In vitro neurotoxic hazard characterisation of dinitrophenolic herbicides

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ABSTRACT

Dinitrophenolic compounds are powerful toxicants with a long history of use in agriculture and industry. While (high) human exposure levels are not uncommon, in particular for agricultural workers during the spraying season, the neurotoxic mechanism(s) that underlie the human health effects are largely unknown. We therefore investigated the in vitro effects of two dinitrophenolic herbicides (DNOC and dinoseb) on a battery of neurotoxicity endpoints in (dopaminergic) rat PC12 cells.

Cell viability, mitochondrial activity, oxidative stress and caspase activation were assessed using fluorescence-based bioassays (CFDA, alamar Blue, H_2DCFDA and Ac-DEVD-AMC, respectively), whereas changes in intracellular $[Ca^{2+}]_i$ were assessed using single-cell fluorescence microscopy with Fura-2AM. The combined results demonstrate that exposure to both DNOC and dinoseb is linked to calcium release from the endoplasmic reticulum and activation of caspase-mediated apoptotic pathways. In subsequent experiments, immunofluorescent labelling with specific antibodies was used to determine changes in intracellular α-synuclein levels, demonstrating that both DNOC and dinoseb increase levels of intracellular α-synuclein. The combined results indicate that in vitro exposure to DNOC and dinoseb activates pathways that are not only involved in acute neurotoxicity but also in long-term effects as seen in neurodegeneration.

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1. Introduction

Humans are exposed to a plethora of chemicals that are potentially neurotoxic. While different in their presumed primary modes of action, many classes of chemicals disturb intracellular calcium homeostasis as a common mode of action. Our previous research has demonstrated that e.g., PCBs (Langeveld et al., 2012; Westerink, 2014), PBDEs (Dingemans et al., 2010; Westerink, 2014) and different classes of pesticides, including conazole fungicides (Heusinkveld et al., 2013) and organochlorine, pyrethroid and organophosphate insecticides (Heusinkveld and Westerink, 2012; Meijer et al., 2014) all target intracellular calcium homeostasis. In several instances, disturbance of the intracellular calcium concentration ($[Ca^{2+}]_i$) already occurs at concentrations that do not yet affect the presumed primary mode of action, highlighting the importance of this common endpoint. Despite the presence of a large number of pesticides in the list of compounds that can affect $[Ca^{2+}]_i$, there is currently relative limited data on the potential disturbance of calcium homeostasis by herbicides.
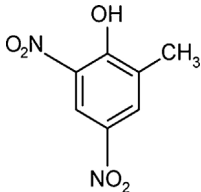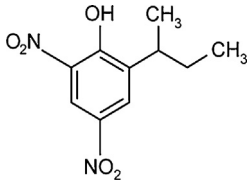
The dinitrophenolic compounds 4,6-dinitro-o-cresol (DNOC; CAS No. 534-52-1; Table 1) and 2-(1-methylpropyl)-4,6-dinitro-phenol (dinoseb; CAS No. 88-85-7; Table 1) are derivatives of 2,4-dinitrophenol (2,4-DNP; CAS No. 51-28-7). Human exposure to DNOC and dinoseb is mainly occupational due to the use as defoliating agents in potato culture, as herbicide targeting grass and broadleaf weeds and as insecticide in grape production (Whitacre et al., 2004). Furthermore, dinitrophenolics have a long history of use in polymer industry and in the manufacture of dyes and explosives. These compounds are easily absorbed via various routes of exposure due to their lipophilicity (DNOC log K_{ow}: 2.56; dinoseb log K_{ow}: 3.56; Parker et al., 1951). Despite a ban on the use of DNOC and dinoseb as pesticide in Europe in the late 1990's, both

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Table 1
Molecular structure and chemico-physical properties of DNOC and dinoseb.

Chemical name	2-Methyl-4,6-Dinitrophenol (DNOC)	2-(1-Methylpropyl)-4,6-Dinitrophenol (Dinoseb)
CAS number	534–52-1	88–85-7
Mol. weight (g/mol)	198.1	240.2
Log K_{OW}	2.56	3.56

compounds have been found consistently in the environment (see e.g. Duyzer, 2003; Quaghebeur et al., 2004; Schummer et al., 2009), which is at least partly due to their continuing use in (plastic) industry but also to their slow environmental degradation (Schummer et al., 2009). Continued use of these compounds in other parts of the world also results in local detection in surface and drinking water (Li et al., 2012).

Dinitrophenolic herbicides are powerful toxicants in plants, animals and humans with high acute toxicity (e.g. mammalian LD_{50} dinoseb: oral = 37 mg/kg body weight, dermal = 200 mg/kg body weight; Puls, 1988). Toxicity is thought to be related primarily to uncoupling of mitochondrial phosphorylation (uncoupling in mouse-brain mitochondrial preparation EC_{100}: DNOC 20 μM, dinoseb 0.5 μM; Ilivicky and Casida, 1969), leading to increased oxidative metabolism and depletion of cellular ATP, subsequently resulting in cell and tissue damage (Ilivicky and Casida, 1969; Palmeira et al., 1994). Considering the dependence of the nervous system on ATP homeostasis, neuronal cells may thus be particularly vulnerable to the toxic effects of dinitrophenolic herbicides, especially since in vivo experiments indicated that dinitrophenolic compounds cross biological membranes, such as the blood-brain and the placental barrier (Gibson and Rao, 1973).

