cultured in RPMI-1640 media (Gibco, Waltham, MA, USA) containing 100 I.U. penicillin and 100 µg/ml streptomycin (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA), 10% FBS (Hyclone, GE Healthcare Life Sciences), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) at 37°C in a humidified incubator with 5% CO_2 in air. THP1 cells (passages 3-10) were differentiated into macrophage-like cells by exposing them to 25 ng/ml PMA (Sigma-Aldrich) for 48 h (1). Differentiated THP1 cells are similar to macrophages in that they lose their ability to proliferate, increase phagocytic activity, express macrophage markers, and increase the ability to release cytokines (1). All experiments were conducted using differentiated THP1 cells.

Differentiated THP1 cells were exposed to either parathion, chlorpyrifos, or diazinon (1-100 µM), or with the oxon metabolites of these pesticides (paraoxon, chlorpyrifos oxon, diazoxon; 0.001–100 µM), or with the inactive OP metabolites (DEP, DETP, DEDTP; 10-300 µM) for 24 h. Control cells were exposed to 0.1-0.2% DMSO (vehicle).

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Quantitative polymerase chain reaction (qPCR). THP1 RNA was isolated (RNeasy; Qiagen, Valencia, CA) and reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA, USA). cDNA (in duplicate) was amplified using QuantiTect SYBR Green (Qiagen) on the Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Specific 18S, TNFα, IL-1β, PDGF, and TGFβ primers were synthesized (Table 1; Integrated DNA Technologies, Coralville, IA, USA). PCR products were quantified on the 7500 Fast Real-Time PCR System (Applied Biosystems). For each gene, the relative concentration of mRNA was calculated using a linear regression equation obtained from a serial dilution of a random sample (2) and normalized to 18S rRNA.

Enzyme-linked immunosorbent assay (ELISA). Conditioned media was collected from THP1 cells. Human TNFα, IL-1β, and PDGF were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). Detection limits are 15.6 pg/ml for TNFα, 3.9 pg/ml for IL-1β, and 15.6 pg/ml for PDGF. Absorbance was measured at 450 nm using the VersaMax Plate reader (Molecular Devices, Sunnyvale, CA, USA). Protein concentration was calculated from the slope of a standard curve.

Cytotoxicity MTT Assay. THP1 cells were treated with OPs for 24 h and then with 500 µg/ml MTT at 37°C in 5% CO_2 for 3 h. MTT was then removed and 5% Triton X-100 was added for 2 h. Absorbance was measured at 562 nm using the VersaMax Plate reader.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay. THP1 cells were treated with OPs for 24 h and the supernatant was evaluated for LDH activity using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI) according to the company's protocol. Results were read out at 560nm/590nm on a SpectraMax spectrophotometer (Molecular Devices). 4 independent experiments were analyzed with 6 replicates per condition.

Live/Dead Assay. THP1 cells were treated with OPs for 24 h. After exposure, live/dead assay was performed with 1 µM calcein-AM/1 µg/ml H-33342 for 30 min at 37 °C. Images were acquired

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immediately after the assay using a 20× objective on the ImageXpress MicroXL high-content screening system (Molecular Devices). Images were analyzed using MetaXpress software. A custom data analysis module was created using Custom Module Editor to calculate the percent of live cells labeled simultaneously by calcein (green) and Hoechst 33342 (blue), and excluding the cells labeled only with Hoechst. Nine sites were imaged per well, in 8 wells per condition, in each of 2 independent experiments.

Measuring intracellular Ca^{2+} response. THP1 cells were plated in black wall, optical bottom plates (Becton Dickinson, Franklin Lakes, NJ, USA) for 48 h. Cells were then washed once with Locke's buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, and 0.0001 mM glycine, pH 7.4) supplemented with probenecid (Invitrogen) at 2.5 mM, and loaded with 4 µM Fluo4 (Invitrogen) in the same buffer for 1 h at 37 °C. Probenecid was used to block the efflux of Fluo4 since macrophages express anion transporters in their cell membranes. Cells were rinsed 3 times with Locke's buffer. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) were measured using the Fluorescence Laser Plate Reader (FLIPR; Molecular Devices). Baseline recordings were acquired in Locke's buffer for 2 min prior to adding parent OP compounds (3-100 µM) or their oxon metabolites (0.1-100 µM) using a programmable 96-channel pipetting robotic system. Intracellular Ca^{2+} was monitored for an additional 10 min. Ionomycin (1 µg/ml; Sigma-Aldrich) was added at the end of each treatment to verify cell responsiveness. Changes in intracellular calcium were calculated by measuring changes in dye fluorescence intensity ($\Delta F/F_0$= fluorescence) acquired during exposure minus baseline fluorescence in 6 independent wells per treatment. From the measured $\Delta F/F_0$, the area under the curve (AUC) was calculated.

