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Original Contribution

Compartmentalized oxidative stress in dopaminergic cell death induced by pesticides and complex I inhibitors: Distinct roles of superoxide anion and superoxide dismutases

Humberto Rodriguez-Rocha [a,b], Aracely Garcia-Garcia [a,b], Chillian Pickett [a,b], Sumin Li [a,b], Jocelyn Jones [d], Han Chen [c], Brian Webb [e], Jae Choi [e], You Zhou [a,b,c], Matthew C. Zimmerman [a,d], Rodrigo Franco [a,b,(*)]

[a] Redox Biology Center, University of Nebraska–Lincoln, Lincoln, NE 68583-0905, USA
[b] School of Veterinary Medicine and Biomedical Sciences, University of Nebraska–Lincoln, Lincoln, NE 68583-0905, USA
[c] Center for Biotechnology, University of Nebraska–Lincoln, Lincoln, NE 68583-0905, USA
[d] Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE 68198-5850, USA
[e] Thermo Scientific, Research and Development, Rockford, IL 61105, USA

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ABSTRACT

The loss of dopaminergic neurons induced by the parkinsonian toxins paraquat, rotenone, and 1-methyl-4-phenylpyridinium (MPP^+) is associated with oxidative stress. However, controversial reports exist regarding the source/compartmentalization of reactive oxygen species (ROS) generation and its exact role in cell death. We aimed to determine in detail the role of superoxide anion ($O_2^{\bullet-}$), oxidative stress, and their subcellular compartmentalization in dopaminergic cell death induced by parkinsonian toxins. Oxidative stress and ROS formation were determined in the cytosol, intermembrane (IMS), and mitochondrial matrix compartments, using dihydroethidine derivatives and the redox sensor roGFP, as well as electron paramagnetic resonance spectroscopy. Paraquat induced an increase in ROS and oxidative stress in both the cytosol and the mitochondrial matrix prior to cell death. MPP^+ and rotenone primarily induced an increase in ROS and oxidative stress in the mitochondrial matrix. No oxidative stress was detected at the level of the IMS. In contrast to previous studies, overexpression of manganese superoxide dismutase (MnSOD) or copper/zinc SOD (CuZnSOD) had no effect on alterations in ROS steady-state levels, lipid peroxidation, loss of mitochondrial membrane potential ($\Delta\Psi m$), and dopaminergic cell death induced by MPP^+ or rotenone. In contrast, paraquat-induced oxidative stress and cell death were selectively reduced by MnSOD overexpression, but not by CuZnSOD or manganese-porphyrins. However, MnSOD also failed to prevent $\Delta\Psi m$ loss. Finally, paraquat, but not MPP^+ or rotenone, induced the transcriptional activation of the redox-sensitive antioxidant response elements (ARE) and nuclear factor kappa-B (NF-κB). These results demonstrate a selective role of mitochondrial $O_2^{\bullet-}$ in dopaminergic cell death induced by paraquat, and show that toxicity induced by the complex I inhibitors rotenone and MPP^+ does not depend directly on mitochondrial $O_2^{\bullet-}$ formation.

Introduction

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1]. Postmortem PD brains have elevated levels of nucleic acid, protein and lipid byproducts from oxidation [2–4], supporting a role for oxidative stress in dopaminergic cell loss. However, the molecular events and mechanisms involved remain unknown. Over 90% of PD cases occur most commonly in a sporadic (idiopathic) with a pathogenesis likely linked to environmental causes. [5,6].

A dysfunction in the electron transport chain (ETC) has been found in PD brains. Thus, inhibitors of complex I activity are well accepted toxicological models for understanding dopaminergic

Abbreviations: ARE, antioxidant response elements; DHE, dihydroethidium; ETC, electron transport chain; CuZnSOD, copper/zinc SOD; IMS, intermembrane space; MnSOD, manganese SOD; MPP^+, 1-methyl-4-phenylpyridinium; $\Delta\Psi m$, mitochondrial membrane potential; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin tetratosylate hydroxide; $NO^{\bullet}$, nitric oxide; NF-κB, nuclear factor kappa-B; PD, Parkinson's disease; $ONOO^-$, peroxynitrite; PI, propidium iodide; ROS, reactive oxygen species; roGFP, reduction–oxidation-sensitive green fluorescence protein; SOD, superoxide dismutase; SNpc, substantia nigra pars compacta; $O_2^{\bullet-}$, superoxide anion

(*) Corresponding author at Redox Biology Center and School of Veterinary Medicine and Biomedical Sciences, 114 VBS 0905, University of Nebraska–Lincoln, Lincoln, NE 68583. Fax: +1 402 472 9690.
E-mail address: rfrancocruz2@unl.edu (R. Franco).

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cell death pathways [7]. Recent epidemiological data also suggests a link between the exposure to environmental toxicants such as paraquat and rotenone and an increased risk in developing PD [8]. Dopaminergic cell death induced by parkinsonian toxins has been reported to be tightly linked to the generation of ROS, primarily $O_2^{\bullet-}$ formation. However, contradictory results exist regarding the role of oxidative stress in dopaminergic cell death induced by these toxins. For example, MPP^+/MPTP toxicity has been reported to be inhibited by SOD mimetics [9,10], and overexpression of CuZnSOD [11,12] and MnSOD [13], while MnSOD or CuZnSOD deficiency increases its toxicity [14,15]. In contrast, several studies also show that MPP^+/MPTP toxicity is mediated, at least in part, by a mechanism independent from inhibition of complex I [16] and the generation of ROS [17–23]. Similar conflicting results have been found with respect to the role of complex I inhibition and ROS formation in rotenone-induced toxicity [16,17,22,24–26]. Dopaminergic cell death induced by paraquat is largely ascribed to the generation of ROS and oxidative stress [27]. However, while some studies demonstrate that mitochondria are the primary site of ROS formation on paraquat exposure [28–30], other reports suggest that the cytoplasm is where ROS are primarily generated [31,32].

Based on the controversies summarized above, we aimed to determine the role of superoxide anion ($O_2^{\bullet-}$), oxidative stress, and its compartmentalization in dopaminergic cell death induced by the parkinsonian toxins. The results presented here clearly distinguish, for the first time, a selective role of mitochondrial $O_2^{\bullet-}$ in dopaminergic cell death induced by paraquat, and show that toxicity induced by the complex I inhibitors rotenone and MPP^+ does not depend directly on mitochondrial $O_2^{\bullet-}$ formation.

Materials and methods

Cell culture and treatments

Human dopaminergic neuroblastoma cells (SK-N-SH) and human IMR-32 neuroblastoma cells (ATCC; Manassas, VA, USA) were cultured as indicated by the provider. Cell culture reagents were obtained from Thermo Scientific/Hyclone (Logan UT) or Invitrogen/GIBCO (Carlsbad, CA). Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), 1-methyl-4-phenylpyridinium iodide (MPP^+), and rotenone were obtained from Sigma/Aldrich (St. Louis, MO).

Recombinant adenoviral vectors

Replication-deficient recombinant adenoviruses (Ad5CMV-MnSOD and Ad5CMV-CuZnSOD were used to overexpress MnSOD or CuZnSOD and have been described previously [33,34]. Adenovirus containing only the CMV promoter (AdEmpty) was used as negative control. Adenoviruses were amplified and titered in HEK293T cells as described previously [35,36]. Cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 0.15 and treated with experimental conditions at 24 h postinfection.

Cytotoxicity assay (mitochondrial activity)

Mitochondrial activity was assessed as a marker of cytotoxicity by measuring the conversion of the tetrazolium salt MTT to formazan as described in [36]. 7500 cells per well (96-well format) were initially plated. The amount of formazan dye produced is directly proportional to the number of metabolically active cells and indicates the reducing potential of the cell.

Cell death determination (loss of plasma membrane integrity)

Loss of cell viability was determined using flow cytometry (FACS, fluorescence activated cell sorting) by measuring either propidium iodide (PI, 1 μg/ml) or Sytox Blue uptake (5 nM) (Invitrogen/Molecular Probes) as markers for plasma membrane integrity loss. PI and Sytox Blue were detected using FL-3 (488 nm excitation, 695/40 nm emission) or L2-4 (561 nm ex, 615/25 nm em), and 407 nm ex, 450/50 nm em respectively, in a BDFACSort (Cytek-DxP-10 upgrade). Data were analyzed using FlowJo 7.6.5 software. When indicated, cells were pretreated with Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) or Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin tetratosylate hydroxide (MnTMPyP). MnTBAP was resuspended in 0.1 mM NaOH. Alternatively, cell death was assessed using a Calcein retention assay which measures both the number of cells attached and the integrity of the plasma membrane. Cells were incubated with 1 μg/ml Calcein-AM for 30 min. Then, cells were washed and analyzed at 485 nm ex and 520 nm em in a FLUOstar OPTIMA plate reader (BMG Labtech, Cary, NC).

ROS formation and oxidative stress in mitochondrial and cytosolic compartments

ROS production was determined using dihydroethidine (DHE) (Invitrogen/Molecular Probes), which after its oxidization to ethidium intercalates within the DNA and exhibits bright red fluorescence. ROS production in the mitochondria was assessed using MitoSOX Red (Invitrogen/Molecular Probes), a derivative of DHE with a cationic triphenylphosphonium substituent responsible for the electrophoretic uptake into actively respiring mitochondria. Cells were incubated with DHE or MitoSOX (5 μM) for 15 min and their oxidation was monitored by flow cytometry using FL-2 (488 nm ex, 585/42 nm em) or L3-2 (407 nm ex, 545/30 nm emission).