Acute toxicity of dinitrophenolic compounds is characterized by delirium, dizziness, loss of consciousness and dysregulation of body temperature that often leads to fatal hyperthermia (Estuardo et al., 2006; Grundlingh et al., 2011). Although there is no known remedy, successful administration of dantrolene has been reported (Siegmueller and Narasimhaiah, 2010). Since dantrolene is an antagonist of ryanodine receptors that prevents calcium release from the endoplasmic reticulum (Van Winkle, 1976), this indicates that changes in calcium homeostasis may comprise part of the mode of action of dinitrophenolic compounds. In the central nervous system, calcium plays a pivotal role in many inter- and intraneuronal processes ranging from normal neuronal functioning to differentiation and degeneration (Spitzer et al., 2004; Westerink, 2006; Mattson, 2007).

Dopaminergic rat pheochromocytoma cells PC12; (Greene and Tischler, 1976) are a well-known, extensively characterized model for neurotoxicity studies (Westerink and Ewing, 2008; Westerink, 2014). These cells express different types of voltage-gated calcium- and sodium channels and display several characteristics of mature dopaminergic neurons such as synthesis, storage and vesicular release of dopamine (DA) (Shafer and Atchison, 1991; Westerink and Ewing, 2008). PC12 cells have thus proven suitable for the functional study of calcium-related neurotoxicity of compounds in vitro (Heusinkveld et al., 2013; Westerink, 2014). In the current study, we therefore used PC12 cells to study the effects of acute exposure to two common dinitrophenolic herbicides (DNOC and dinoseb) on intracellular calcium homeostasis and in vitro neurotoxicity.

2. Materials and methods

2.1. Chemicals

Fura-2-AM, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) and 2,7-dichlorodihydrofluorescein diacetate (H_2-DCFDA) were obtained from Molecular Probes (Invitrogen, Breda, The Netherlands); DNOC and dinoseb were obtained Pestanal® grade, 99.8% purity (Riedel de Haën, Seelze, Germany); all other chemicals were obtained from Sigma (Zwijndrecht, The Netherlands), unless otherwise noted. Saline solutions, containing (in mM) 125 NaCl, 5.5 KCl, 2CaCl$_2$, 0.8 MgCl$_2$, 10HEPES, 24 glucose and 36.5 sucrose (pH 7.3), were prepared with de-ionized water (Milli-Q®; resistivity >18 MΩ·cm). Stock solutions of 2 mM ionomycin in DMSO were kept at -20 °C. Stock solutions of 0.1–100 mM DNOC and dinoseb (Pestanal® grade, 99.8% purity, Riedel de Haën, Seelze, Germany) were prepared in DMSO and diluted in saline to obtain the desired concentrations just prior to the experiments (all solutions used in experiments, including control experiments, contained 0.1% DMSO).

2.2. Cell culture

PC12 cells (Greene and Tischler, 1976) were grown for up to 10 passages in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 5% fetal calf serum and 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands) in a humidified incubator at 37 °C and 5% CO$_2$ as described previously (Heusinkveld et al., 2013). For Ca^{2+} imaging experiments, cells were subcultured in poly-L-lysine coated glass-bottom dishes (MatTek, Ashland, MA, USA) as described previously (Heusinkveld et al., 2013). For cell viability and caspase experiments cells were sub-cultured in poly-L-lysine coated 24-wells plates (Greiner Bio-one, Solingen, Germany) at a density of 5×10^5 cells/well. For experiments assessing oxidative stress, i.e. enhanced production of reactive oxygen species (ROS), cells were seeded in poly-L-lysine coated 48-wells plates at a density of 2.5×10^5 cells/well. For alpha-synuclein immunostaining cells were subcultured on poly-L-lysine coated coverslips at a density of 5×10^5 cells per coverslip.

2.3. Absorbance spectra dinitrophenolics

To assess potential interference of DNOC and dinoseb with the excitation and emission wavelengths used in different fluorescence assays, absorbance spectra (300–600 nm) of

increasing concentrations (0.1–100 μM) of DNOC and dinoseb have been measured spectrophotometrically (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland).

2.4. Intracellular Ca^{2+} imaging

Changes in cytosolic $[Ca^{2+}]_i$ upon exposure to DNOC (1–30 μM) and dinoseb (0.3–30 μM) were measured on a single-cell level using fluorescence microscopy in PC12 cells loaded with the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM as described previously (Heusinkveld et al., 2013). Involvement of intracellular calcium stores in effects on basal $[Ca^{2+}]_i$ was assessed using pharmacological manipulation with thapsigargin 1 μM; to empty the ER; see also: (Dingemans et al., 2010).

2.5. Mitochondrial activity and cell viability assay

The effects of DNOC and dinoseb on mitochondrial activity and cell viability were measured using a combined alamar Blue/CFDA-AM assay as described earlier (Heusinkveld et al., 2013), which is used to assess respectively mitochondrial activity and membrane integrity. Briefly, following exposure for 24 or 48 h in phenol red- and serum-free medium, cells were incubated for 30 min with 12,5 μM and 4 μM CFDA-AM. Resorufin was measured spectrophotometrically at 540/590 nm (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland), whereas hydrolysed CFDA was measured spectrophotometrically at 493/541 nm.

2.6. ROS measurement using H_2-DCFDA

The involvement of oxidative stress in the observed reduction in cell viability was investigated using the fluorescent dye H_2-DCFDA as described earlier (Heusinkveld et al., 2013). Briefly, cells were loaded with 1.5 μM H_2-DCFDA for 30 min at 37 °C. Cumulative ROS production upon exposure to 0.1, 1, 10 or 100 μM DNOC or dinoseb was measured for different exposure durations up to 24 h, fluorescence was measured spectrophotometrically at 488/520 nm (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland). Rotenone (100 μM) was included as positive control for oxidative stress (Radad et al., 2006).