Data analysis. All data were analyzed by Shapiro-Wilk and D'Agostino & Pearson normality tests. mRNA expression, protein concentration, and MTT absorbance of treated cells was normalized to vehicle controls in each experiment, since baseline cytokine expression varied in control cells depending on passage. Data were analyzed by Kruskal-Wallis (nonparametric one-way ANOVA) and corrected by the Dunn's Multiple Comparison Test (Prism 7, GraphPad, La Jolla, CA, USA). LDH activity and Live/Dead Assay raw data

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were analyzed by one-way ANOVA using Dunnet's multiple comparison test. To analyze changes in intracellular calcium, the area under the curve (AUC) was analyzed by one-way ANOVA and presented as whisker box plots. Statistical probability of $p \leq 0.05$ was considered significant in the ANOVA and post hoc tests. Data are represented as mean ± standard error of the mean (SEM).

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SUPPLEMENTAL FIGURE LEGENDS

Figure E1. Effects of OPs on cytokine and growth factor protein levels in THP1 conditioned media. Differentiated THP1 cells were treated with parathion or paraoxon (A, D), chlorpyrifos or chlorpyrifos oxon (B, E), or diazinon or diazoxon (C, F) for 24 h. Conditioned media were collected from THP1 cells and quantified by ELISA to quantify the amount of IL-1β (A-C) or PDGF (D-F) protein released by cells into the media. The effect of OPs on protein release was expressed as a fold change over protein release from vehicle controls (0.1% DMSO) within each experiment. Data are presented as the mean + SEM (each exposure was performed in triplicate wells; n= 4-10 separate experiments for each exposure). *Significantly different from vehicle control at $p \leq 0.05$.

Figure E2. OPs do not cause cytotoxicity at concentrations that modulate cytokine and growth factor expression. Differentiated THP1 cells were treated with parathion or paraoxon (A, D, G, J), chlorpyrifos or chlorpyrifos oxon (B, E, H), or diazinon or diazoxon (C, F, I) for 24 h. Cell viability was determined by MTT assay, lactate dehydrogenase (LDH) activity assay, and live/dead cell assay. For the MTT assay (A, B, C), values obtained for OP-exposed cells were normalized to vehicle controls (0.1% DMSO) within each experiment and then expressed as a fold change over vehicle control. Each exposure was performed in triplicate wells in 4-10 separate experiments. LDH results (D, E, F) correspond to read out at 560nm/590nm of 4 independent experiments analyzed with 6 replicates per condition, per experiment. The live/dead assay (G, H, I) shows live cells labeled with calcein AM (green) plus Hoescht (blue) (arrows; J) while dying cells are depicted with Hoescht labeled nuclei only (arrowheads; J). Nine sites were imaged per well, in 8 wells per condition, in each of 2 independent experiments. Analysis of the live/dead assay graphs show the % of live cells normalized to vehicle controls. Data are represented as mean + SEM. *Significantly different from vehicle control at $p \leq 0.05$.

Figure E3. Influence of phosphorothioate vs. phosphate OP metabolites on growth factor mRNA expression in THP1 cells. Differentiated THP1 cells were treated with 100 µM chlorpyrifos (CPF) or the

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OP metabolites diethyl dithiophosphate (DEDTP; 10–300 μM), diethyl thiophosphate potassium salt (DETP; 10–300 μM), or diethyl phosphate (DEP; 10–300 μM) for 24 h. The effects of CPF vs. OP metabolites on PDGF (A) and TGFβ (B) mRNA expression was expressed as a fold change over expression in vehicle controls (0.1% DMSO) within each experiment. Data are presented as the mean + SE (each exposure was performed in triplicate wells; n = 4 separate experiments). *Significantly different from vehicle control at $p \leq 0.05$.

