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Capillary Electrophoresis Monitors Enhancement in Subcellular Reactive Oxygen Species Production upon Treatment with Doxorubicin

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Abstract

This study investigated the role of doxorubicin (DOX) accumulation in the ROS production detected in individually electrophoresed organelles, including mitochondria, acidic organelles, and peroxisomes. While bulk measurements of ROS production in cells and organelles are not capable of discriminating between the effects of preparative procedures on measured ROS production, capillary electrophoresis with dual laser-induced detection of individual organelles demonstrated a difference in the measured ROS production as a result of various preparative procedures. Using this technique, the three different types of detected organelles: (i) produce ROS and do not have detectable levels of DOX, (ii) contain detectable DOX but do not produce ROS, or (iii) produce ROS and accumulate DOX. The third type displays two sub-populations of organelles, one of which demonstrated a direct relationship between DOX uptake and subsequent ROS production, corresponding most likely to mitochondria, and a second one with low DOX uptake but large variation in ROS production, corresponding most likely to acidic organelles.

Keywords

reactive oxygen species; doxorubicin; MEKC; laser-induced fluorescence; capillary electrophoresis

1. Introduction

The development of methods to probe the toxicological impact of xenobiotics on cellular systems has applications in a variety of areas, including pharmaceutical research. By simultaneously monitoring a pharmaceutical agent and a marker of cellular distress, the relationship between insult and response may more clearly define mechanisms of toxicity than traditional methods of cellular analysis. An example of such a system is the relationship between the anthracycline doxorubicin (DOX) and reactive oxygen species (ROS); it has long been hypothesized that a direct relationship between DOX accumulation and ROS production exists. However, available technologies were not capable of testing this hypothesis.

The emergence of cumulative, dose-dependent, cardiotoxicity has been attributed to long-term use of the antineoplastic doxorubicin.¹ Despite the efficacy of this drug in the treatment of leukemia and solid tumors, the life-time dose of DOX does not exceed 500 mg/m² (approximately 27 treatments) in clinical settings, in order to minimize the risk of developing this deleterious side effect.² Analysis of DOX in *in vitro* and *in vivo* systems has been readily facilitated by the native fluorescence of this molecule,³ allowing for strong correlations to be made between treatment levels and the emergence of side effects.

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The formation of ROS in the mitochondria have been implicated as the primary mechanism by which DOX causes the cardiotoxic phenotype.^{4,5} DOX is capable of undergoing redox cycling at Complex I of the mitochondrial respiratory chain, resulting in the formation of a semiquinone radical species.^{6,7} This reactive intermediate then proceeds through one of several pathways: (i) reduction to hydroquinone (ii) adduct formation with proteins or DNA (iii) oxidation by transferring the unpaired electron to an electron acceptor, resulting in a more stable radical intermediate.⁸ In the mitochondria, molecular oxygen is the electron acceptor of most interest to the medical and biochemical community. Redox cycling between DOX and O₂ results in the formation of superoxide, which can be further reduced to H₂O₂ (Figure 1).⁸ These ROS can then react with heme catalysts to form hydroxyl radicals which, in turn^{9,10} (i) react with mtDNA to form adducts that impede mitochondrial function¹¹ (ii) modify proteins¹² (iii) diffuse out of the mitochondria, oxidizing lipids on the plasma membrane and increasing membrane fluidity.¹³ Because DOX treatments are attributed to ROS production in cellular environments (e.g. mitochondria, cytosol, nuclei) and the damaging effects that result from this production, it is desirable to investigate the effects of DOX on ROS production within a given subcellular environment.

Fluorimetric probes (e.g. hydroethidine, Amplex Red, dihydrorhodamine 123) have been widely used to detect ROS in cellular and abiotic environments.¹⁴ While hydroethidine exhibits specificity to superoxide,¹⁵ the remaining listed reporters do not exhibit specificity to a particular ROS, and no probes available for the detection of ROS by fluorimetric methods are specific to a particular subcellular environment. Furthermore, most of the available fluorimetric cannot be used in conjunction with DOX analyses due to significant overlap of their emission spectra. One exception is 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which has an emission spectrum that shows minimal overlap with the DOX emission spectrum.^{4,5} Upon entering the cell, DCFH-DA is deacylated by cellular esterases and oxidized in the presence of heme catalysts by ROS to form 2',7'-difluorescein (DCF) (Figure 2).^{23,24} While not specific to a particular ROS (i.e. it responds to the presence of hydrogen peroxide,^{16,17} hydroxyl radicals,^{18,19} and other endogenous ROS),²⁰ this probe transforms rapidly into its fluorescent product (<30 min)²¹ and has a high quantum yield (0.97).²²