2.7. Caspase activation assay

To investigate the role of apoptosis in the DNOC and dinoseb-induced reduction in cell viability, the effect of DNOC and dinoseb exposure (6, 24 and 48 h) on activation of the effector caspase-3 was determined using a fluorescent caspase-3 substrate (Ac-DEVD-AMC) according to the manufacturer's protocol. Fluorescence of cleaved caspase substrate was assessed spectrophotometrically at 360/460 nm (Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland). Staurosporine (1 μM, 6 h exposure) was included as a positive control. Fluorescence data was corrected for cell number using a fluorescamine-based assay (Udenfriend et al., 1972) to quantify the protein content of the sample.

2.8. Immunofluorescence staining

To assess the effect of exposure to DNOC (1–100 μM) and dinoseb (0.3–30 μM) on intracellular α-synuclein levels, α-synuclein was labelled using specific fluorescent antibodies for subsequent analysis using confocal laser-scanning microscopy. Exposure concentrations were chosen based on the observed effects in calcium imaging, including for both compounds a concentration with no, intermediate and maximal Ca^{2+} influx observed. Briefly, cells were exposed for 24 h or 48 h and subsequently fixed (4% para-formaldehyde) and permeabilized (0.1% saponin). Aspecific binding was blocked using bovine serum albumin (BSA). α-Synuclein was labelled using a polyclonal sheep-anti rat antibody (ab6162; 1:200; Abcam, Cambridge, UK). For fluorescent detection, a fluorochrome-conjugated secondary antibody was used (Alexa488 donkey-anti-sheep; 1:200; Invitrogen, Breda, The Netherlands). Immunostained coverslips were visualized using confocal laser-scanning microscopy (Leica DMI4000 equipped with TCS SPE-II), full preparation Z-stack images (0.5 μm) were analysed using LAS AF Lite software (version 2.6.0).

2.9. Data-analysis and statistics

Data on mitochondrial activity, cell viability and ROS production is presented as mean% ± standard error of the mean (SEM) of ≥3 independent experiments (N) with ≥4 wells (n) per experiment compared to (time-matched) controls. Data from single-cell fluorescence microscopy is presented as mean% increase of $[Ca^{2+}]_i$ over baseline (± SEM) from the number of individual cells (n) indicated, obtained from 4 to 9 independent experiments (N) (Heusinkveld and Westerink, 2011). Data on intracellular α-synuclein levels from confocal images is quantified using ImageJ software v1.47C (Wayne Rasband, National Institutes of Health, USA) and presented as% α-synuclein compared to control. Data on caspase activation is presented as% increase in fluorescence (± SEM) compared to the time-matched control, corrected for protein level (≥3 independent experiments (N) with ≥4 wells (n) per experiment). Statistical analyses were performed using GraphPad Prism v6.04 (GraphPad Software, San Diego, California, USA). Continuous data were compared using One-way ANOVA with post-hoc Bonferroni test where applicable. A p-value ≤0.05 is considered statistically significant.

3. Results

3.1. Absorbance spectra dinitrophenolic herbicides

As high concentrations of DNOC or dinoseb result in an intense yellow colouration of the media and saline solutions, we investigated whether the presence of DNOC and dinoseb in the medium interfered with the yield in the different fluorescent assays. The results from the absorbance spectra demonstrate that increasing concentrations of DNOC and dinoseb (>10 μM) concentration-dependently quench the wavelengths used in fluorescent calcium imaging (Fura-2: 340 and 380 nm) differently (for spectra see: Supplemental Data). This is in line with findings of Hutanu and co-workers (Hutanu et al., 2013), who demonstrated quenching of fluorescence by various dinitrophenolic compounds. As this could lead to misinterpretation of $[Ca^{2+}]_i$ data through an artefact in the calculation of F340/F380 ratios, results from the fluorescent calcium imaging are presented for concentrations ≤30 μM. The other assays, using (single) wavelengths >480 nm, are not subject to absorbance-induced artefacts. In those assays therefore, concentrations up to 100 μM are presented.

