Published in final edited form as:

Cardiovasc Toxicol. 2017 April ; 17(2): 140–149. doi:10.1007/s12012-016-9367-x.

Environmentally Persistent Free Radicals cause apoptosis in HL-1 Cardiomyocytes

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Abstract

Samples of environmental particulate matter (PM) contain environmentally persistent free radicals (EPFRs) capable of sustained generation of oxygen radicals. While exposure to EPFRs produces cardiac toxicity and oxidative stress in experimental animals, the underlying mechanisms are largely unknown. To determine whether EPFRs could directly damage cardiomyocytes, cultured mouse cardiomyocytes (HL-1) and primary rat adult left ventricular myocytes (ALVM) were incubated with an EPFR consisting of 1, 2-dichlorobenzene chemisorbed to CuO-coated silica beads (DCB230). Treatment with DCB230 killed both HL-1 and ALVM in a dose- and time-dependent manner. The cytotoxic effects of DCB230 were significantly attenuated by treatment with α -tocopherol. One to 2 hours after exposure to DCB230, there were significant reductions in mitochondrial membrane potential and significant increases in cleaved caspase-9, but no significant increases in DNA damage or cell death. After 8 hours of treatment, there were significant increases in caspase-3, caspase-9, DNA damage and PARP cleavage associated with significant cell death. Together, these data indicate that DCB230 kills HL-1 myocytes by inducing oxidative stress that initiates apoptosis, with the intrinsic or mitochondrial pathway acting early in the apoptotic signaling process.

Keywords

Nanoparticles; particulate matter; oxidative stress; apoptosis

INTRODUCTION

Epidemiology studies consistently link human exposure to particulate air pollution with increased cardiovascular morbidity and mortality (1-3). In spite of this association, the

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CONFLICT OF INTEREST

None declared

mechanisms underlying particle-mediated cardiac toxicity are largely unknown; however, systemic inflammation, autonomic and vascular dysfunction are thought to play important roles (3). There is also evidence that fine (<2.5 μm diameter) and ultrafine (<0.1 μm diameter) particulates can traverse the lung to the systemic circulation, raising the possibility that the particles can contact various tissues and organs producing localized toxicity (3-5).

Environmentally persistent free radicals (EPFRs) are a unique pollutant-particle system that forms when polyaromatic hydrocarbons covalently bind to the surface of transition metal oxide coated particles (6). The hydrocarbon reduces the metal to form an organic radical that is capable of sustained (days to weeks) redox cycling, producing large quantities of reactive oxygen species, potentially causing oxidative stress. Inhalation and intratracheal instillation of EPFRs also reduces cardiac left ventricular function in otherwise healthy adult rats and attenuates the heart's ability to compensate for ischemia reperfusion injury (7-9). These cardiac deficits are associated with systemic inflammation, oxidative stress in ventricular tissue and increased pulmonary arterial pressure (7, 8). Diesel exhaust particles (DEP), which contain EPFRs, produce endothelial damage and accelerate atherosclerotic disease in mice (10). Inhalation of EPFRs produces pulmonary toxicity in neonatal rats and mice (11, 12). EPFRs produce oxidative stress and cellular toxicity/death in a number of endothelial, epithelial, fibroblast and macrophage culture systems (12-15). Whether EPFRs can similarly produce toxicity when in direct contact with cardiomyocytes is unknown. Since oxidative stress is known to induce apoptosis, a well-known contributor to cardiac pathology, we sought to determine whether EPFRs are cytotoxic to cardiomyocytes, and if so, whether the toxicity is driven by oxidative stress-mediated apoptosis utilizing intrinsic or extrinsic signaling pathways.