Fluorimetry and flow cytometry have both been used to investigate the effect of DOX on ROS production by using fluorimetric probes, specifically DCFH-DA.^{5,25} These studies have all demonstrated increased production of ROS upon exposure to DOX; however, these studies are fairly limited from several perspectives. First, standard fluorimetric assays are only capable of reporting an average of ROS production in the cellular population. Second, previous analyses by fluorimetry have correlated the amount of DOX administered to ROS production, but have not examined the effect of DOX uptake in a given cell to subsequent ROS production.²⁵ Third, while flow cytometry is capable of discriminating between individual cells' DOX uptake and ROS production, these analyses provide no information on the subcellular localization of either DOX or endogenous ROS when performed on intact cells;²⁶ in individual organelles, flow cytometry requires extensive fixing procedures to be completed prior to analysis. However, such fixing procedures would allow for diffusion of fluorimetric probes, thereby decreasing sensitivity. As stated, because the localization of ROS directly impacts the cellular effect observed after DOX treatment, quantification of ROS and DOX within a given organelle type is of interest.

An ideal assay would (i) simultaneously detect DOX and a fluorimetric ROS reporter (ii) allow for subcellular analyses of ROS and/or DOX within a given cell population (iii) have low limits of detection for quantification of both DOX and ROS. Rather than correlating ROS production to the amount DOX administered to a system, it is more accurate to report the effect on the amount of DOX taken up on the amount of ROS produced. Therefore, it is necessary to be able to simultaneously detect both DOX and ROS in the same environment (e.g. organelle). For

example, it is the accumulation of DOX and production of ROS in the mitochondria that may lead to appearance of 8-OHdG mutations,²⁷ which impede mitochondrial function and ultimately lead to cardiotoxic phenotypes. Finally, methods with low limits of detection will allow for quantification of the low levels of DOX and ROS believed to accumulate in organelles.

The use of capillary electrophoresis with dual laser-induced fluorescence detection (CE-dual-LIF) takes advantage of the unique electrophoretic mobility of individual organelles to temporally separate and detect individual organelles, while simultaneously detecting DOX and a ROS fluorescent reporter in a given organelle. Here, we report on the use of CE-LIF to demonstrate enhanced ROS production in individually electrophoresed organelles after treatment of the immortalized human leukemia cell line CCRF-CEM with acute doses of DOX.

2. Experimental

DOX was donated by Dr. Antonio Suarato (Pharmacia, Milan, Italy). Sodium borate decahydrate was purchased from EM Science (Gibbstown, NJ, USA). Sodium dodecyl sulfate (SDS) Ultrapure Bioreagent was purchased from J.T. Baker (Phillipsburg, NJ, USA). Methanol was purchased from Pharmco (Brookfield, CT, USA). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Sucrose and D-mannitol were purchased from Fischer Scientific (Pittsburg, PA, USA). A stock (10%) solution of PBS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT salt), EDTA, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), and 0.4% Trypan Blue solution were purchased from Sigma Aldrich (St. Louis, MO, USA). All buffers were made using 18 mΩ water obtained from a Millipore purification system (Billerica, MA, USA). The pH of all buffers and solutions was adjusted using either 0.1 M HCl or 0.1 M NaOH after the addition of solutes. After pH adjustment, buffers were filtered through a 0.22 μm Nalgene filter and stored up to one month. Buffers containing sucrose were stored at 4°C, whereas buffers without sucrose were stored at room temperature. Micellar Electrokinetic Capillary Chromatography (MEKC) buffer consisted of 10 mM borate, 10 mM SDS at pH 9.38 (BS buffer). Mitochondrial isolation buffer consisted of 210 mM D-mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM EDTA at pH 7.4 (M buffer). Respiration buffer consisted of 220 mM sucrose, 50 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 10 mM HEPES at pH 7.4 (R buffer).²⁸ Separation buffer consisted of 250 mM sucrose, 10 mM HEPES at pH 7.4 (SH buffer).

Stock solutions of DOX were prepared in 100% MeOH at 1 mM. Stock solutions of DCFH-DA were prepared in 100% DMSO at 1 mM. The stock solutions were stored at -20°C and used up to 1 month post-preparation. Working solutions were prepared the day of analysis.

2.2 Cell Culture Maintenance

Passages 17–22 of CCRF-CEM human leukemia cells (American Type Culture Collection (ATCC), Manassas, VA, USA), cultured at 37°C and 5% CO₂ in RPMI 1640 medium (Sigma, St. Louis, MO, USA), supplemented with 10% bovine calf serum (Invitrogen, Corp., Carlsbad, CA, USA) were used for these studies. Cells were maintained by splitting every 3–4 days through the addition of fresh medium.