3.2. Dinitrophenolic herbicides disturb $[Ca^{2+}]_i$ via store-mediated release of Ca^{2+}

Since proper calcium homeostasis is essential for neuronal development, function and survival (Spitzer et al., 2004; Westerink, 2006; Mattson, 2007), we measured effects of DNOC and dinoseb on $[Ca^{2+}]_i$ using single-cell fluorescence microscopy in Fura-2-loaded PC12 cells. In control cells ($n = 85$), basal $[Ca^{2+}]_i$ was low (97 ± 2 nM) with only minor fluctuations, whereas depolarization with high-K^+-containing saline evoked a strong, transient increase in $[Ca^{2+}]_i$ amounting up to 2.0 ± 0.1 μM ($n = 85$). Cells
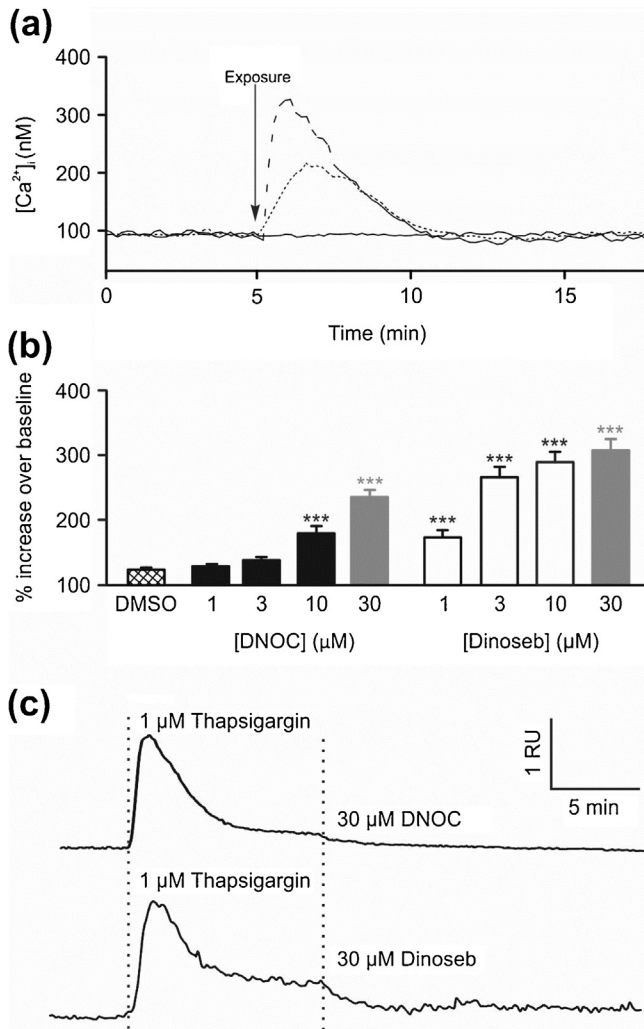
Fig. 1. Both DNOC and dinoseb concentration-dependently increase basal $[Ca^{2+}]_i$. **A** Example traces of basal $[Ca^{2+}]_i$ upon exposure to DMSO (solid line), 30 μM DNOC (dotted line) and 30 μM dinoseb (dashed line). **B** Bar graph displaying the concentration-response curve of DNOC and dinoseb. Bars display average data (±SEM) from at least 28–80 individual cells (4–9 experiments per concentration). Notably, the data shown for 30 μM DNOC and dinoseb (grey bars) can be slightly distorted as a result of optical interference (see Supplemental data) **C** Example traces of basal $[Ca^{2+}]_i$ upon exposure to DNOC (30 μM) or dinoseb (30 μM) following thapsigargin (1 μM) pre-treatment under Ca^{2+}-free conditions. Thapsigargin pretreatment completely abolishes the DNOC and dinoseb-induced increase in $[Ca^{2+}]_i$. Difference from control *** $p \leq 0.001$.

acutely (20 min) exposed to DNOC or dinoseb for up to 100 μM did not show changes in depolarization-evoked $[Ca^{2+}]_i$ (data not shown). However, both DNOC and dinoseb induced an increase in basal $[Ca^{2+}]_i$ (Fig. 1A). Although exposure of cells to 1 or 3 μM DNOC did not result in a significant increase in basal $[Ca^{2+}]_i$ compared to control cells, cells exposed to 10 μM DNOC induced a transient increase of basal $[Ca^{2+}]_i$ with an amplitude amounting to $186 \pm 10\%$ ($n = 79$, $p \leq 0.001$; Fig. 1B). This increase was larger following exposure to 30 μM DNOC ($236 \pm 9\%$, $n = 108$; $p \leq 0.001$; Fig. 1B).

Similarly, cells exposed to 0.3 μM dinoseb did not show a significant alteration of the basal $[Ca^{2+}]_i$ (data not shown), whereas cells exposed to 1 μM dinoseb showed a transient increase of basal $[Ca^{2+}]_i$ with an amplitude amounting to $173 \pm 8\%$ ($n = 62$; $p \leq 0.001$; Fig. 1B). This increase was larger following exposure to 3 μM dinoseb ($266 \pm 15\%$, $n = 62$, $p \leq 0.001$; Fig. 1B) and apparently already maximal as exposure to 10 or 30 μM did not result in a

further increase of basal $[Ca^{2+}]_i$ ($293 \pm 14\%$, $n = 57$ and $311 \pm 17\%$, $n = 67$ respectively; Fig. 1B).

Additional experiments were performed to identify the origin of the observed increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was still observed when cells were exposed to DNOC or dinoseb under Ca^{2+}-free conditions, indicating that the increase originates from intracellular organelles (data not shown). To assess whether Ca^{2+} release from the endoplasmic reticulum (ER) plays a role, the ER was emptied under Ca^{2+}-free conditions using 1 μM thapsigargin and cells were subsequently challenged with DNOC or dinoseb. As the thapsigargin treatment abolished both DNOC and dinoseb-induced increases in $[Ca^{2+}]_i$, the results demonstrate that the increase in $[Ca^{2+}]_i$ originates from the ER (Fig. 1C).