MATERIALS AND METHODS

Reagents and equipment

Unless otherwise noted, all reagents were reagent/molecular biology grade or better. Liberase TH was purchased from Roche. Gentamicin (#15710-072) was obtained from Life Technologies (Carlsbad, CA). Primary antibodies against pro-caspase 9 (#9508), pro-caspase 3 (#9665), cleaved caspase 3 (#9664), and pro/cleaved PARP1 (#9542) were purchased from Cell Signaling Technologies (Boston, MA). Horseradish peroxidase-linked secondary antibodies against mouse (#NXA931) and rabbit (#NA934V) were from GE Healthcare (Pittsburgh, PA). SDS-PAGE pre-cast gels, 0.2 μm PVDF membrane (#162-0177) and protein molecular weight standards (#161-0374) were purchased from Bio-Rad (Hercules, CA). Amersham ECL reagent and LAS4000 ImageQuant Imager were purchased from GE Healthcare (Pittsburgh, PA). For the membrane potential assay, MitoTracker Green was obtained from Life Technologies (Carlsbad, CA) and tetramethylrhodamine ethyl ester perchlorate (TMRE) was obtained from Sigma Aldrich (St. Louis, MO). Laser scanning confocal microscopy was performed using Lab-Tek II four-chamber #1.5 German coverglass (#155382) from Nunc/Thermo Fisher (Rochester, NY) and a Leica TCS SP2 AOBS system mounted on a Leica DM IRE2 inverted microscope (Mannheim, Germany).

DCB230

These studies used a surrogate EPFR, DCB230, which was synthesized by chemisorption of 1,2-dichlorobenzene to CuO-coated (5%) fumed amorphous silica beads (0.2 μm mean diameter) at 230°C using previously published methods (16). DCB230 is an environmentally-relevant pollutant particle that produces an electron paramagnetic resonance spectrum indicative of an oxygen centered semi-quinone radical (6, 16). Our group reported that DCB230 produces large numbers of hydroxyl radicals in saline, biological fluids (bronchoalveolar lavage fluid) and cultures of pulmonary epithelial cells (13). Control particles (<200 nm) consisted of Cab-O-sil EH-5 silica beads (Cabot, Alpharetta, Georgia), or CuO-coated (5%) silica beads (CuO/Si; 0.2 μm mean diameter) that do not contain EPFRs.

Cell culture and treatments

HL-1 cardiomyocytes were generously provided by William Claycomb (LSUHSC, New Orleans, LA). HL-1 is a mouse atrial cardiomyocyte line which possesses cardiac electrophysiological properties, such as spontaneous contraction (17). HL-1 cells were cultured at 37°C with 5% CO₂ in Claycomb medium (Sigma Aldrich, St. Louis, MO) with 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM l-glutamine, and 50 $\mu\text{g}/\text{mL}$ gentamicin. Culture flasks or chambered coverglass were pre-coated with 0.02% gelatin and 5 $\mu\text{g}/\text{mL}$ fibronectin before use. For all experiments, each well or dish was seeded with equal number of cells and the cells were then grown to full confluence to ensure proper contractile behavior. Quiescence was induced by either 4 hour or overnight serum deprivation in DMEM/F-12 (1:1) medium with 1% fetal bovine serum, 0.1 mM norepinephrine, 2 mM l-glutamine, and 50 $\mu\text{g}/\text{mL}$ gentamicin. Experiments were performed with fresh serum deprived medium as described above. After 2 or 8 hour treatments, cells were lysed with 0.1% Triton X-100 in 50 mM Tris and 150 mM NaCl, pH 7.4 for the LDH activity assay. For western blots, this was followed by adding an equal volume of 2x RIPA lysis buffer and incubating for 15 minutes at 4°C to improve protein extraction. Cell lysates were collected from the supernatant after 10 minutes, 10,000 RCF centrifugation at 4°C, and protein concentration was quantified using a BCA protein assay.

Isolation of adult rat cardiomyocytes

Adult left ventricular myocytes (ALVM) were enzymatically isolated from 3 male Sprague-Dawley rats (Harlan, Indianapolis, IN) as described previously (18). Briefly, rats were anesthetized with a mixture of ketamine and xylazine (100/10 mg/kg, i.p.), hearts were removed and retrograde perfused via the aorta using perfusion buffer (Alliance for Cell Signaling, Protocol PP00000125) followed by digestion buffer (perfusion buffer containing 12.5 μM CaCl₂, 0.14 mg/ml trypsin and 0.25 mg/ml of Liberase TH). After digestion, the left ventricle was removed and minced in perfusion buffer with 12.5 μM CaCl₂ and 5% BSA. The concentration of CaCl₂ in the solution was gradually increased over 20 minutes to achieve a final concentration of 1 mM. Equal numbers of ALVM were plated in each well with Minimal Essential Media (MEM) with Hanks' salts and 2 mM L-glutamine, supplemented with 5% calf serum, 10 mM 2, 3-butanedione monoxime (BDM) and 100 U/ml penicillin. After 1 hour incubation, ALVM were rinsed and placed in culture medium

(serum-free MEM with 0.1% BSA, 100 U/ml penicillin and 2 mM L-glutamine). Particles and/or α -tocopherol (50 μ M) were added to the ALVM in serum-free MEM with 0.1% BSA, 100 U/ml penicillin and 2 mM L-glutamine and incubated for 12 hours.