2.3 Cell Viability

Cell viability was determined by Trypan Blue exclusion, and measuring the activity of Complex I by MTT assay. Following DOX treatment, cells were assayed by the MTT assay in parallel to Trypan blue exclusion.

Briefly, the MTT assay was conducted by incubating MTT with cells for 4 hrs after the completion of DOX treatments.²⁹ Cells were separated from suspension by centrifugation at

600 g for 10 min and were resuspended in acidified cell medium, and the formazan product was solubilized by the addition of isopropanol. The resulting suspension was monitored by UV-Vis spectroscopy using a test wavelength of 570 nm and reference wavelength of 630 nm.

Trypan Blue exclusion was completed by mixing cell suspension, Trypan Blue, and 1 $\times$ PBS in a 1:1:1 ratio and counting the percentage of viable cells following a 5 min incubation at 37°C.

2.4 Sample Preparation

2.4.1 DOX Treatment and DCFH-DA Exposure—Figure 3 depicts the preparative procedures. DOX treatments began 10 h after passaging cells. Treatment consisted of a 10 μ M dose and lasted 12 h, resulting in > 80% viability as determined by Trypan Blue exclusion and the MTT assay.

In the final analysis, cells were removed from medium by centrifugation at 600 g for 10 min immediately following DOX treatment, washed twice with 1 $\times$ PBS, and resuspended in R buffer. DCFH-DA was added to the suspension at a final concentration of 20 μ M. The cell suspension was placed in the dark at 37°C for 30 min to allow deacylation and oxidation of DCFH-DA. Two control samples included cells only treated with DOX (no DCFH-DA) and untreated cells exposed to DCFH-DA (no DOX).

2.4.2 Preparation of Mitochondria in Bulk—For preliminary studies involving bulk mitochondria, cells exposed to DCFH-DA were centrifuged 10 min at 600 g and resuspended in M buffer. The plasma membrane was selectively disrupted by one cycle Nitrogen cavitation at 0°C for 20 min at 600 psi (resulting in >95% lysis). The plasma membrane, nuclei and intact cells were removed from suspension by differential centrifugation at 600 g for 10 min. A mitochondrial enriched fraction was isolated from the resulting supernatant by centrifugation at 14.1 $\times 10^3$ g for 20 min.³⁰ The resulting pellet was dissolved in BS buffer for analysis (c.f. Figure 3).

2.4.3 Preparation of Individual Mitochondria—The isolation procedure remained identical to those described above for individual mitochondrial studies; however, the final mitochondrial pellet was resuspended in SH buffer (c.f. Figure 3).

2.5 CE-LIF Set-up

The design and set-up of the separation system with post-column LIF detection has been previously described.³¹ The 488 nm line of an Argon-ion laser (JDS Uniphase, San Jose, CA, USA) was used for excitation of fluorescent analytes. A dichroic mirror (CP-AR 585, CVI Laser, Albuquerque, NM) was used to transmit emitted light with wavelengths higher than 585 nm to one photomultiplier tube (PMT) and reflect emission with wavelengths lower than 585 nm to another. DOX fluorescence emission was monitored using a filter transmitting in the range of 607.5 – 662.5 nm (635DF55, Omega Optical, Brattleboro, VT, USA). DCF emission was monitored using a filter transmitting in the range of 500 – 540 nm (Bright Line, Ringstead, UK). Scattering caused by air bubbles and/or organelles was reduced by placing a 505 nm long-pass filter (Omega Optical) in front of the interference filters.

2.6 CE-LIF Analysis

As previously described, separations were carried out using a 50 μ m i.d. poly-AAP coated capillary,³² this coating reduces interactions between organelles and the capillary wall, as well as suppresses electroosmotic flow. Prior to sample analysis, the detector was aligned by continuously injecting 10⁻⁹ M fluorescein and optimizing the position of the capillary outlet with respect to the laser and photomultiplier tube (PMT). The limit of detection (LOD) for the

separation and detection system was determined to be 3.1 ± 0.4 zeptomoles, based on triplicate injections of 10^{-10} M fluorescein.

CE separations of individual organelles were carried out at -400 V/cm in SH buffer following a 5 s siphoning injection at 2.7 kPa, using a previously reported procedure.³³ For analysis of bulk organelle samples and for construction of DOX and DCF calibration curves, separations were carried out in uncoated fused-silica capillaries at $+400$ V/cm following electrokinetic injections at $+50$ V/cm. Analyses of bulk organelle samples were carried out in BS buffer. The uniformity of the detector's response to individual particle detection was determined by calculating the RSD of fluorescence intensities of 1- μ m Fluoresbrite YG microspheres that were introduced by continuous electrokinetic injection. The RSD was 14.5% for 532 events.