Alterations in the redox state of both cytosol and mitochondrial compartments were monitored using the reduction–oxidation-sensitive green fluorescent protein (roGFP). roGFP sensor allows the noninvasive monitoring of intracellular thiol-disulfide equilibrium, and its fluorescence is determined by the oxidation state of two cysteines introduced into the structure of the GFP. Expression vectors encoding roGFP1 and roGFP1 with a mitochondrial targeting sequence (Mito-roGFP containing the pyruvate dehydrogenase E1α subunit leader sequence) were obtained from Dr. Remington's lab (University of Oregon, Eugene, OR) [37]. roGFP was targeted to the intermembrane space (IMS) using the IMS-targeting signal at the N-terminal amino acid sequence (residues 10–57) from Smac/DIABLO (RSVCSLFRYRQRFPVLANSKKRCFSELIKPWHKTVLTGFGMTLCAVPI), an approach that has been shown to target functional fluorescent proteins into the mitochondrial intermembrane space. This sequence was kindly provided by Dr. Takeaki Ozawa (Department of Chemistry, University of Tokyo, Tokyo, Japan) [38]. All plasmids were linearized with *Nde I* and transfected into SK-N-SH cells using FuGENE HD (Promega, Madison, WI, USA). Stable overexpressing cells were selected in complete medium containing 0.3 mg/ml G418 and GFP positive cell sorting was carried out in a BD FACSAria cell sorter (BD Biosciences, San Jose, CA). roGFP oxidation results in reciprocal changes in emission intensity when excited at two different wavelengths. Changes in roGFP fluorescence were monitored at the single cell level by flow cytometry using ratiometric analysis of roGFP by its dual excitation at 407 and 488 nm, and 530 nm emission.

Protein extraction and Western immunoblotting

Western immunoblots were performed as explained in [36]. Blots were incubated with the corresponding primary antibody overnight (1:1000): MnSOD (Abnova, Taipei, Taiwan); CuZnSOD,

VDAC, and GAPDH (Cell Signaling, Danvers, MA). Blots were reprobed to verify equal protein loading.

Confocal microscopy

Cells were grown on glass bottom dishes (MatTek, Ashland, MA) coated with poly-D-lysine (0.1 mg/ml) and incubated with MitoTracker Deep Red (500 nM, 15 min) to label mitochondria. MitoTracker was removed after incubation and live cells were analyzed using an inverted (Olympus IX 81) confocal microscope. (Mito-/IMS-)roGFP was visualized using 488 nm ex laser wavelength and 505–525 nm em. MitoTracker Deep Red was analyzed using 633 nm ex and 660IF emission filter.

Isolation of mitochondrial and cytosolic fractions

Mitochondrial fraction was isolated as described in [39]. Briefly, cells were harvested by trypsinization and then resuspended and homogenized in ice-cold isolation buffer-1 (IB-1: 225 mM mannitol, 75 mM sucrose, and 30 mM Tris•Cl, pH 7.4, 0.1 mM EGTA), using a Teflon-glass homogenizer. Samples were centrifuged twice (600 *g*) to remove the nuclei and debris and the supernatant was centrifuged again (7000 *g*). Supernatants containing plasma membrane, lisosomes, microsomes, and cytosol fractions were collected for further analysis. Pellets were washed twice in IB-2 (225 mM mannitol, 75 mM sucrose, and 30 mM Tris-HCl) and centrifuged at 7000 *g* and 10,000 *g*. Crude mitochondria pellet was resuspended in 2 ml of mitochondria resuspension buffer (MRB: 250 mM mannitol, 5 mM Hepes, pH 7.4, and 0.5 mM EGTA) and then layered on top of Percoll solution (225 mM mannitol, 25 mM sucrose, 1 mM EGTA, and 30% Percoll, v/v) in polypropylene tubes for SW41 Ti rotor (Beckman). Additional MRB was layered on top of mitochondria. After centrifugation (95,000 *g*), a band containing purified mitochondria was isolated and diluted 10 times in MRB. After centrifugation (6300 $g \times 10$ min, 4 °C), mitochondria pellet was gently resuspended in MRB.

Transmission electron microscopy (TEM)

Cells were fixed with 2% glutaraldehyde in 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.4) for 2 h at room temperature. Fixed cells were collected and postfixed with 1% OsO_4 in 0.2 M Hepes, and stained in 2% uranyl acetate [40]. The pellets were dehydrated through a graduated ethanol series and embedded in Epon 812 (Electron Microscopic Sciences, Fort Washington, PA) for sectioning, and subsequently observed under a transmission electron microscope (Hitachi H7500). A series of ultrastructural images were collected with a bottom-mount digital camera for the analysis of mitochondria.

Electron paramagnetic resonance (EPR) spectroscopy

After treatment, cells were loaded with the $O_2^{\bullet-}$-sensitive cyclic hydroxylamine cell permeable spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) (200 μM; 30–60 min) in EPR buffer (Krebs-Hepes buffer, pH 7.4, consisting of [in mM]: 99 NaCl, 4.69 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.03 KH_2PO_4, 5.6 D-glucose, 20 Hepes and supplemented with the metal chelators DETC [5 μM] and deferoxamine [25 μM pH 7.4]). After incubation, cells were collected and placed into a Bruker e-scan EPR spectrometer (Billerica, MA). The following EPR settings were used for all experiments: field sweep width, 60.0 G; microwave frequency, 9.75 kHz; microwave power, 21.90 mW; modulation amplitude, 2.37 G; conversion time, 10.24 ms; time constant, 40.96 ms. Levels of $O_2^{\bullet-}$ were normalized to the number of cells in each sample.

Luciferase reporter assays

The ARE, NFkB, XRE-Gaussia, and -Cypridina Luc reporter plasmids were constructed by cloning each response element plus minimal promoter into Pierce promoterless pMCS-Luciferase Reporter Plasmids (Part No. 16146, Part No. 16149, Part No. 16152).Three plasmids (ARE-Gaussia, ,NFkB-Cypridina, and CMV-Red Firefly) were transfected into 1.0×10^5 cells for 24 h using Pierce TurboFect Transfection Reagent. At 24 h post-transfection, cells were treated with the indicated neurotoxins for 4 to 5 h . The media (Gaussia activity) was carefully collected for activity measurement. Then, the cells were lysed with Pierce Luciferase Cell Lysis Buffer. Cypridina and Red Firefly activities in lysate were determined using the luciferase dual assay reagents (Part No. 16181). Gaussia and Cypridina activities were normalized by Red Firefly activities. Bioluminescence signals (RLUs) were detected using a Thermo Scientific Varioskan Flash Luminometer equipped with reagent injectors (signal integration time = 1 s) and filter sets in the ranges of 425 to 525 nm for Cypridina Luc and 615 nm LP for Red Firefly Luc.

Lipid peroxidation

Lipid peroxidation was determined using the ratiometric probe BODIPY C11 (Invitrogen/Molecular Probes), whose fluorescence emission at 590 nm (bright red fluorescence) decreases in response to oxidation with a concomitant increase in bright green fluorescence (530 em) [41,42]. Cells were incubated for 30 min prior to FACS analysis with BODIPY C11 (2.5 μM) and its oxidation was monitored by flow cytometry using FL-1 (488 nm ex, 530/30 nm em) and L2-3 (561 nm ex, 590/20 nm). Results were analyzed ratiometrically (530/590 nm em) normalized with control values.

Mitochondrial membrane potential (ΔΨm)

Alterations in ΔΨm were evaluated using tetramethylrhodamine (TMRM, Invitrogen/Molecular Probes), whose accumulation in the mitochondria is driven by the ΔΨm. Cells are incubated with 50 nM TMRM 15 min prior to FACS analysis and TMRM fluorescence was measured using 561 nm ex, and 580/20 nm em.

Statistical analysis

Experiment replicas were independent and performed on separate days. Collected data were analyzed according to statistical criteria by using paired or unpaired *t* test, one-way ANOVA or two-way ANOVA, and the appropriate parametric or nonparametric normality post-test using Sigma-Plot/Stat package. A probability value of $P<0.05$ was considered as statistically significant. Data were plotted as mean values of at least three independent experiments ± standard error of the mean (SE) using the same statistical package for data analysis. Flow cytometry plots and Western blots presented are representative of at least three independent experiments.