3.3. Effects of dinitrophenolic herbicides on cell viability and ROS production

Considering the important role of ER calcium release in cellular survival (Orrenius et al., 2011), we investigated the effects of DNOC and dinoseb on mitochondrial activity, cell viability and ROS production (Fig. 2A–B). The results from the alamar Blue assay demonstrate that exposure of dopaminergic PC12 cells to 10 μM DNOC for 24 h induced a significant increase in mitochondrial activity compared to control, amounting to $131 \pm 8\%$ (N = 4; $p \leq 0.001$; Fig. 2A1). However, upon exposure to 100 μM DNOC mitochondrial activity decreased to $89 \pm 5\%$ compared to control (N = 4; $p \leq 0.001$; Fig. 2A1), indicative for cell death at this high concentration. This is confirmed by the results from the CFDA assay that demonstrate a concentration-dependent decrease of the fraction intact cells following 24 h exposure to 10 or 100 μM DNOC, which reduced membrane integrity to $84 \pm 5\%$ (N = 3, $p \leq 0.001$; Fig. 2A2) and $72 \pm 2\%$ (N = 3, $p \leq 0.001$; Fig. 2A2) of control, respectively.

Exposure for 24 h to dinoseb already increased mitochondrial activity at 0.1 and 1 μM (Fig. 2A1), amounting to $114 \pm 10\%$ (N = 4; $p \leq 0.01$) and $127 \pm 4\%$ (N = 4; $p < 0.01$) of control, respectively. Upon exposure to 10 or 100 μM dinoseb, a decrease in mitochondrial activity was observed, amounting to respectively $89 \pm 6\%$ (N = 4; Fig. 2A1) and $70 \pm 7\%$ (N = 4; $p \leq 0.001$; Fig. 2A) of control, indicative for cell death at these higher concentrations. In line with these findings, the results from the CFDA assay demonstrate that 24 h exposure to 10 μM dinoseb reduced membrane integrity to $80 \pm 3\%$ (N = 3; $p < 0.001$; Fig. 2A2) of control. Membrane integrity decreased further to $70 \pm 7\%$ (N = 3; $p < 0.001$; Fig. 2A2) of control upon exposure to 100 μM dinoseb.

Since increased ROS production is a well-known cause for decreased cell viability (Franco et al., 2009), effects of exposure to DNOC and dinoseb for up to 24 h on cumulative ROS production were assessed in PC12 cells loaded with the fluorescent H_2-DCFDA dye. Rotenone (100 μM) was used as a positive control and showed a profound increase in ROS production over time. The results demonstrate that exposure to DNOC (10–100 μM) did not increase ROS production compared to controls, whereas exposure to dinoseb produced a small increase in ROS production over time compared to control, although only at the highest concentration dinoseb tested (100 μM) which reached significance only after 24 h exposure ($132 \pm 7\%$ of control; $p \leq 0.001$; N = 3; Fig. 2B).

The combined results thus demonstrate that both DNOC and dinoseb concentration-dependently alter mitochondrial activity and cell viability in dopaminergic PC12 cells and that these effects appear not directly related to increased production of ROS.

3.4. Dinitrophenolic herbicides activate caspase-mediated apoptosis

Since the effects of DNOC and dinoseb on cell viability are apparently not related to enhanced production of ROS, we assessed
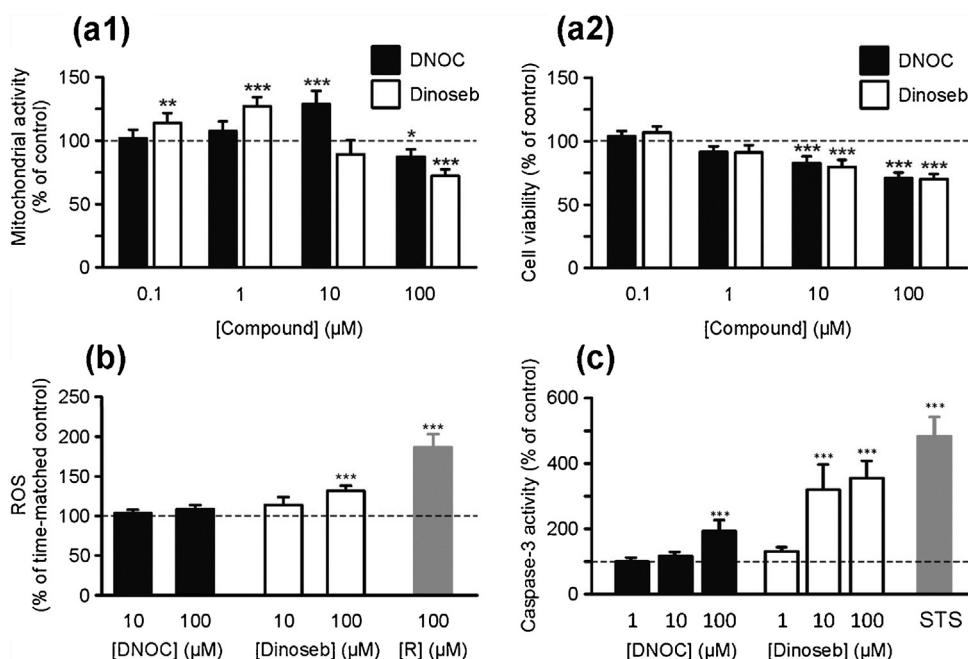
Fig. 2. DNOC (black bars) and dinoseb (white bars) concentration-dependently change parameters of cell viability, oxidative stress and apoptosis in PC12 cells upon exposure for 24 h. **A1** Bar graph displaying the DNOC and dinoseb-induced upregulation of mitochondrial activity ($\geq$10 μM and $\geq$0.1 μM respectively). **A2** Bar graph displaying the DNOC and dinoseb-induced decrease in membrane integrity (both $\geq$10 μM). Difference from control (dashed line): ** $p \leq 0.01$, *** $p \leq 0.001$. **B** Bar graph displaying the results from the H_2-DCFDA oxidative stress assay in PC12 cells following 24 h exposure to 10 or 100 μM DNOC or dinoseb. Only exposure to dinoseb (100 μM) is related to a modest increase in oxidative stress compared to control. Rotenone (R; 100 μM) was included as positive control. Difference from control (dashed line): *** $p \leq 0.001$. **C** DNOC and dinoseb concentration-dependently induce caspase-3 activity. Staurosporine (STS; 1 μM, 6 h) was included as positive control. Difference from control (dashed line) *** $p \leq 0.001$. All bars display average data ($\pm$ SEM) from $\geq$3 independent experiments.

