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).

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

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

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).

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

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 $p \leq 0.05$.

SUPPLEMENTAL REFERENCES

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Figure E1

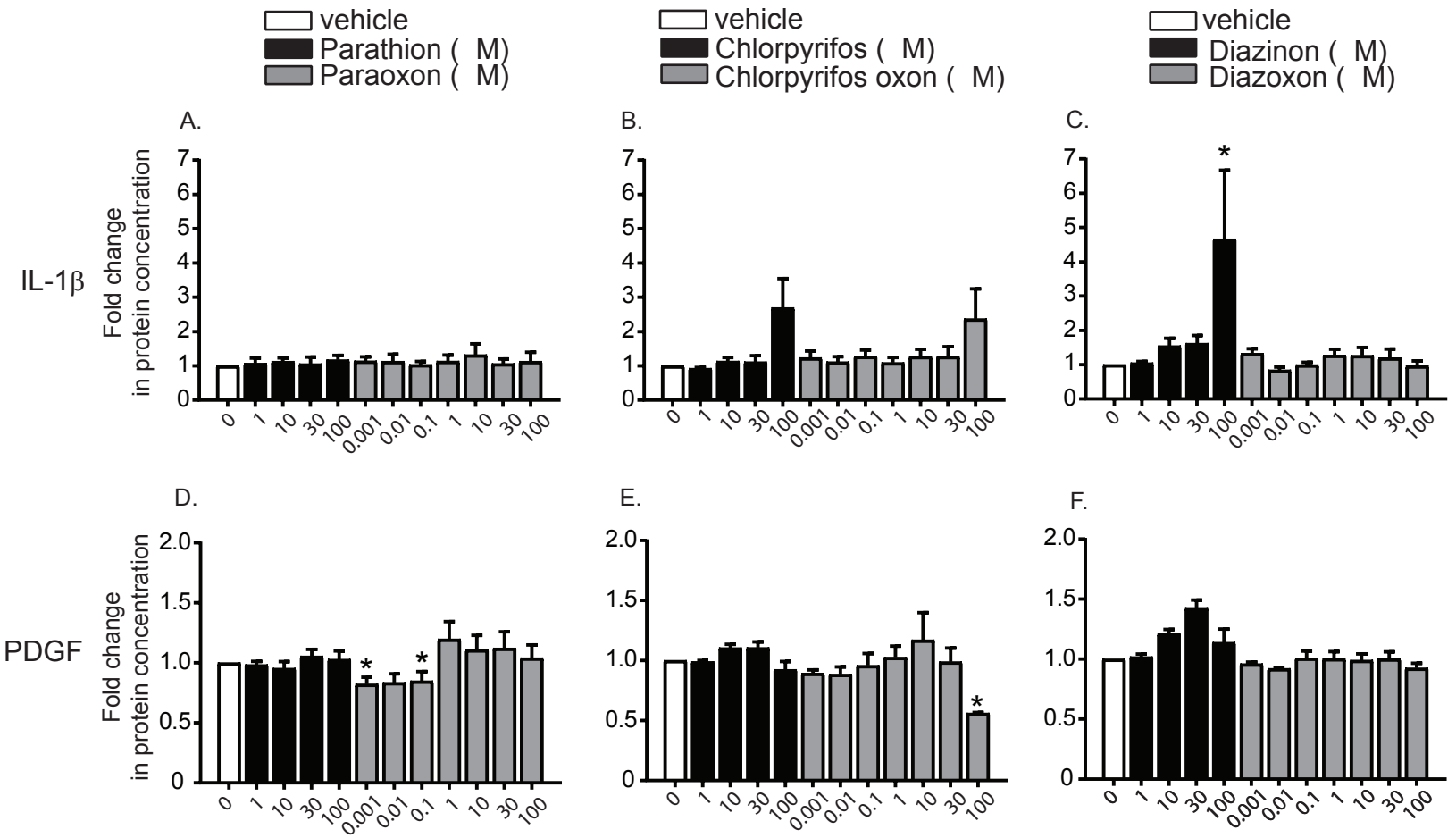
vehicle
Parathion (M)
Paraoxon (M)
vehicle
Chlorpyrifos (M)
Chlorpyrifos oxon (M)
vehicle
Diazinon (M)
Diazoxon (M)
A.
B.
C.
D.
E.
F.
IL-1β
PDGF
Fold change
in protein concentration
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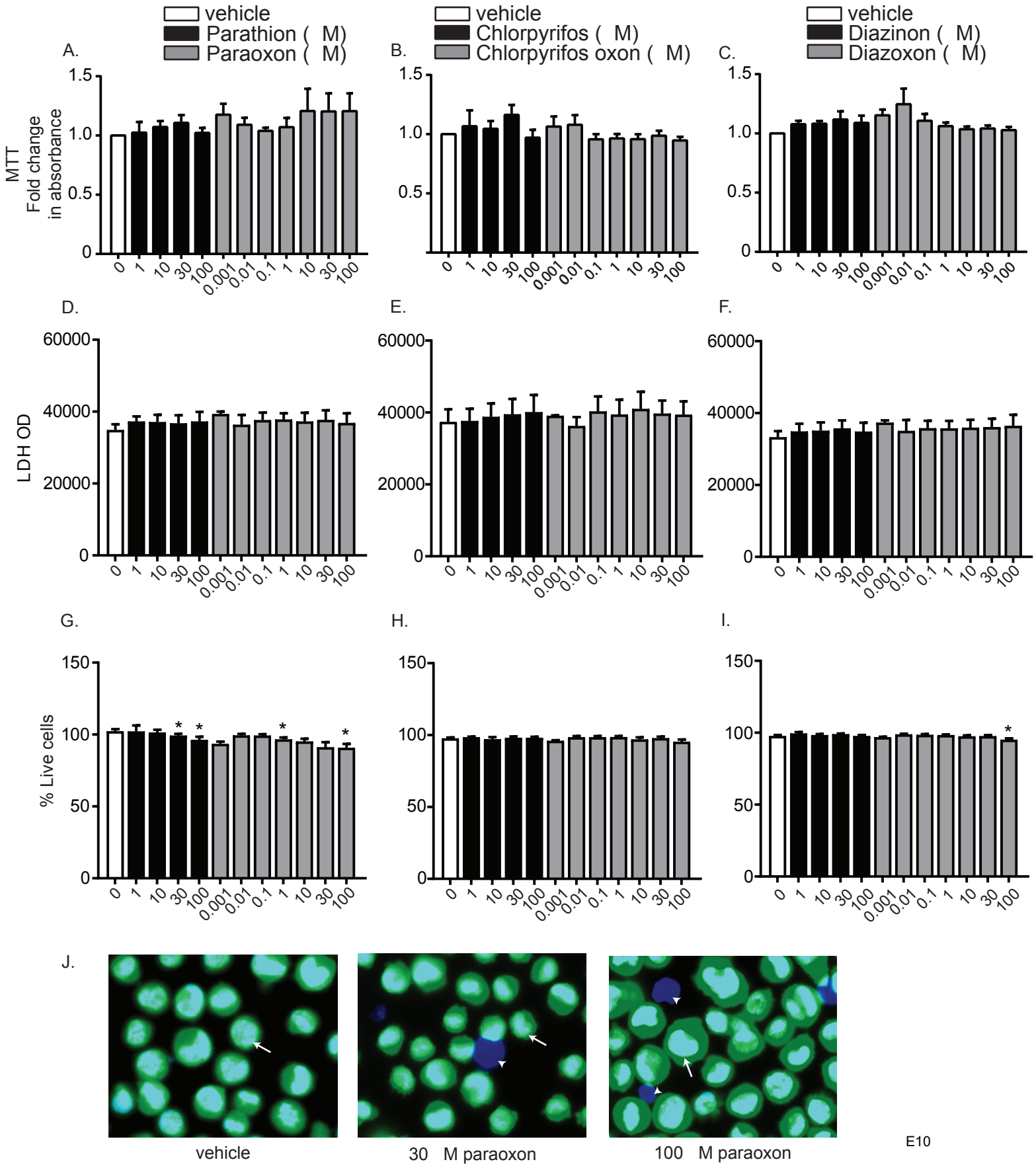
Figure E2