Results

Mitochondrial $O_2^{\bullet-}$ formation in response to parkinsonian toxins

Paraquat toxicity is largely ascribed to the generation of $O_2^{\bullet-}$. However, while some studies demonstrate that mitochondria are the primary source of ROS formation on paraquat exposure [28–30,43,44], other reports suggest that oxidative stress mainly happens in the cytosol [31,32]. We first evaluated whether paraquat induces ROS production, specifically in the cytosol or mitochondria using the dihydroethidium derivatives DHE and MitoSOX,

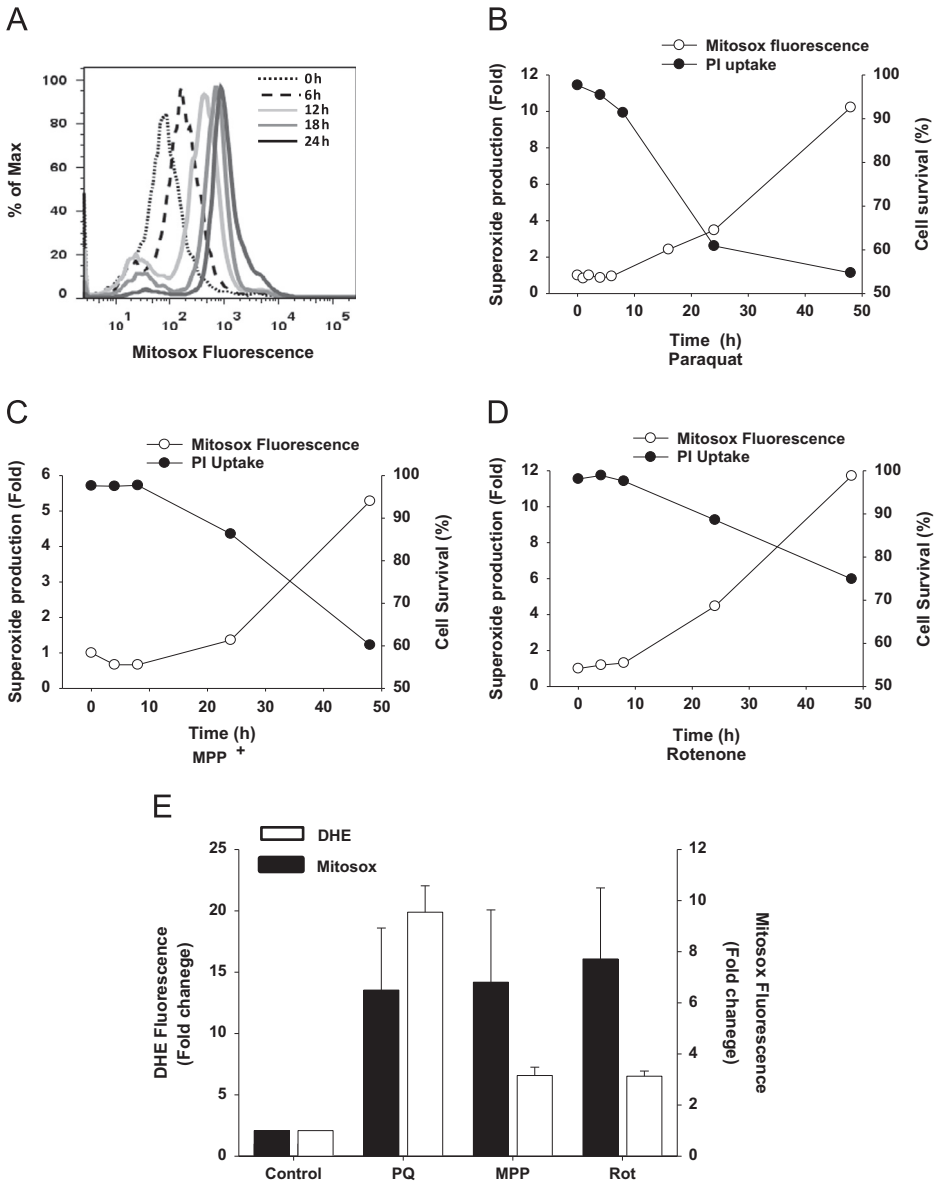

Fig. 1. ROS detection using dihydroethidium derivatives. In A–D, SK-N-SH cells were treated with 0.5 mM paraquat, 2.5 mM MPP^+, or 4 μM rotenone for the time indicated. Then, cells were stained with MitoSOX (mitochondrial ROS) or propidium iodide (cell death), and analyzed by FACS. Histogram in A depicts the changes in the MitoSOX mean fluorescence in response to paraquat. In B–D, data are expressed as ROS production (MitoSOX fluorescence) and cell survival (% of cells with low propidium iodide staining) normalized against control values. Experiments and plots in A–D are representative of at least three independent experiments. In E, cells were treated with 0.5 mM paraquat, 2.5 mM MPP^+, or 4 μM rotenone for 48 h. Then, cells were costained with DHE (cytosolic ROS) or MitoSOX (mitochondrial ROS) and analyzed by FACS. Results are represented as the increase in DHE or MitoSOX mean fluorescence with respect to controls and are means ± SE of three replicas.

respectively. Fig. 1A, B, and E demonstrate that paraquat induces both cytosolic and mitochondrial ROS formation, which parallels cell death. MPP^+ and rotenone treatment also resulted in an increase in cell death and ROS production in a time-dependent manner, which was primarily restricted to the mitochondrial compartment (Fig. 1C, D, and E). These results demonstrate that while these parkinsonian neurotoxins induce to a similar extent mitochondrial ROS production, paraquat induces major cytosolic ROS accumulation.

SODs are metalloenzymes that catalyze the conversion of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). CuZnSOD has primarily cytoplasmic localization, although it can also be found in the peroxisomes, lysosomes, nucleus, and mitochondrial intermembrane space (IMS), while MnSOD is located in the mitochondrial matrix [45]. We overexpressed MnSOD and CuZnSOD using adenoviral vectors (Fig. 2A) and determined their effect on ROS formation induced by paraquat, MPP^+, or rotenone. MnSOD prevents the increase in mitochondrial ROS steady-state levels induced by paraquat, while CuZnSOD had no effect on oxidative stress (Fig. 2B). In contrast, neither MnSOD nor CuZnSOD overexpression prevented the increase in ROS levels induced by complex I inhibitors (Fig. 2C). Paraquat, MPP^+, and rotenone significantly altered mitochondrial morphology assessed by electron microscopy including swelling, matrix thinning, and cristea breakdown/disruption (Fig. 2D). These results demonstrate that although paraquat, MPP^+, and rotenone act as mitochondrial toxins and induce mitochondrial oxidative damage, only MnSOD is able to reduce ROS accumulation induced by paraquat.

Effect of overexpression of SODs on dopaminergic cell death induced by parkinsonian toxins

We next evaluated the effect of SOD overexpression on dopaminergic cell death induced by paraquat, MPP^+, and rotenone by flow cytometry analyzing both changes in PI staining and cell