whether exposure to these dinitrophenolic compounds induced caspase-3 activity following 24 h exposure using the fluorescent caspase-3 substrate (Ac-DEVD-AMC). Staurosporine exposure (1 μM, 6 h exposure) served as positive control and increased caspase-3 activity to $478 \pm 93\%$ (N = 9; $p < 0.001$; Fig. 2C) of control. The results indicate a significant increase of caspase-3 activity following 24 h exposure to DNOC (100 μM) and dinoseb (10 μM) amounting to $190 \pm 31\%$ (N = 8; $p < 0.001$; Fig. 2C) and $316 \pm 87\%$ (N = 3; $p < 0.001$; Fig. 2C) of control, respectively. No further increase was observed upon exposure to 100 μM dinoseb ($358 \pm 51\%$ (N = 7; $p < 0.001$; Fig. 2C)) of control.

These data indicate that exposure to DNOC and dinoseb at low micromolar concentrations increased caspase-3 activity, which may underlie the observed effects on cell viability.

3.5. Dinitrophenolic herbicides increase intracellular α-synuclein levels

ER-related changes in $[Ca^{2+}]_i$ and caspase-activation are linked to neurodegeneration (Nath et al., 2011; Mattson, 2012). A clear hallmark of dopaminergic degeneration is an increase in α-synuclein and eventually the occurrence of α-synuclein-containing cytoplasmic aggregates (Breydo et al., 2012). We therefore investigated α-synuclein levels in cells exposed to DNOC (1–100 μM) and dinoseb (0.3–30 μM). Cells were exposed for 24 or 48 h and subsequently intracellular α-synuclein was labelled using specific antibodies. Confocal microscopy images of cells exposed to the solvent control (DMSO; Fig. 3A1–2) show a weak α-synuclein stain. Quantification of the α-synuclein fluorescence revealed no significant increase in intracellular α-synuclein upon 24 h exposure (data not shown). However, cells exposed to DNOC (Fig. 3B1–2) and dinoseb (Fig. 3C1–2) for 48 h revealed a more intense α-synuclein stain. Quantification of the α-synuclein fluorescence revealed a concentration-dependent increase in intracellular

α-synuclein upon 48 h exposure to DNOC (LOEC: 1 μM) and dinoseb (LOEC: 0.3 μM), amounting to a maximum of $203 \pm 5\%$ (100 μM; n = 10, $p \leq 0.001$; Fig. 3D) and $183 \pm 5\%$ (30 μM; n = 28, $p \leq 0.001$; Fig. 3D) compared to control, respectively.

4. Discussion

The present results demonstrate that exposure to the dinitrophenolic herbicides DNOC and dinoseb induces an acute and transient increase in basal intracellular calcium levels ($[Ca^{2+}]_i$; LOEC DNOC: 10 μM; LOEC dinoseb: 1 μM; Fig. 1) which originates from the ER (Fig. 1)

The extent to which ER-mediated calcium release occurs differs between cells. Calcium release from the ER is related to the activation of apoptosis pathways and cell death (Orrenius et al., 2011). Correspondingly, exposure to the dinitrophenolic herbicides DNOC and dinoseb induces an increase in mitochondrial activity (Fig. 2A1), activation of caspase-mediated apoptosis (Fig. 2C; LOEC DNOC 24 h: 100 μM; LOEC dinoseb 24 h: 10 μM) and a mild reduction in cell viability ($\sim$25%; Fig. 2A2) in dopaminergic PC12 cells. Neither a further reduction in cell viability nor a further increase in caspase-3 activation is observed when the exposure is prolonged to 48 h (data not shown).