2.7 Data Analysis

The output of the PMT was electronically filtered ($RC = 0.01$ s) and digitized by an I/O board, driven by Labview (National Instruments, Austin, TX, USA) software. Data were acquired at 100 Hz and stored as binary files. Analysis was completed using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). The raw electropherograms consisted of both multiple narrow spikes and one/two broad bands. These broad bands correspond to free DCF or DOX (i.e. not found within organelles) in the sample that leak from disrupted organelles during the preparation procedure and not after organelle isolation. These bands are eliminated from the final electropherogram by subtracting a background trace (produced by Median filtering a copy of the raw electropherogram) from the respective raw electropherogram. Fluorescence intensities and migration times of individual events in each electropherogram were determined using PickPeaks, an in-house program previously described,³⁴ which initially selects events with intensities higher than three times the standard deviation of the background. In order to reduce the number of false positives (i.e. events that are detected due to high scattering by the detected particle), the initially selected events are divided into a "pre-migration window" and a "migration window". Since the events in the "pre-migration window" correspond to false positives, a new threshold was established that resulted in elimination of 95% of pre-migration events. Events detected in the DOX and DCF detectors that had the same migration time (± 0.01 s) were selected using the Igor Program Coincidence analysis, written in-house. Spectral cross-talk was not observed between the DOX and DCF detector, when analyzing individual organelles and it was found to be $<0.1\%$ from the DOX to the DCF detector when analyzing bulk (dissolved) mitochondria.

Estimation of DOX and DCF in intact organelles required determination of (i) detector sensitivity to DOX and DCF in BS buffer ($s = 2.96 \times 10^{-18}$ and 9.50×10^{-18} mole/area-unit for DOX and DCF, respectively) (ii) detector efficiency ($d = 0.88$, amount fluorophore detected over amount injected) (iii) average peak area to height ratio ($R = 0.0475$ area-unit/V) and (iv) relative response of intact over BS-solubilized organelles ($F = 0.115$ and 0.061 area-unit/area-unit for DOX and DCF, respectively).

These parameters are related by the equation:

$$m = \left(\frac{sRd}{F} \right) h \quad (1)$$

where m (mole) is the fluorophore content in an individual organelle with peak intensity h .

A detailed description of the methodologies used to determine the numerical value of each of the above parameters has been previously reported.³³

2.8 Statistical Analysis

For bulk analyses, the peak area of DCF was compared, both between replicates of the same sample and different samples (e.g. DOX vs. untreated, between two untreated controls). A one-tailed *t*-test, assuming equal sample variance, was performed using Microsoft Excel. The null hypothesis, “there is no difference between DCF peak areas” was tested at a confidence level of 95% and rejected when $P < 0.05$.

3. Results and Discussion

3.1 CE-LIF Analysis of ROS

Because previous studies regarding DOX-induced ROS production have been completed on whole cells in bulk and using standard fluorimetry, it was necessary to validate the use of a separation technique, such as CE-LIF, to quantitate ROS in cellular systems. Therefore, method development was initially conducted on bulk organelle samples in order to ascertain whether CE-LIF resulted in similar observations to those reported using fluorimetry. In addition, no work has been reported on the measurement of subcellular production of ROS upon exposure to DOX; by comparing the results of ROS production in organelles (i.e. mitochondria) in bulk and comparing these results to previously reported data, it was possible to determine whether similar trends are observed, thereby validating the use of subcellular analyses.

The bulk, solubilized, organelles from two untreated controls were analyzed by MEKC-LIF. As these samples were not treated with DOX prior to exposure to DCFH-DA, fluorescence in these samples was considered to be normal ROS production, a known byproduct of cellular respiration and organelle isolation procedures. Because the effects of isolation procedures on measured ROS production were not known, exposure to DCFH-DA was completed at different timepoints in the sample handling procedure to evaluate whether or not preparative procedures had a measurable impact on ROS production as defined by fluorescence attributed to DCF. One untreated control consisted of untreated, intact, cells that were exposed to 20 μM DCFH-DA for 30 min, with subsequent isolation, disruption, and analysis of organelles. The other untreated control consisted of isolated organelles exposed to 20 μM DCFH-DA for 30 min, followed by organelle disruption and subsequent analysis. The lysate of the organelles was analyzed by MEKC-LIF in BS buffer. Figure 4 demonstrates representative electropherograms (i.e. fluorescence intensity versus migration time) of the ROS detector for bulk, solubilized, organelles from untreated (Figure 4 (i)) and DOX treated (Figure 4 (ii)) cells. The tall, broad (5.2 ± 0.4 s), peak at 288 s in trace i corresponds to dichlorofluorescein (DCF), the product resulting from reaction with DCFH-DA and ROS. Comparison of DCF peak areas from the two different untreated controls (Figure 5) demonstrates that bulk organelle analysis is not capable of detecting differences in the effect of preparative procedures on measured ROS production as defined by fluorescence.