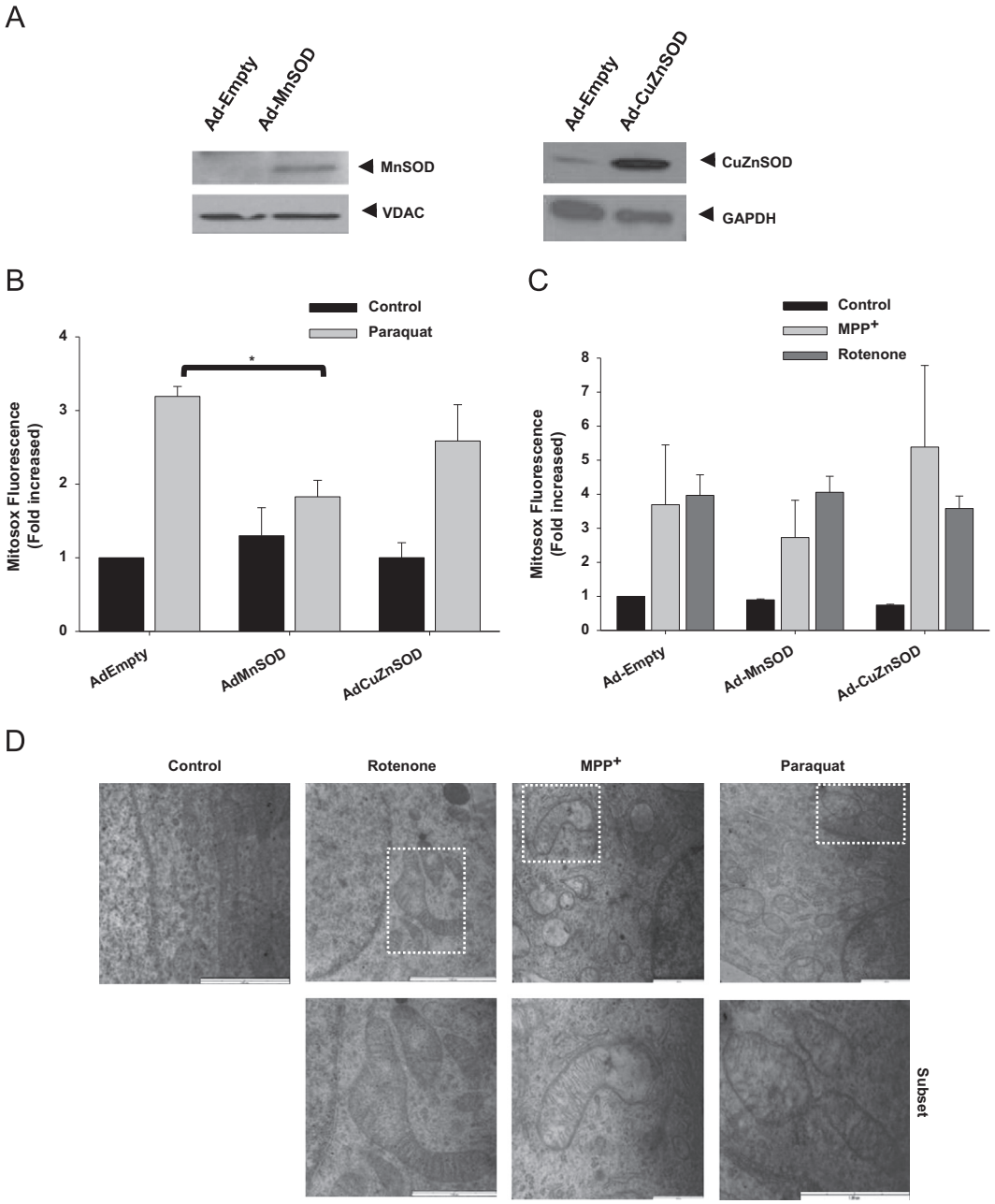

Fig. 2. Mitochondrial damage and effect of SOD overexpression in ROS formation. Cells were transduced with Ad-MnSOD, Ad-CuZnSOD, or Ad-Empty. In A, isolation of mitochondrial and cytosolic fractions was performed by differential centrifugation. Western blot analysis demonstrates the increased expression of SOD enzymes. VDAC and GAPDH signal was used as loading control for mitochondrial and cytosolic fractions, respectively, and to evaluate cross-contamination of subcellular fractionation (not shown). In B and C, cells transduced with viruses were treated with 0.5 mM paraquat, 2.5 mM MPP^+, or 4 μM rotenone for 48 h. Mitochondrial $O_2^{\bullet-}$ was determined using MitoSOX as explained in Fig. 1. In D, cells were treated with parkinsonian toxins as explained above for 24 h and then fixed with 2% glutaraldehyde. Fixed cells were processed for TEM. Blots (A) and images (D) are representative experiments, and data in B and C are means ± SE of at least three independent replicas. *$P<0.05$ vs Ad-Empty + paraquat values.

shrinkage (a marker for apoptosis) (Fig. 3A). Parkinsonian neurotoxins induced a decrease in the population of PI negative cells with normal cell size (see %s in Fig. 3A). MnSOD but not CuZnSOD overexpression significantly increased cell survival in the presence of paraquat (Fig. 3B). Similar results were observed when mitochondrial activity was determined by MTT assay (Fig. 3C). No additive effect was observed by coexpression of both MnSOD and CuZnSOD. In contrast to previous studies [11–15], overexpression of either CuZnSOD or MnSOD did not decrease MPP^+- or rotenone-induced cell death (Fig. 3D). Manganese-porphyrins, MnTBAP and MnTMPyP, failed to reduce dopaminergic cell death (Calcein retention assay)induced by paraquat, and mitochondrial ROS detection (Fig. 3E, and supplementary Fig. 5C). These results demonstrate that while paraquat-induced cell death is directly dependent on the generation of $O_2^{\bullet-}$ in the mitochondria, the mechanism by which MPP^+ and rotenone induce toxicity might involve additional events besides mitochondrial $O_2^{\bullet-}$ formation.

Alterations in the redox state of cytosol and mitochondria compartments in response to parkinsonian neurotoxins

To study more in detail the alterations in the redox state induced by paraquat, MPP^+, or rotenone, we use the redox sensor roGFP. Cells were stably transfected with roGFP, Mito-roGFP, and IMS-roGFP. The expression, localization, and functionality of the redox sensors were determined by confocal microscopy and flow

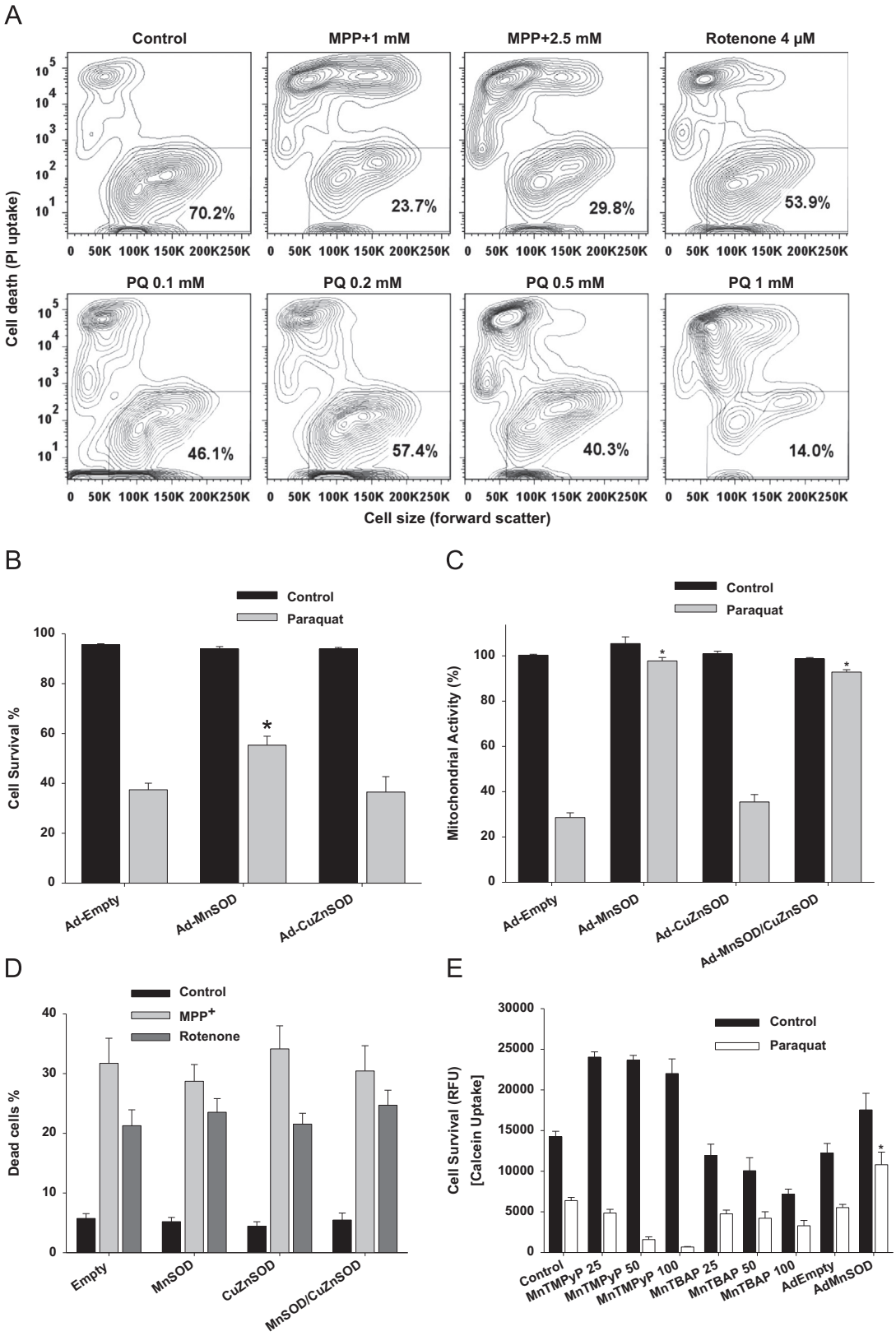

Fig. 3. Effect of overexpression of SODs on dopaminergic cell death induced by parkinsonian toxins. In A, cells were treated with paraquat, MPP^+, or rotenone for 48 h and stained with PI. Cell death was analyzed in a forward scatter vs PI contour plot to depict cells with both a decrease in cell size (a marker for apoptosis) and a loss of plasma membrane integrity. %s represent the population of cells with low PI and normal cell size (healthy cells) depicted in the lower right region. In B–D, cells were transduced with Empty, MnSOD, or CuZnSOD adenoviruses 24 h prior exposure to parkinsonian toxins. Cell survival (B) or cell death (D) was determined analyzing the % of cells with low or high PI staining, respectively. In C, alterations in mitochondrial activity were determined using the MTT assay. The effect of MnTBAP and MnTMPyP porphyrins on the survival of cells exposed to the parkinsonian mimetics was quantified by Calcein retention (E). Porphyrins were present throughout the experiment and concentrations used are indicated in μM. Data in B–E represent means ± SE of at least three replicas. *$P<0.05$ vs Ad-Empty + paraquat values.