Dinitrophenolic herbicides are primarily known to act as uncouplers of mitochondrial oxidative phosphorylation via their protonophoric properties, thereby disturbing the proton gradient across the mitochondrial membrane (Judah, 1951; Ilivicky and Casida, 1969). This leads to an increase in mitochondrial activity to restore the proton gradient and upon a sufficiently high level of exposure this will result in a concentration-dependent deficiency in ATP production related to cell damage. Uncoupling of mitochondrial phosphorylation in these experiments (minimum uncoupling concentration in mouse-brain mitochondrial preparation DNOC 20 μM, dinoseb 0.5 μM; (Ilivicky and Casida, 1969)) is
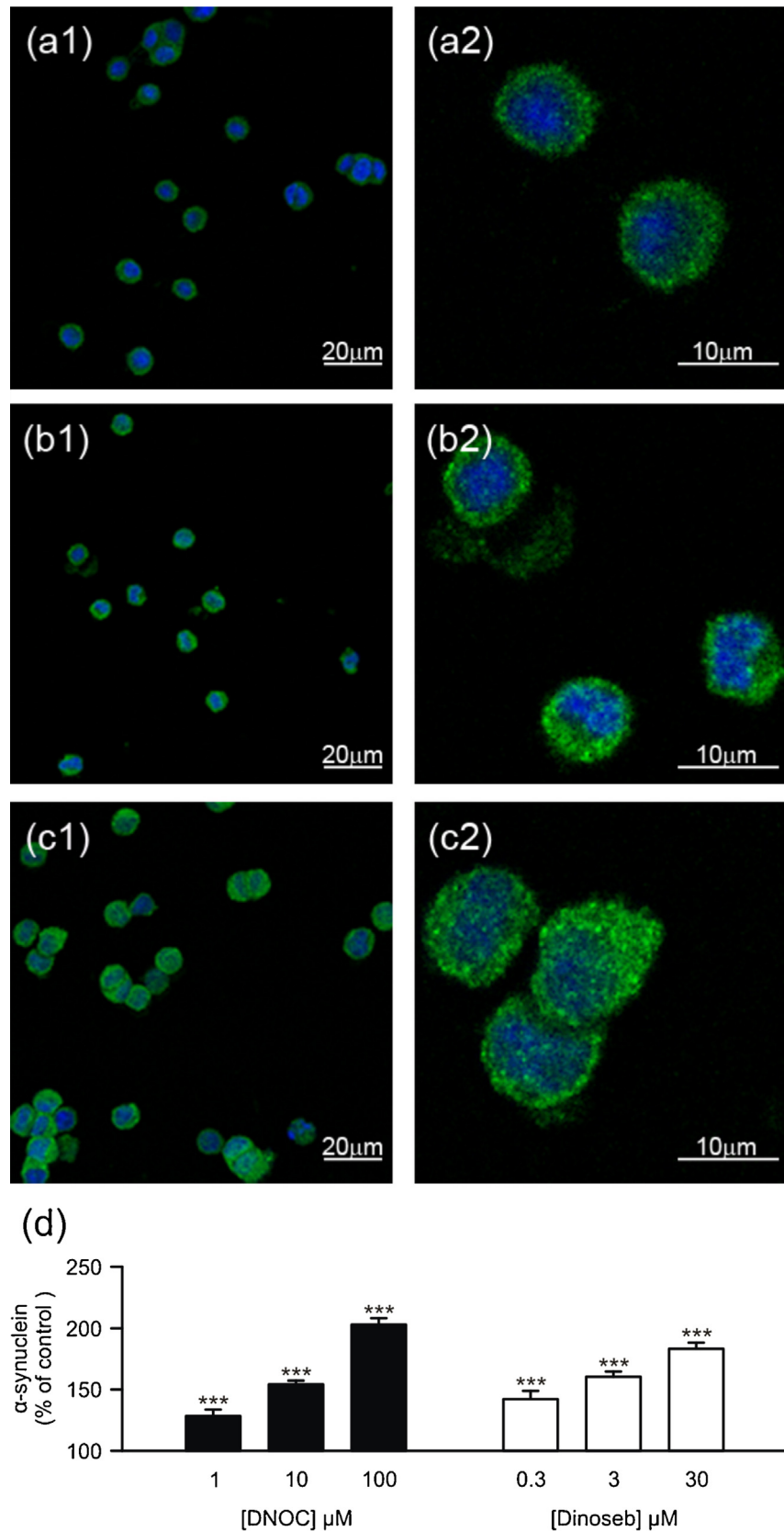
Fig. 3. Exposure of PC12 to DNOC and dinoseb for 48 h concentration-dependently increases intracellular α-synuclein levels. **a–c.** Example 1-stack confocal fluorescence micrographs showing α-synuclein immunostaining (green) and DAPI nuclear stain (blue) upon exposure to DMSO (**a1**, detail: **a2**), 100 μM DNOC (**b1**, detail: **b2**) and 30 μM dinoseb (**c1**, detail: **c2**). **d.** Bars display quantified fluorescence data (average ± SEM) from 10 to 52 individual cells stained on at least 2 independent coverslips. Difference from control: *** $p \leq 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

most likely indicated by the increase in mitochondrial activity as observed in the alamar Blue assay. However, the mild nature of this uncoupling activity is illustrated by the absence of ROS production. Nevertheless, as ER-mediated apoptotic cascades will ultimately also involve mitochondria (for review see: Circu and Aw, 2012), it remains to be determined what the effect of the compound-induced uncoupling is on the observed activation of apoptotic pathways. An increase of ROS production is limited to the highest concentration of dinoseb tested (Fig. 2B) at which cytotoxicity is already evident (Fig. 2A2). Consequently, the increase in oxidative stress appears not to be causative for the observed decrease in cell viability in PC12 cells.

In dopaminergic cells, ER-mediated Ca^{2+} release is linked to changes in intracellular α-synuclein levels (Mattson, 2007; Jiang et al., 2010; Marques and Outeiro, 2012). Indeed, a concentration-dependent increase in intracellular α-synuclein levels (Fig. 3) is observed. However, this increase is not observed following 24 h exposure but only becomes apparent after 48 h exposure and already at exposure levels below the observed effect concentration for increases in basal $[Ca^{2+}]_i$ (DNOC ($\geq$1 μM) or dinoseb ($\geq$0.3 μM); Fig. 1). It can be hypothesized that this is due to the spatial and temporal resolution of the applied fluorescence microscopy that, despite its high level of detail, does not allow for detection of small and fast oscillations in $[Ca^{2+}]_i$ or highly localized Ca^{2+} waves that relate to store-mediated signaling pathways (Eilers et al., 1995). These rather small and fast changes may precede the bigger calcium release events as observed at higher concentrations, but will remain undetected. However, it cannot be excluded that other, yet unknown, mechanisms also play a role.