Further examination of fluorescence peak areas corresponding to ROS (Figure 5) demonstrate increased ROS production in DOX treated cells. This is in agreement with previously reported data in which fluorimetry was utilized to examine ROS production in whole cells.^{5,25} Increased DCF fluorescence in DOX treated cells is not attributed to direct oxidation of DCFH-DA, as neither DOX, nor other pharmacological agents with similar redox properties, have been shown to directly interact with this probe.³⁵ Because the density (and number) of cells from which organelle fractions were isolated were the same for both DOX treated ($2.0 \pm 0.4 \times 10^6$ cells/mL, 10.00 mL) and untreated ($2.0 \pm 0.5 \times 10^6$ cells/mL, 10.00 mL) cells, it can be concluded that increased production of DCF is the result of increased ROS production, rather than a difference in the number of cells or organelles in the sample. While the data are in agreement with previously reported results, this is a unique study from the standpoint that MEKC-LIF allows for temporal resolution of DCF and DOX, allowing for complete

quantitation of the two compounds. Examination of Figure 4, trace ii reveals two peaks: in the offset trace, the peak corresponding to DCF is found at 288 s; a small peak (spectral cross-talk from the saturated DOX detector; cross-talk < 0.1%) at 380 s corresponds to DOX.

However, the data in Figure 5 represent an average ROS production by all organelle types included in the subcellular fraction. As described below, each organelle type is expected to have different response to DOX treatment.

3.2 Classification of Organelles

The fractionation procedures used in this study produce enriched organelle fractions containing mitochondria, acidic organelles, and peroxisomes.³⁰ Both mitochondria and acidic organelles are known to accumulate DOX;^{33,37,39} similarly, all three organelle types are capable of producing ROS.³⁰ Using CE-LIF, it is possible to temporally resolve individual organelles and spectrally resolve DOX and a ROS fluorescence reporter (i.e. DCF). Figure 6 is an example of a narrow region of an electropherogram of organelle fractions of DOX-treated cells, exposed to DCFH-DA. Each spike observed in the electropherogram corresponds to the fluorescence produced by one organelle passing through the laser beam of the LIF detector. In comparison with bulk measurements (c.f. Figure 4) that have wide peak widths (e.g. 5.2 ± 0.4 s), the spikes in Figure 6 have narrow widths (e.g. 0.15 ± 0.02 s). The upper trace in Figure 6 (trace i) corresponds to DCF (ROS) production in individual organelles; the lower trace (trace ii) corresponds to DOX accumulation. Three types of events are detected in the electropherograms of DOX treated cells exposed to DCFH-DA: (A) DCF only, indicating an organelle producing ROS but that has not accumulated detectable levels of DOX (e.g. a peroxisome); (B) DOX only, indicating a mitochondria or acidic organelle that has accumulated the drug but is not actively producing ROS; and (C) both DCF and DOX (dual detector event), indicating an organelle that has both accumulated drug and is producing ROS.

It has been well-documented that mitochondria are cellular targets of DOX;⁸ specifically, DOX is known to form complexes with cardiolipin, a phospholipid exclusive to the inner membrane of mitochondria and that shows considerable variability in content from one mitochondrion to the next.³⁴ Both morphological³⁶ and functional differences in mitochondria, are all expected to impact the degree of ROS production. An increase in either size or cardiolipin content within a given mitochondrion would allow for more DOX uptake, which leads to higher ROS production.⁸

DOX is also known to accumulate in the lysosomes (i.e. lysosomes, late endosomes) as a consequence of a pH gradient between the cytosol and the lumen of these organelles.^{37,39} Similarly, these organelles are known to produce ROS⁴⁰ and have documented heterogeneity.³⁸ However, no relationship between DOX accumulation and ROS production is known to exist in these organelle types.

Lastly, it is expected that peroxisomes produce ROS but do not show significant accumulation of DOX. Below, the analysis of individual organelles describes the effect of the inherent variability of DOX accumulation of a given organelle population on ROS production.

Based on the dual-detection system, the detected organelles were classified as shown in Table 1. These data indicate that the majority of organelles in the enriched subcellular fraction are organelles that have produced ROS, but are not accumulating DOX at detectable levels (DCF only). Further evidence that different organelle types are present comes from their electrophoretic mobility profiles (supplementary data). While those organelles containing both DOX and DCF have a median electrophoretic mobility of $-1.01 \pm 0.02 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$, those that contain only DCF have a median mobility of $-2.25 \pm 0.06 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$, and those that contain only DOX have a median mobility of $-2.31 \pm 0.07 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$.