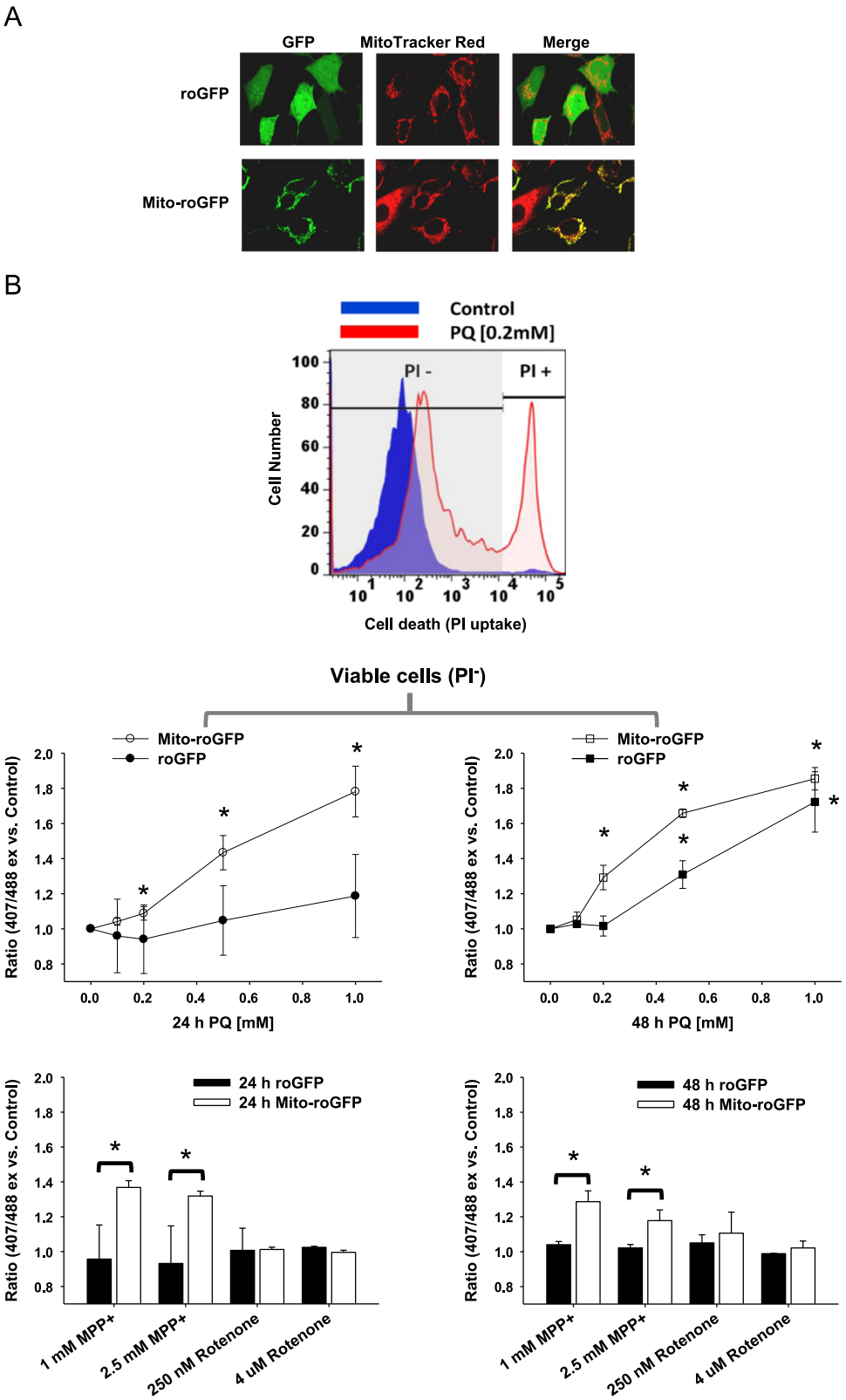

Fig. 4. Alterations in the redox state of cytosol and mitochondrial compartments in response to parkinsonian neurotoxins. In A, stable cells overexpressing roGFP and Mito-roGFP were stained with MitoTracker Red to depict mitochondrial localization of Mito-roGFP. In B, roGFP and Mito-roGFP cells were treated with paraquat, MPP^+, or rotenone as indicated. Cells were costained with PI and only viable cells were analyzed (see population of PI- cells in the gray region of the histogram). Alterations in the redox state were determined by ratiometric analysis of changes in (Mito-)roGFP fluorescence at 407/488 ex and 530 em normalized with respect to control values. Data in graphs represent means ± SE of at least five independent experiments. $^*P<0.05$ vs control values.

cytometry. While roGFP was distributed throughout the cell, Mito-roGFP (Fig. 4A) and IMS-roGFP (Supplementary Fig. 2A) colocalized with mitochondria staining (Mitotracker). Ratiometric analysis at 407/488 nm ex and 530 nm em demonstrated the functionality and sensitivity of theroGFP sensors stably overexpressed, to oxidizing (H_2O_2) and reducing conditions (dithiothreitol, DTT) (Supplementary Fig. 1). To determine alterations in the cellular redox balance occurring prior to cell death, we only analyzed viable cells that have not lost the plasma membrane integrity (PI- cells depicted in the histogram of Fig. 4B). Paraquat and MPP^+ significantly increased oxidative stress in the mitochondrial matrix at 24 h (Fig. 4B, left panels). At 48 h, there was a dose–response increase in both mitochondrial matrix and cytoplasmic oxidative stress (Fig. 4B, right panels) on paraquat exposure. Neither paraquat nor MPP^+ increased oxidative stress in the intermembrane space (IMS-rogfp, Supplementary Figs. 2B and C). Rotenone failed to induce alterations in the cellular redox state measured by roGFP and Mito-roGFP. However a slight increase in

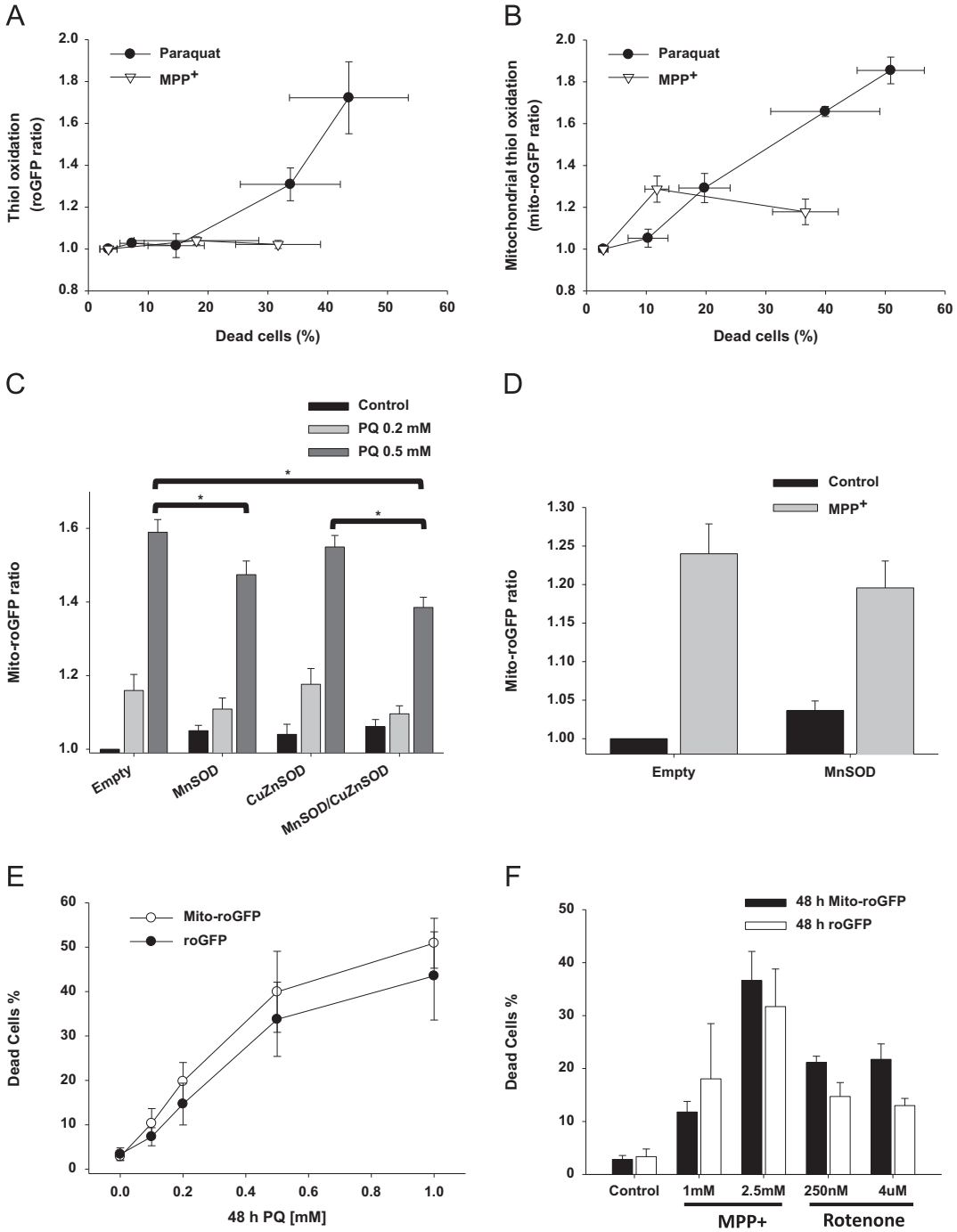

Fig. 5. Relationship between cell death and alterations in the redox state of cytosol and mitochondria compartments, and effect of SOD overexpression. In A and B, stable cells overexpressing roGFP and mito-roGFP were treated with 0.5 mM paraquat and 2.5 mM MPP^+ for 48 h. Cells were costained with PI and only viable cells (PI-) were analyzed for alterations in (Mito-)roGFP fluorescence as explained in Fig. 4. Cell death was plotted against (Mito-)roGFP ratio to depict the relationship between cell loss and alterations in the redox balance of the cytosol and mitochondrial compartments. In C and D, cells were transduced with Empty, MnSOD, or CuZnSOD adenoviruses and treated 24 h after infection. Alterations in Mito-roGFP fluorescence were determined as explained before. In E and F, stable cells overexpressing roGFP and Mito-roGFP were treated with paraquat, MPP^+, or rotenone as indicated. Cells were costained with PI and cell death was quantified as explained in Fig. 3. Graphs represent means ± SE of at least five independent experiments. $^*P<0.05$.