Figure E4. OP effects on cytokine expression in THP1 cells is not mediated by increased levels of intracellular calcium. Differentiated THP1 cells were loaded with Fluo-4 calcium indicator dye. The OPs parathion or paraoxon (A), chlorpyrifos or chlorpyrifos oxon (B) or diazinon or diazoxon (C) were added to the cells and images were recorded for 10 min on FLIPR (Molecular Devices). Ionomycin (1ug/ml) was added at the end of each treatment to confirm cell and system responsiveness. Changes in intracellular calcium $[Ca^{2+}]_i$ resulting from OP exposure were calculated by measuring changes in dye fluorescence intensity ($\Delta F/F_0$). Data is presented as the mean area under the curve (AUC) ± SE (each exposure was performed in triplicate wells; n = 2 separate experiments for each exposure). The horizontal line in each box represents the mean; lower and upper box limits, the 25th and 75th percentile, respectively; whiskers, the 1–99th percentile. *Significantly different from vehicle control at $p \leq 0.05$.

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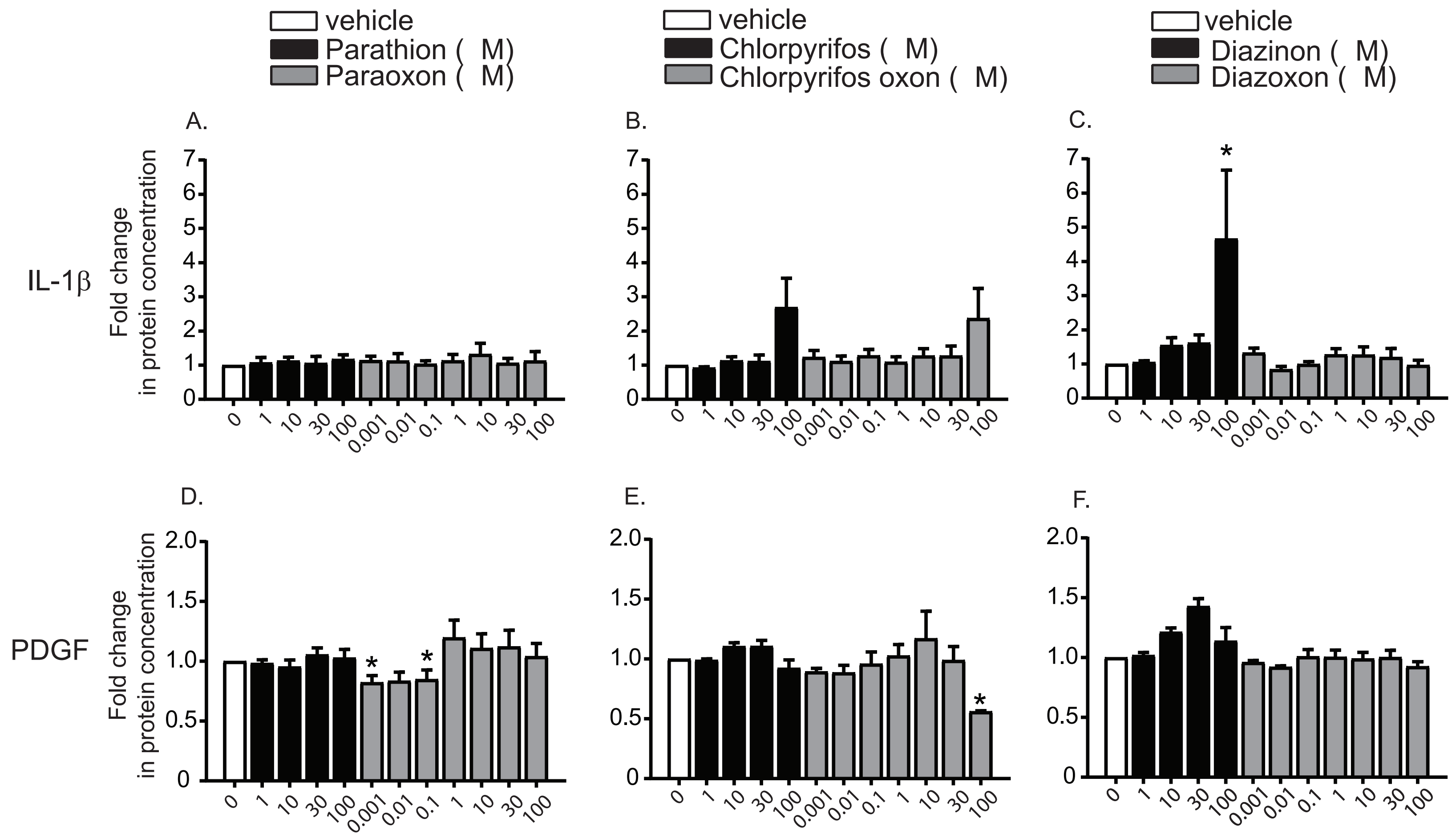
Figure E1