While these values cannot be directly compared to other methods of cellular analysis, these differences in mobility distributions arise from alterations in the surface charge of individual organelles, which is indicative that different organelle types are being detected.

3.3 Accumulation of DCF in Individual Organelles

Because bulk analyses did not account for inter-organelle variability, studies examining the impact of preparative procedures on ROS production were repeated, this time investigating the impact in individually electrophoresed organelles. As shown previously (c.f. Figure 6), organelle events appear as narrow spikes, rather than as a broad zone that would be characteristic of the migration of multiple events. Because organelles in these electropherograms result from cells that were not treated with DOX, only events in the DCF detector need be considered for analysis.

In order to draw comparisons between the electropherograms of individually electrophoresed organelles, intensity histograms were generated by plotting the normalized number of events (e.g. organelles) versus the observed fluorescence intensity. Comparison of the resulting fluorescence intensity histograms (Figure 7) of events in the two untreated controls demonstrates that exposure of organelles to the DCFH-DA probe in intact cells, prior to organelle isolation, while respiration is still active, results in higher ROS levels. As seen in Figure 7, there is a slightly higher abundance of high intensity events when intact cells are exposed to DCFH-DA, followed by organelle isolation (Fig. 7A), than when isolated organelles are exposed to DCFH-DA (Fig. 7B); this is best exemplified by plotting the difference in fluorescence intensity (Fig. 7C). Residual fluorescence observed in isolated organelles exposed to the probe is attributed to non-enzymatic degradation of DCFH-DA by H_2O_2 -Fe(2+)-derived oxidant,²³ rather than enzymatic deacylation and subsequent oxidation by ROS. Consequently, further analyses, in which DOX treatments were conducted, utilized the preparative procedure in which DCFH-DA exposure was accomplished in intact cells, prior to organelle isolation.

DCFH-DA is capable of accumulating in any subcellular environment and being oxidized by ROS localized in these environments (e.g. cytosol, mitochondria); consequently, flow cytometry, microscopic techniques, and standard fluorimetry are limited to describing the overall ROS content of a cell, as intact cells exposed to this probe appear to be fluorescing throughout the entire cell volume. Because these methods have not been able to discern the subcellular localization of ROS production, the findings described above are exciting from two perspectives. First, the data show that ROS can be detected in individual organelles through use of DCFH-DA. DCFH-DA enters the cell by passive diffusion and is deacylated in the cytosol of cells. From there, it can (i) be oxidized to fluorescent DCF by ROS or (ii) diffuse to other organelles, where oxidation can take place. Therefore, fluorescence associated with DCF, and attributed to ROS production, would be present throughout the cell if no fractionation procedures are used. Using differential centrifugation coupled with CE-LIF, it is possible to conclude that ROS production can be detected in individual organelles.

Furthermore, the data described above (c.f. Figure 7) demonstrate that ROS (DCF) production is not uniform from one organelle to the next, highlighting the expected heterogeneity of the organelle population. This heterogeneity includes variations within an organelle type (e.g. mitochondria), and among organelles that are found in the subcellular fraction which are capable of ROS production (e.g. peroxisomes and acidic organelles). Previous analyses of ROS production in cellular systems have provided temporal resolution (e.g. microscopy, fluorimetry) of ROS production and variability in the ROS production in intact cells (e.g. flow cytometry), but have not accounted for differences in organelles within a given cell population.

3.4 DOX and ROS Content in Individual Organelles

As a first step towards understanding the impact of ROS production in cellular and organelle populations, the estimated content of DOX and ROS in individually electrophoresed organelles was determined. Estimation of DOX and ROS was accomplished using an indirect approach previously reported.^{33,37} Calibration curves were constructed for DOX and DCF standards analyzed by MEKC-LIF in BS buffer. The sensitivity of the detector to DOX ($s = 2.96 \times 10^{-18}$) and DCF ($s = 9.50 \times 10^{-18}$) (mole fluorophore/area unit) was determined from these curves. Since fluorescence quenching,⁴¹ differences in pH,⁴² and interactions with membrane components⁴³ all impact the fluorescence of DOX in intact organelles, a factor ($F = 0.115$) (unit area/unit area) was determined to correlate fluorescence intensity observed in intact organelles to that of the calibration curve.³³ A similar approach was used to determine the response factor for DCF, in order to account for differences in the fluorescence emission of fluorescein derivatives in biological matrices and in electrophoresis buffers containing SDS. Figure 8 shows the emission spectrum of DCF in the organelles from untreated cells after a 30 min exposure. By integrating the peak area from 500 – 540 nm, the spectral response of the interference filter used in the DCF detector, the response factor for DCF ($F = 0.061$) (unit area/unit area) indicates significant alteration of DCF fluorescence emission in intact organelles compared to that observed in solubilized organelles.