IMS-roGFP signal was detected (Fig. 4B, and Supplementary Fig. 2C).

When analyzing the relationship between oxidative stress and cell death we observed that there was a strong correlation between alterations in the redox balance in both cytosolic and mitochondrial compartments, and cell death induced by paraquat (Fig. 5A and B). In contrast, MPP^+ toxicity was only associated with a slight increase in mitochondrial oxidative stress (Fig. 5B). Similar to the observations obtained using dihydroethidium derivatives, overexpression of MnSOD but not CuZnSOD significantly reduced mitochondrial oxidative stress in paraquat-treated cells (Fig. 5C), while overexpression of MnSOD had no effect on mitochondrial oxidative stress induced by MPP^+ (Fig. 5D). These results demonstrate that paraquat induces an early increase in oxidative stress in the mitochondrial matrix associated with $O_2^{\bullet-}$ formation, which is followed by subsequent oxidative stress in the cytosol. Furthermore, these data confirm that mitochondrial oxidative stress induced by MPP^+ cannot be prevented by overexpression of MnSOD, suggesting that alterations in the redox balance induced by MPP^+ might involve a distinct source of oxidative stress besides $O_2^{\bullet-}$. It is important to note that the differences in the kinetic responses among roGFP, Mito-roGFP, and IMS-roGFP cells to distinct concentrations of paraquat, MPP^+, and/or rotenone cannot be ascribed to intrinsic differences between the stable cell lines overexpressing the roGFP sensors, as their sensitivity to cell death was comparable (Fig. 5E and F, and Supplementary Fig. 3).

Detection of free radical formation induced by parkinsonian toxins using EPR

Although EPR has low sensitivity relative to the reactivity to most free radicals, it is considered the primary and most unambiguous technique for detection of free radicals, which makes it an excellent approach for studying oxidative stress in response to parkinsonian toxins. Oxidation of the spin probe CMH was used to detect free radical$^-$ formation. CMH oxidation-dependent EPR signal was increased by exposure to parkinsonian toxins in a time- and dose-dependent manner (Fig. 6A and Supplementary Figs. 4A and B). Overexpression of MnSOD but not CuZnSOD significantly decreased CMH oxidation induced by paraquat (Fig. 6B and C). In contrast, SOD overexpression had no effect on oxidative stress induced by MPP^+ or rotenone (Fig. 6C and Supplementary Figs. 4C and D).

Activation of ARE- and NF-κB-driven reporters and lipid peroxidation induced by parkinsonian neurotoxins

Oxidative stress activates the transcription of a variety of antioxidant genes through *cis*-acting sequences known as antioxidant response elements (ARE). Similarly, oxidative stress has been largely demonstrated to activate NF-κB-dependent transcription of prosurvival genes [46]. We next determined the activation of both ARE- and NF-κB-driven reporters (Fig. 6D) in response to parkinsonian mimetics as a measurement of oxidative stress and cellular antioxidant response. Fig. 6D shows that paraquat, but not complex I inhibitors, triggered transcriptional activity driven by ARE and NF-κB .

Lipids are major targets of ROS and lipid peroxidation is a major consequence of oxidative damage [47]. Thus, we also evaluated the effects of SOD overexpression on lipid peroxidation-induced by parkinsonian mimetics. Fig. 6E demonstrates that paraquat-induced lipid peroxidation is higher compared to MPP^+ and rotenone. Regardless, only MnSOD overexpression reduces paraquat-induced lipid peroxidation. Overall, these results demonstrate that oxidative stress and cell death induced by paraquat are directly linked to $O_2^{\bullet-}$ formation in the mitochondria, while oxidative stress and cell death associated with complex I inhibition involve additional mechanisms besides mitochondrial $O_2^{\bullet-}$.

Loss of mitochondrial membrane potential by paraquat is not prevented by MnSOD

As shown in Fig. 2D (TEM), paraquat, MPP^+, and rotenone induce profound alterations in mitochondrial structure, while overexpression of MnSOD prevents the loss of mitochondrial activity induced by paraquat (Fig. 3C). To further study the role of paraquat-induced $O_2^{\bullet-}$ in mitochondrial function we evaluated alterations in $\Delta\Psi m$ induced by paraquat. Fig. 7A shows that cell death induced by paraquat, MPP^+, or rotenone is paralleled by a decrease in $\Delta\Psi m$ (upper left quadrants in contour plots). A population of viable cells with a decrease in $\Delta\Psi m$ (lower left quadrants) was significantly increased in response to MPP^+ and rotenone treatments (Fig. 7B), suggesting that $\Delta\Psi m$ loss precedes cell death. Overexpression of MnSOD or CuZnSOD had no effect in $\Delta\Psi m$ loss induced by MPP^+ or rotenone. Interestingly, loss of $\Delta\Psi m$ on paraquat exposure was only detected in the population of dead cells (Fig. 7A upper left quadrants). MnSOD overexpression decreased cell death induced by paraquat (Fig. 7A, upper quadrants, and Fig. 3B). However, it did not alter $\Delta\Psi m$ loss and, as a result, induced an accumulation of viable cells with a decrease in $\Delta\Psi m$ (Fig. 7A, lower left quadrants, and Fig. 7B). These results demonstrate that depolarization of $\Delta\Psi m$ in response to paraquat is independent from mitochondrial $O_2^{\bullet-}$ formation.

Discussion

Controversial reports exist regarding the source/compartmentalization of ROS generation and its exact role in dopaminergic cell death induced by complex I inhibitors and pesticides. We have done a thorough analysis to determine the role of $O_2^{\bullet-}$, oxidative stress, and its compartmentalization in dopaminergic cell death induced by parkinsonian toxins. We found that paraquat induced an early increase in mitochondrial $O_2^{\bullet-}$ and oxidative stress, which was followed by further oxidative stress in the cytosol. Oxidative stress and cell death were inhibited by overexpression of MnSOD, but not by CuZnSOD or Mn-porphyrins, demonstrating that mitochondrial $O_2^{\bullet-}$ formation is the primary event regulating oxidative stress and cell death induced by paraquat. In contrast, MPP^+- or rotenone-induced toxicity and oxidative stress were insensitive to MnSOD or CuZnSOD overexpression, suggesting that additional mechanisms besides $O_2^{\bullet-}$ formation are involved in the toxicity induced by complex I inhibition

MPP^+/MPTP toxicity is ascribed to the inhibition of complex I [48]. Overexpression of CuZnSOD [11,12] or MnSOD [13] has been shown to decrease MPTP toxicity, while MnSOD or CuZnSOD deficiencies increase it [14,15]. In this study, MPP^+-induced toxicity was not prevented by MnSOD or CuZnSOD overexpression. The discrepancies might relate to the model system used. Glial cells participate in MPTP and rotenone toxicity as sources of ROS formation and inflammatory responses [49,50]. Thus, the effect of SOD overexpression or deficiency *in vivo* might be ascribed to the regulation of ROS production from glial cells. Our aim was to determine the role of ROS and its compartmentalization specifically in dopaminergic cells, and thus, the use of *in vivo* experimental models or mixed dopaminergic neuronal primary cultures was not suitable for this purpose.

Several studies have shown that MPP^+/MPTP toxicity is mediated by mechanisms independent from complex I inhibition [16] and generation of ROS [17,18,20–23], which might include microtubule depolymerization and energy failure [18,26]. Similar contradictory results have been found regarding rotenone-induced