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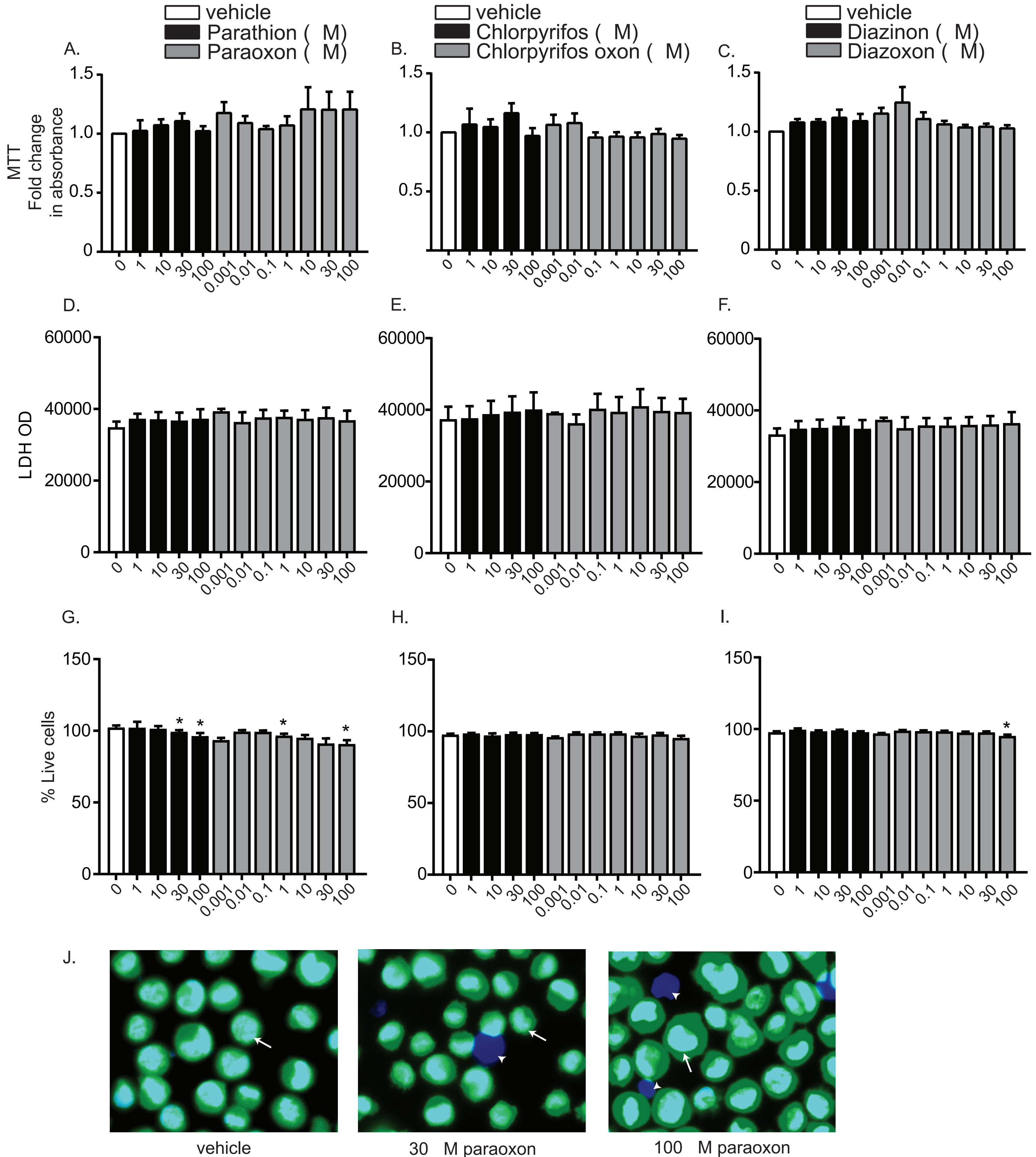