Using the calculated sensitivity (s) and response (F) factors, as well as the peak area-to-height ratio (R) and detector efficiency (d) described in the Experimental, it was possible to estimate the content of both DOX and DCF in individual organelles. Because a 1:1 stoichiometric ratio between ROS and DCF production has been reported,²³ it is possible to extend this analysis to include an estimation of captured ROS in these organelles. Figure 9 A demonstrates the effect of DOX treatment on ROS production in individual organelles, in which the histogram representing the total ROS produced are depicted. Similar to the analysis done in Figure 7, subtracting the histogram corresponding to the untreated cells (not shown), shows that more ROS are produced in organelles from cells exposed to DOX prior to isolation (Figure 9 B). Comparison of Figures 9A and 9B show that organelles producing $< 0.75 \times 10^{-19}$ moles were attributed to ROS production characteristic of untreated cells.

Organelles that contain both DOX and ROS were also of interest. Figure 10 shows that, by plotting the DOX content versus ROS content for coincident events (i.e. those events that occurred simultaneously in both detectors and are indicative of organelles containing detectable levels of DOX and having produced detectable levels of ROS), two sub-populations of organelles emerge. At low levels of DOX accumulation (e.g. 0.02 – 0.05 attomoles DOX per organelle), organelles generally produce up to 1 attomole ROS, as detected using this method (c.f. Figure 10B). Because spectral cross-talk was not observed in individual organelle analysis of controls of organelles with only DOX or only DCF (data not shown), all peaks can be considered the result of low-level DOX accumulation and subsequent ROS production. Just as a wide range of DOX was found to have accumulated in this organelle sub-population, a wide range of ROS production was observed; between 0.01 and 1 attomole ROS is produced per organelle, indicating the intrinsic variability of organelle populations. The wide distribution of ROS production in this sub-population may also indicate that, rather than ROS production being a consequence of DOX accumulation, production of these compounds may represent endogenous ROS, independent of DOX accumulation. Based on this evidence, it is postulated that this subpopulation mainly contains acidic organelles.

A different trend emerges at higher concentrations (Figure 10C). For organelles containing between 0.05 – 10 attomoles DOX per organelle, a directly proportional relationship between DOX accumulation and ROS production is observed, indicating that ROS production in this sub-population is directly linked to DOX accumulation. Given the trends observed in bulk mitochondrial studies conducted in both *in vitro* and *in vivo* systems,¹⁸ this data strongly

suggest that organelles displaying this trend are likely mitochondria. However, while previous studies have demonstrated a correlation between the amount of DOX administered and subsequent ROS production, these data represent the first report of a direct relationship between the amount of DOX accumulating in a cellular environment and the resulting moles of ROS produced.

Further evidence that these are two distinct groups of organelles comes from comparison of electrophoretic mobilities (Supplementary Data). Those defining the pattern (B) have a median mobility of $-0.93 \pm 0.03 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ and those defining pattern (C) have a median mobility of $-1.93 \pm 0.02 \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$. Comparison of electrophoretic mobility distributions with those reported in the literature suggest that the majority of organelles defining pattern (B) are acidic organelles,³⁷ whereas the majority of those defining pattern (C) are mitochondria.³⁴ Finally, the distribution of electrophoretic mobilities confirms the inherent variability of biological (i.e. cellular) systems.

4. Conclusions

Using CE-dual-LIF, it is possible to monitor the effect of DOX treatments on the production of ROS in individually electrophoresed organelles found in an enriched subcellular preparation containing mitochondria, acidic organelles, and peroxisomes. These findings reveal that traditional, bulk, methods of analysis do not detect differences in the effect of preparative procedures on the measured ROS production as defined by fluorescence intensity. Furthermore, we have demonstrated that CE-LIF is capable of differentiating between true ROS production and fluorescence that is attributed to non-enzymatic degradation of the DCFH-DA probe.