According to animal studies, DNOC and dinoseb spread quickly through all tissues in the animal, and easily pass biological membranes such as the blood-brain (Parker et al., 1951) and placental barrier (Gibson and Rao 1973; Parker et al., 1951). Detection of high concentrations of DNOC in cerebrospinal fluid of human poisoning victims indicate that the same is true in humans (Bidstrup and Payne, 1951; Jiang Jiukun et al., 2011). A single subcutaneous dose of 1.5 mg (corresponding with approx. 3–4 mg/kg) in rats resulted in a blood DNOC concentration of 100 μg/ml serum (approx. 270 μM whole blood) and brain concentration of 4 μg/g w.w. (corresponding with approx. 20 μM) without being lethal (Gibson and Rao 1973). Animal studies have shown that systemic clearance of DNOC is rapid in rodents, cats and dogs without signs of accumulation of DNOC in the body ($T_{1/2}$: 24–72 h; Parker et al., 1951), However, a remarkable long half-life has been found in humans (calculated $T_{1/2}$: 5–14 days; Harvey et al., 1951). In the same study, is was demonstrated that in healthy human volunteers following 5 daily doses of 75 mg DNOC (0.92–1.27 mg/kg; peak blood concentration of ~100 μM), the compound was still measurable in reasonable amounts (1–1.5 μg/g blood = 5–8 μM; calculated using average physiological values) after 40 days. This is confirmed by a study from the Netherlands, demonstrating that upon chronic exposure (agricultural workers; 1 month with daily exposure) blood DNOC levels peaked at 11–88 μg/ml serum (corresponding with 30–240 μM; calculated using average physiological values), depending on the level of personal protection (van Noort et al., 1960). Despite differences in clearance between species, the lethal blood concentration DNOC was in all cases ~125 μg/ml (corresponding with a concentration of ~630 μM; calculated using average physiological parameters). Human data on dinoseb exposure and blood levels is not available. However, based on the similarities in the use pattern and the physicochemical properties with a higher lipophilicity of dinoseb compared to DNOC, it can be anticipated that internal dose levels of dinoseb are at least equal, but likely even higher.

Although the use of DNOC and dinoseb has been banned in the EU and the USA, detection of dinoseb in drinking water in e.g. China indicates that it is still in use (Li et al., 2012). Also, DNOC and dinoseb are demonstrated to be persistent in the environment, which is illustrated by the detection of these compounds in drinking- and rainwater (Quaghebeur et al., 2004; Schummer et al., 2009; Li et al., 2012; Rubio et al., 2012). Furthermore, a variety of chemically closely related compounds are used around the globe (see e.g. Schummer et al., 2009).

Notably, exposure to dinitrophenolic compounds can also occur through (illegal) use of these compounds as weight-loss agents in body building. Hospital admissions as a result of dinitrophenol poisoning are seen regularly, also in the EU (Siegmueller and Narasimhaiah, 2010; Petroczi et al., 2015). Often, these poisonings are lethal. In the last decade, also the pharmaceutical use of dinitrophenolic compounds has been suggested as the uncoupling activity of this type of compound is thought to be beneficial and even suggested to be neuroprotective (De Felice and Ferreira, 2006; Modrianský and Gabrielová, 2009). Altogether this indicates that exposure to these compounds or their chemical counterparts is still possible, which further underlines the importance of identifying possible long-term effects.

In conclusion, we demonstrated that in vitro exposure of dopaminergic PC12 cells to both DNOC and dinoseb results in ER-stress, which is followed by activation of apoptotic pathways and loss of cell viability as well as an increase of α-synuclein levels in surviving cells. Although an increase in intracellular α-synuclein levels is a hallmark of dopaminergic neurodegeneration as seen in PD, it may also reflect activation of protective pathways (Harischandra et al., 2015). Therefore, it remains to be determined what the long-term effects of the observed increase may be. Nonetheless, chronic exposure to environmental pollutants, in particular pesticides, has been linked to neurodegeneration and the development of Parkinson's disease (PD) in several epidemiologic studies (see e.g. Elbaz et al., 2009; van der Mark et al., 2012). In this respect it is noteworthy that recent research in a group of Parkinson's patients in an agricultural area of the Netherlands identified exposure to dinitrophenolic herbicides as a common denominator (unpublished results).

Though mechanisms underlying pesticide-induced (dopaminergic) neurodegeneration are largely unknown, disturbance of mitochondrial phosphorylation, changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) and in particular Ca^{2+}-related endoplasmic reticulum (ER) stress are recognized as inducers of tissue damage in dopaminergic brain areas (Mattson, 2007; Bartels and Leenders, 2009; Bezprozvanny, 2009; Heusinkveld et al., 2014). Subsequent protein misfolding and aggregation contributes to the development of cytoplasmic inclusions, such as α-synuclein-containing Lewy bodies that are a common pathophysiological hallmark of PD (Marques and Outeiro 2012; Witt, 2013).

Although the in vitro findings need to be confirmed in animals or even human tissue, we hypothesize exposure to dinitrophenolic herbicides or alike compounds may have long-term neurological consequences. Nevertheless, the current results demonstrate that exposure of dopaminergic cells to concentrations of dinitrophenolic herbicides that are relevant to the human situation can lead to activation of pathways that are considered as part of the pathophysiological cascade that may ultimately lead to neurotoxicity and neurodegeneration.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2016.04.014.

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