vehicle
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Paraoxon (M)
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MTT
Fold change
in absorbance
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1.0
0.5
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
vehicle
Chlorpyrifos (M)
Chlorpyrifos oxon (M)
B.
1.5
1.0
0.5
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
vehicle
Diazinon (M)
Diazoxon (M)
C.
1.5
1.0
0.5
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
D.
LDH OD
60000
40000
20000
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
E.
60000
40000
20000
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
F.
60000
40000
20000
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
G.
% Live cells
150
100
50
0
*
*
*
*
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
H.
150
100
50
0
0 1 10 30 100 0.001 0.01 0.1 1 10 30 100
I.
150
100
50
0
*
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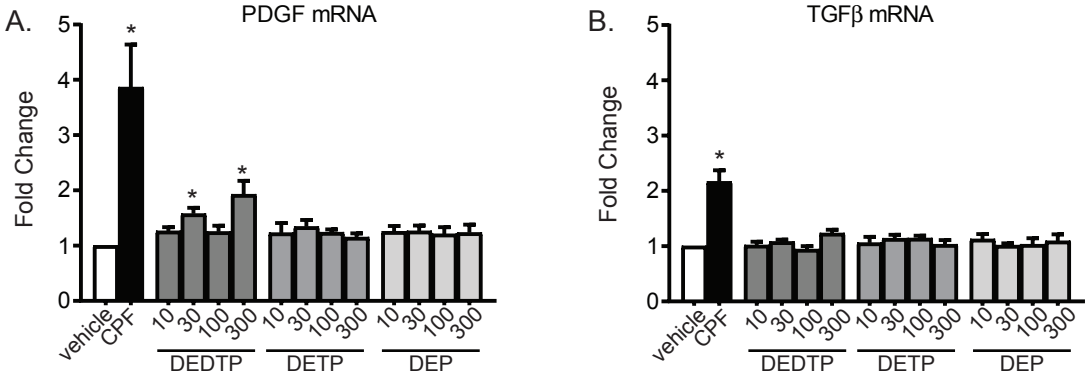
J.
vehicle
30 M paraoxon
100 M paraoxon