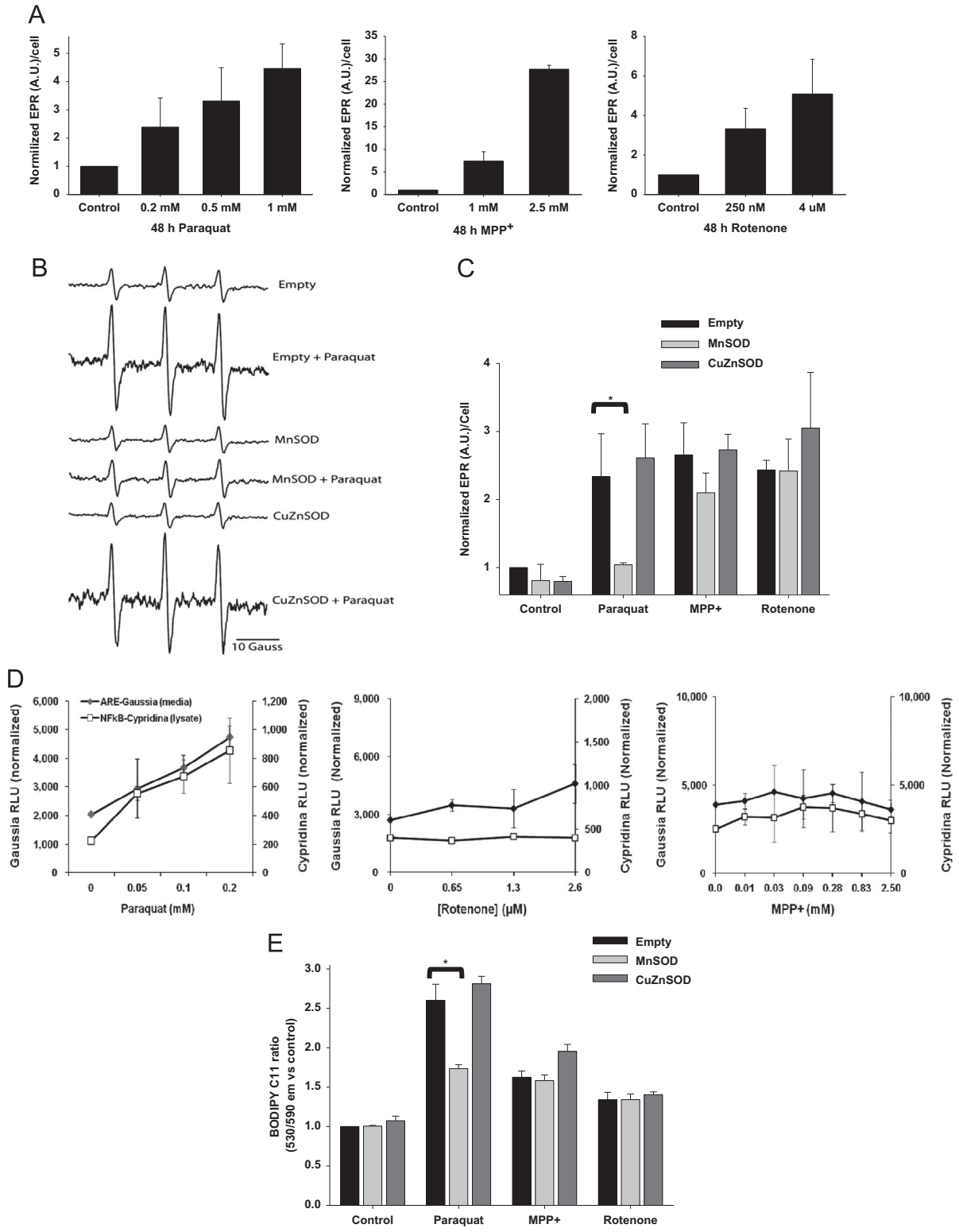

Fig. 6. Determination of free radical formation, activation of ARE- and NF-κB-driven reporters, and lipid peroxidation induced by parkinsonian neurotoxins. In A–C, cells were treated with parkinsonian mimetics as depicted. Then, cells were loaded with the cell-permeable spin probe CMH (200 μM, 60 min). After CMH incubation, cells were collected and EPR signal was detected in a Bruker e-scan EPR spectrometer. In A and C, EPR signal was normalized with the number of cells present after treatment and compared to control values. In B and C, cells were transduced with Empty, MnSOD, or CuZnSOD adenoviruses and 24 h after infection, cells were treated with parkinsonian toxins (0.5 mM paraquat, 2.5 mM MPP^+, or 4 uM rotenone) for 48 h. Data in B are representative EPR spectra from experiments in C. In D, human IMR-32 neuroblastoma cells were transfected with ARE-Gaussia, NF-κB-Cyrpidina, and CMV-Red Firefly reporter plasmids. At 24 h after transfection, cells were treated for 5 h with paraquat, rotenone, or MPP^+ at the concentrations indicated. Then, luciferase activity was assessed in media (ARE) and lysate (NF-κB). Results are normalized by Red Firefly activity and expressed as RLUs. In E, lipid peroxidation was determined using the ratiometric probe BODIPY C11, whose emission fluorescence at 590 nm decreases concomitant with the fluorescent spectra at 530 nm in response to oxidation. Cells were treated as explained in C and incubated for 30 min prior to FACS analysis with BODIPY C11 (2.5 μM). Results were analyzed ratiometrically 530/590 em normalized with control values. Graphs represent means ± SE of at least 5 independent experiments. $^*P<0.05$ vs Ad-Empty values.

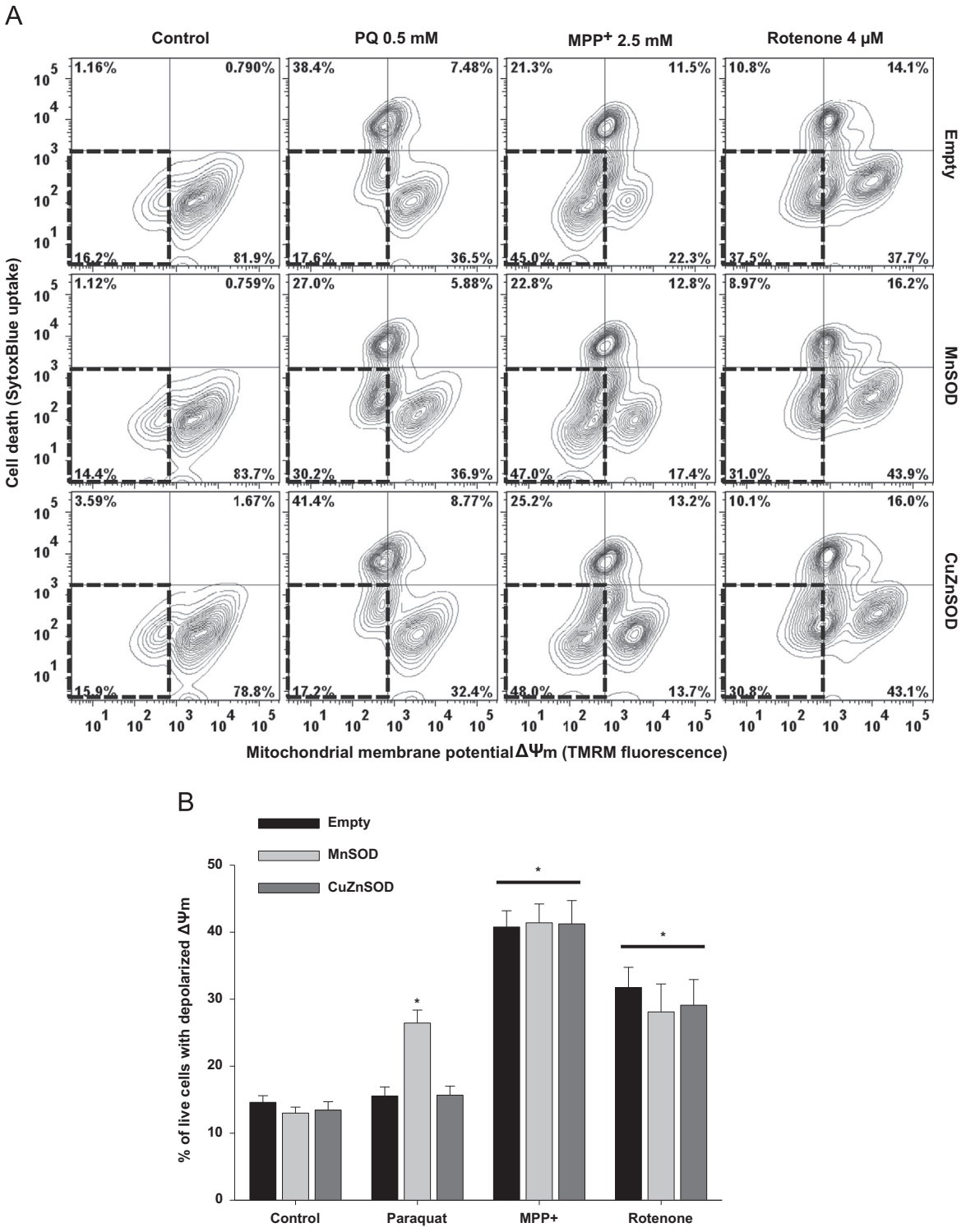

Fig. 7. MnSOD overexpression decreases cell death but not mitochondrial membrane potential (ΔΨm) loss induced by paraquat. Cells were transduced with Empty adenovirus or adenovirus encoding MnSOD or CuZnSOD for 24 h prior exposure to parkinsonian toxins. In A, cells were treated with paraquat, MPP^+, or rotenone for 48 h and stained with TMRM (50 nM) and Sytox Blue (5 nM) 15 min prior to FACS analysis. Loss of ΔΨm and cell death were simultaneously analyzed in a TMRM vs Sytox Blue contour plot to depict cells with a decrease in ΔΨm (low TMRM fluorescence, left quadrants) and loss of plasma membrane integrity (increased Sytox Blue uptake, upper quadrants). In B, % of cells with low Sytox Blue fluorescence and reduced TMRM signal depict live cells with ΔΨm depolarization (from broken line, lower left quadrants in A). Data in B represent means ± SE of at least three replicas. *$P<0.05$ vs Empty in the absence of treatment.

toxicity [16,17,22,24,25,51]. In our work, overexpression of SODs did not protect against MPP^+ or rotenone toxicity. We cannot rule out a role for oxidative stress in dopaminergic cell death induced by complex I inhibition as the overexpression of SODs had no effect on oxidative stress induced by MPP^+ or rotenone (EPR and Mitosox data) . However, our results strongly argue against a direct role of $O_2^{\bullet-}$ formation. There is a possibility that overexpressed MnSOD fails to scavenge $O_2^{\bullet-}$. However, this is unlikely because MnSOD overexpression inhibited cell death induced by paraquat, which is paralleled by a higher degree of mitochondrial ROS formation and oxidative stress (lipid peroxidation) (See Figs. 1B, 5B, and 6E).