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Figure E3

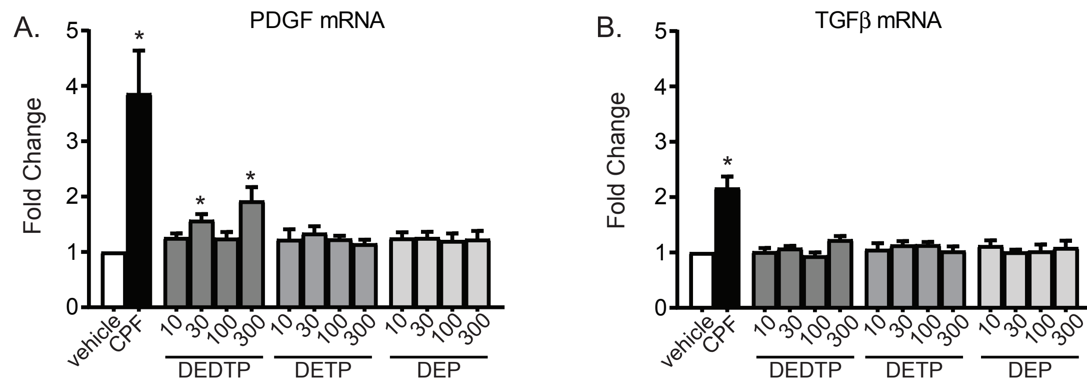

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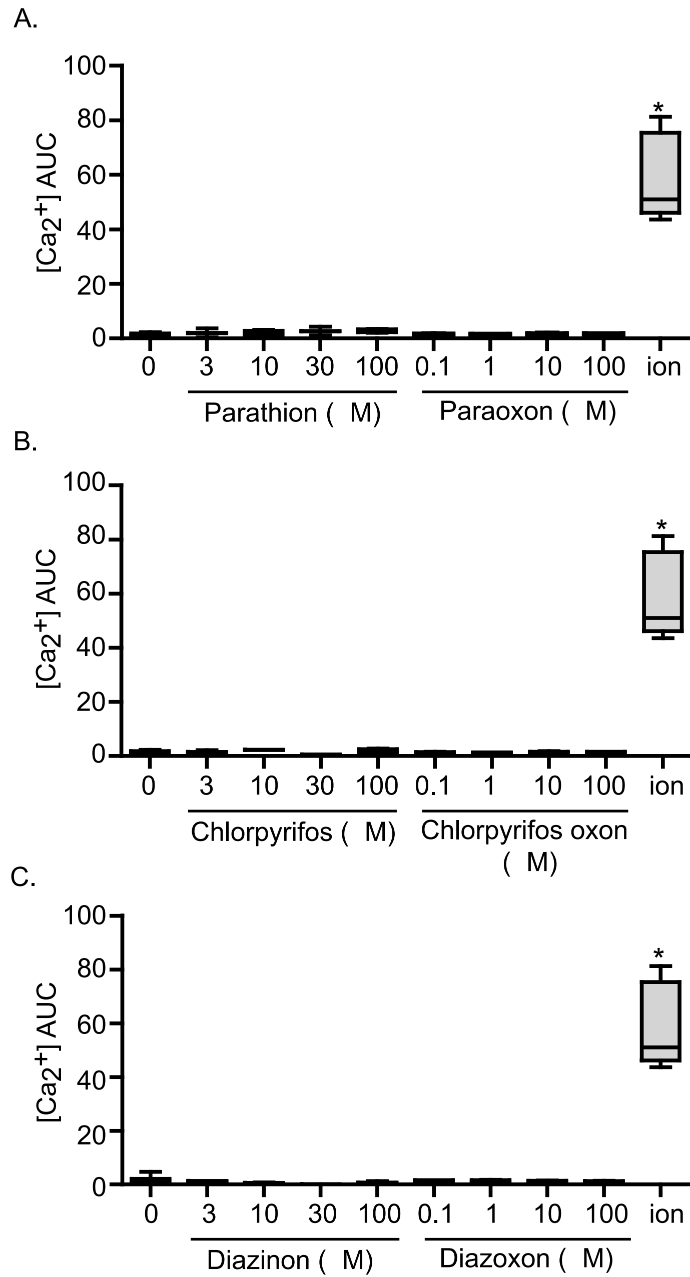
Figure E4



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