Variability in ROS production was observed in organelles isolated from both DOX treated and untreated cells, which is expected for any biological system. In one organelle sub-type ROS production is low and independent of the DOX content. Based on our findings, we propose that this organelle subtype comprises acidic organelles. In a second sub-type of individually electrophoresed organelles, ROS production was proportional to DOX treatment. Similarly, we propose that this subtype corresponds to mitochondria. These findings are novel in that they demonstrate the feasibility of CE-LIF to detect differences and increases in ROS production in individual organelles upon exposure of cells to a xenobiotic. Since differences such as enzymatic activity and cardiolipin content from one mitochondrion to the next impact both DOX accumulation and mitochondrial function, differentiation of ROS production provides an important parameter for describing the effect of DOX treatment on the resulting subcellular side effects, like mtDNA mutations, that result in cardiotoxicity.²⁷

Further developments to investigate the role of ROS production in individually electrophoresed organelles will require the synthesis of fluorescent reporters that are (i) specific to an organelle type (e.g. mitochondria), (ii) specific to a particular ROS (e.g. superoxide, hydrogen peroxide) and (iii) exhibit no spectral overlap with DOX. Despite low specificity of DCFH-DA to a particular ROS and lack of specificity of this probe to a particular subcellular environment, this study provides an important first step in developing methodologies that are capable of probing the impact of xenobiotics on cellular and subcellular systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DOX	Doxorubicin
ROS	reactive oxygen species
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MeOH	Methanol
DCFH-DA	2',7'-dichlorofluorescein diacetate
DCF	dichlorofluorescein

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Figure 1.

Formation of ROS from DOX. (A) Redox cycling of DOX with the mitochondrial respiratory chain. Superoxide ($\text{O}_2^{\bullet -}$) reduces to H_2O_2 through superoxide dismutase (B) or cycles with DOX (A).

Figure 2.
Cellular dependent formation of DCF.

Figure 3. Sample preparation procedures used for analysis of ROS in isolated organelles. Cells were either exposed to both (i) DOX (procedure a) and DCFH-DA (procedure b), (ii) DOX (only procedure a), or (iii) DCFH-DA (only procedure b; untreated). One additional control (untreated) was exposure to DCFH-DA after isolation (procedure b instead of procedure c).

Figure 4. Bulk DCF detection. Electropherograms of isolated organelles: (i) untreated cells and (ii) DOX treated cells. Traces have been y-offset for clarity. See Figure 3 for an outline of the bulk preparation procedure. Samples injected at +50 V/cm for 5 s and separated at +400 V/cm in BS buffer.

Figure 5.

Bulk comparison of DCF production in DOX treated and untreated organelles. Untreated control is DCF exposure prior to organelle isolation (procedure b in Figure 3). Untreated* control is DCF exposure after organelle isolation (procedure c in Figure 3). Error bars represent the standard deviation of triplicate measurements.

Figure 6.

CE-LIF detection of individual organelles isolated from DOX-treated cells. (i) ROS (DCF) detector (ii) DOX detector. A 20 s window shows examples of (A) organelles accumulating ROS but with little or no DOX, (B) organelles that contain DOX but that do not have detectable ROS levels, and (C) organelles containing DOX with detectable ROS levels. Separation completed at -400 V/cm using SH buffer following 5 s siphoning injection.

Figure 7.

Histograms of individual DCF fluorescence intensities of untreated controls. Fluorescence intensity distributions of DCF detector: (A) in organelles exposed to DCFH-DA before isolation (procedure b in Figure 3), (B) in organelles exposed to DCFH-DA after isolation (procedure c in Figure 3); and (C) the increase in DCF production in (A) over (B). Data are the average and standard deviation of three replicates (thick bars represent the mean, whereas thin bars represent standard deviation).

Figure 8.

DCF emission spectrum. Spectrum (A) corresponds to untreated organelles solubilized in BS buffer. Data has been corrected for 100-fold dilution. Spectrum (B) corresponds to untreated organelles suspended in SH buffer.

Figure 9.

Increase in ROS levels of individual organelles. (A) Distributions of individual DCF intensities (procedure b, Figure 3) from DOX-treated cells; (B) Net increase in individual DCF intensities obtained by subtracting the DCF intensity distributions of untreated cells from the corresponding DOX-treated distribution. Data are expressed as moles ROS; the average and standard deviation of three replicates (thick bars represent mean, whereas thin bars represent standard deviation). The x-axis truncated at 0.5 attomoles ROS for clarity.

Figure 10.

Simultaneous DOX accumulation and ROS production in individual organelles. ROS accumulation in (A) organelles containing 0.02 – 10 attomoles DOX; (B) 0.02 – 0.05 attomoles DOX; and (C) 0.05 – 10 attomoles DOX.

Table 1

Classification of detected organelles

	DOX Only	DCF Only	DOX and DCF
Fraction Events (%) ^a	9.3 ± 4.4	88.6 ± 4.3	2.1 ± 0.7

^aData represent an average and standard and deviation of triplicate injections. The total number of events was 1252 ± 45.