$O_2^{\bullet-}$ reacts with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$) three times faster than $O_2^{\bullet-}$ dismutation by SOD [52] and MnSOD has been reported to be inactivated by tyrosine nitration [53]. Because $NO^{\bullet}$ and $ONOO^-$ participate in MPP^+/MPTP and rotenone toxicity [54–57], $O_2^{\bullet-}$ could mediate its toxic effects by $ONOO^-$ formation. Another possibility is that dismutation of $O_2^{\bullet-}$ by SODs leads to accumulation of H_2O_2. However, when CuZnSOD or MnSOD were overexpressed together with catalase or mitochondria targeted catalase using adenoviral vectors [58], there still was no protection against MPP^+ or rotenone toxicity (data not shown). In addition, high extracellular GSH (25 mM), *N*-acetylcysteine (25 mM), or cell-permeable GSH-ester (1 mM) failed to prevent toxicity induced by complex I inhibition (data not shown). These results would suggest that energy failure but not oxidative stress mediates dopaminergic cell death induced by complex I inhibition as previously suggested by other research groups [17,18,20–23]. Our next step would be to study and compare independently the role of energy failure vs mitochondrial ROS formation in dopaminergic cell death induced by complex I inhibitors but this requires extensive additional experimental work.

Oxidative stress induced by intracellular dopamine oxidation but not mitochondrial ROS formation has been proposed as a major contributor to MPP^+ toxicity [59]. A protective role for both cytosolic peroxiredoxin 2 (Prx2) and mitochondrial Prx3 against MPP^+/MPTP toxicity has been demonstrated [60,61]. We observed that MPP^+ and rotenone-induced toxicity was associated with only a minor, but significant, increase in oxidative stress in the cytosol (Figs. 4B and 5A). In addition, MPP^+ and rotenone failed to activate redox-sensitive ARE or NF-κB reporters (Fig. 7). Our results seem to suggest then oxidative stress induced by inhibition of complex I is primarily restricted to the mitochondrial matrix.

While some studies demonstrate that mitochondria are the primary source of ROS formation on paraquat exposure [28–30], other reports suggest that the cytoplasm is the major site for ROS generation [31,32]. We demonstrated that paraquat induces an early increase in mitochondrial oxidative stress that precedes the rise in cytoplasmic ROS levels. This was demonstrated by experiments demonstrating that Mito-roGFP is more sensitive to low doses of PQ compared to roGFP. This difference was not related to alterations in the cellular sensitivity to parkinsonian toxins induced by the expression of the redox sensors. Furthermore, the ratiometric nature of (mito-)roGFP analyses rules out differences ascribed to expression levels of roGFP and/or alterations in pH. Paraquat also induced the transcriptional activation of ARE- and NF-κB reporters and lipid peroxidation. Cell death and oxidative stress induced by paraquat were inhibited by MnSOD but not CuZnSOD overexpression, demonstrating that mitochondria are the primary source for $O_2^{\bullet-}$ formation. Interestingly, overexpression of MnSOD does not prevent the loss of $\Delta\Psi m$ induced by paraquat, suggesting that this event is independent from mitochondrial $O_2^{\bullet-}$. Paraquat redox cycle involves its reduction by cellular levels of NADPH, before becoming oxidized by an electron receptor to produce $O_2^{\bullet-}$, and inducing concomitant NADPH depletion [43,62]. A recent study reports that that knockdown of nicotinamide nucleotide transhydrogenase (NNT) involved in mitochondrial NADPH generation induces $\Delta\Psi m$ loss [63]. Thus, we speculate that paraquat-induced $\Delta\Psi m$ depolarization might be associated with depletion of NADPH levels and not $O_2^{\bullet-}$ formation.

Metalloporphyrins MnTBAP and MnTMPyP failed to reduce mitochondrial ROS accumulation induced by paraquat, rotenone, or MPP+, and protect against their toxicity (Supplementary Fig. 5C). MnTBAP has been shown to decrease dopaminergic apoptosis induced by paraquat *in vivo* [64], but whether this effect is ascribed to a reduction in ROS accumulation in neuronal or glial cells also known to contribute to paraquat toxicity was not studied [65,66]. In addition, MnTBAP is an anionic porphyrin with a reduction potential more negative than the potential for oxidation of $O_2^{\bullet-}$ [67,68]. MnTMPyP is fairly prone to accept electrons. However, MnTMPyP might induce toxicity by its association with nucleic acids [67]. Manganese porphyrins also perform cycles of oxidant detoxification by taking electrons from the respiratory chain, and then, reducing oxidants such as $O_2^{\bullet-}$ and $ONOO^-$ [69,70]. In addition, manganese porphyrins increase oxidative stress and cell death [67,71,72]. In our study, MnTMPyP increased paraquat toxicity.

To understand the source of ROS formation and their compartmentalization we have used a variety of complementary experimental approaches to: (1) determine ROS formation and oxidative stress (dihydroethidium derivatives); (2) determine alterations in subcellular redox balance/potential (roGFP sensors); (3) free radical formation (EPR); (4) redox signaling (ARE-driven reporters) and oxidative damage (lipid peroxidation). We consider that it is important to summarize and discuss here the limitations ascribed to these assays. Dihydroethidium derivatives are widely used probes for detecting intracellular $O_2^{\bullet-}$. However, direct oxidation of DHE to ethidium (Etd^+) is mediated by ROS such as $ONOO^-$, hydroxyl radical ($OH^{\bullet}$), and H_2O_2, but not by $O_2^{\bullet-}$. $2OH\text{-}Etd^+$ formed from the reaction of DHE with $O_2^{\bullet-}$ can be measured by analytical techniques [73]. Because we aimed to characterize oxidative stress in live cells, this type of analysis could only be done by FACS. Robinson et al. demonstrated that 405 nm ex enhances the fluorescence of $2OH\text{-}Etd^+$, reducing spectral overlap with Etd^+ [74]. In our experiments, DHE and MitoSOX fluorescence increased primarily at 488 nm ex compared to 407 nm ex (data not shown), suggesting that they mostly detect overall oxidative stress and not only $O_2^{\bullet-}$. In order to detect the formation of free radicals induced by parkinsonian mimetics, we used detected the oxidation of CMH by EPR spectroscopy [75]. However, this approach was recently reported to lack specificity regarding the chemical identity of the oxidant/free radical that oxidizes the probe [76]. Regardless, the fact that overexpression of MnSOD did not prevent ROS accumulation (detected by MitoSOX and EPR spectroscopy) and oxidative damage (lipid peroxidation) induced by complex I inhibition demonstrates that mitochondria $O_2^{\bullet-}$ is not the primarily mediator of oxidative stress and dopaminergic cell death induced by MPP^+ and rotenone.

To our knowledge, this is the first report characterizing alterations in redox balance of distinct subcellular compartments in response to parkinsonian mimetics using roGFP sensors. A previous study using Mito-roGFP transgenic mice reported increased oxidative stress under basal conditions in dopaminergic neurons of the SNpc [77]. We now demonstrate that alterations in the redox balance induced by MPP^+ are primarily restricted to the mitochondria. Oxidation of roGFP in response to paraquat was delayed when compared to mito-roGFP suggesting that mitochondrial ROS production precedes cytosolic oxidant production. roGFP oxidation can be promoted by distinct ROS, and roGFP sensors equilibrate with the intracellular thiol-disulfide balance [78]. Thus, alterations in roGFP fluorescence are an indication of oxidative stress but cannot be associated with the detection of a specific ROS. Interestingly, mitochondrial ROS formation induced by rotenone could be detected by MitoSOX but not Mito-roGFP. This discrepancy is most likely associated with the nature of the ROS or prooxidant condition generated. Supplementary Fig. 5A shows that overexpression of SODs does not prevent roGFP oxidation, which might be explained by the increase production of H_2O_2. However, MnSOD does reduce significantly DHE oxidation (Supplementary Fig. 5B). Although more experiments are required to clearly identify whether $O_2^{\bullet-}$ or H_2O_2 mediate roGFP oxidation, our observations indicate that an important component of

cytosolic oxidative stress is mediated by mitochondrial ROS formation. However, in the cytosol, paraquat most likely also generates ROS. Regardless, only overexpression of MnSOD but not CuZnSOD protects against paraquat toxicity, demonstrating that mitochondria are the primary source of ROS on paraquat exposure.

All together, our results clearly distinguish for the first time a selective role of mitochondrial $O_2^{\bullet-}$ in dopaminergic cell death induced by paraquat, and show that toxicity induced by the complex I inhibitors rotenone and MPP^+ involves additional factors other than mitochondrial $O_2^{\bullet-}$ formation.

Acknowledgments

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2013.04.021.

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