Figure E3


A.
PDGF mRNA
Fold Change
0
1
2
3
4
5
*
*
*
vehicle
CPF
10
30
100
300
10
30
100
300
10
30
100
300
DEDTP
DETP
DEP
B.
TGFβ mRNA
Fold Change
0
1
2
3
4
5
*
vehicle
CPF
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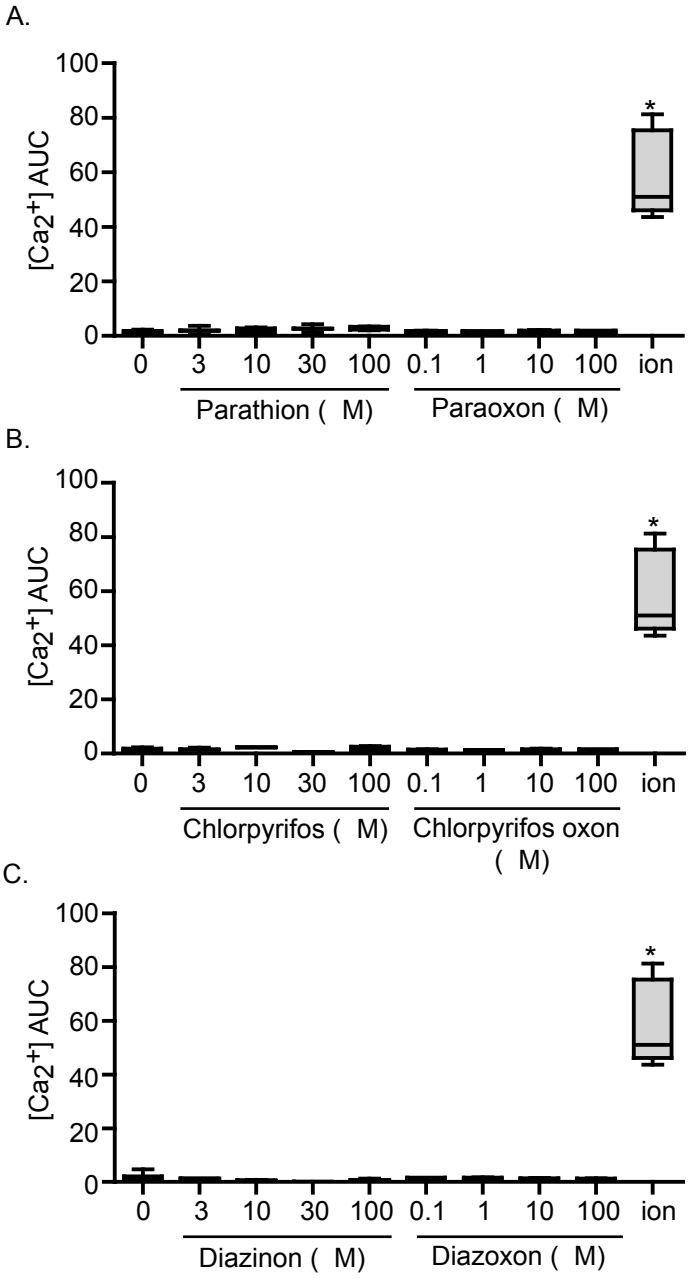
30
100
300
10
30
100
300
10
30
100
300
DEDTP
DETP
DEP

Figure E4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60
40
20
0
[Ca2+] AUC
*
0 3 10 30 100 0.1 1 10 100 ion
Parathion (M)
Paraoxon (M)
B.
100
80
60
40
20
0
[Ca2+] AUC
*
0 3 10 30 100 0.1 1 10 100 ion
Chlorpyrifos (M)
Chlorpyrifos oxon
(M)
C.
100
80
60
40
20
0
[Ca2+] AUC
*
0 3 10 30 100 0.1 1 10 100 ion
Diazinon (M)
Diazoxon (M)