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) enzymatic activity was used to determine cell viability. The assay was adapted from a method previously described (19). Extracellular LDH inactivation by the EPFR treatments produced artifactually low viability results. Therefore, cell viability was calculated as the ratio of cytosolic LDH activity between the treatment and control groups.

MTT Assay

In some experiments, cell viability was measured in HL-1 cells using a MTT assay kit (Promega, G4100), following the manufacturer's instructions. Briefly, the assay was performed in a 24-well cell culture plate containing equal numbers of HL-1 cells with various treatments. A dye solution was added to each culture well, incubated at 37° C for 2 hours. During the incubation, living cells convert the tetrazolium component of the dye solution into a formazan product. The stop solution was then added to the culture wells to solubilize the formazan product. After 1 hour incubation at room temperature, the absorbance at 570 nm was recorded using a plate reader and used for determination of live cell number.

DNA damage

DNA oxidative damage was assessed by measuring apurinic/aprimidinic (AP) sites. AP sites are DNA lesions formed during the excision and repair of oxidized, deaminated or alkylated bases and are an indicator of DNA damage. To assess DCB230-mediated DNA damage, HL-1 cardiomyocytes were treated with DCB230 (25, 100 or 200 μ g/ml) or silica (200 μ g/ml) for 1, 2 or 8 hours. After treatment, cells were harvested in PBS and centrifuged at 500 g for 5 minutes. The pellets were then stored at -80°C before performing the AP assay. DNA was isolated from the cell pellets using a genomic DNA isolation kit (BioVision, K281-50). DNA damage, as indicated by increases in the number of AP sites/10⁵ base pairs, was measured using a DNA Damage Quantification Colorimetric Kit (BioVision, K253-25) per the manufacturer's instructions.

Western blots

Denaturing, reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weights. An equal mass of protein from each sample was loaded into either 12% or 4-15% gels and wet transferred onto PVDF membranes. Ponceau S stain was used to ensure that equal amounts of protein were loaded into each lane, band intensity in the treatment groups is expressed relative to that of vehicle. Membranes were blocked in tris-buffered saline with 0.05% Tween 20 (TBST) containing 3% bovine serum albumin and 0.02% NaN₃ for 1 hour. Membranes were then probed with primary antibodies in blocking buffer overnight (1:1000 dilution, 4°C), washed 3 times with TBST, probed with corresponding secondary antibodies in TBST for 1 hour (1:10,000

dilution), and washed 3 more times with TBST. Finally, membranes were incubated with ECL reagent and imaged with an LAS 4000 ImageQuant set to standard/auto mode to avoid over- or under-exposures.

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed using TMRE and laser scanning confocal microscopy according to a protocol adapted from (20). HL-1 cells were seeded onto gelatin/fibronectin-coated chambered coverglasses, allowed to adhere for 4-6 hours, and then serum deprived overnight. Cells were pre-loaded with 250 nM MitoTracker Green and 250 nM TMRE for 30 minutes, treated with 100 µg/mL DCB230 in 500 µL of fresh medium, then imaged. Argon-krypton (488 nm) or helium-neon (568 nm) lasers were used to excite the fluorophores. Laser power was set at less than 20% via an acousto-optical tunable filter to minimize photo bleaching. A water-immersion 63x objective (HCX PL APO 63.0×1.20 IMM BD) was used for imaging. Resolution was set to 12-bit and 1024×1024, while scanning was set to 400 speed, bi-directional, and 8-line average. The airy unit was set to 1 for optimal balance between signal and resolution. MitoTracker Green was imaged at 488 nm excitation and 530 nm band pass emission. TMRE was imaged at 568 nm excitation and 590 nm long pass emission. MitoTracker Green to TMRE fluorescence resonance energy transfer (FRET) was imaged at 488 nm excitation and 590 nm long pass emission. TIFF images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data were analyzed using GraphPad Prism 5 (La Jolla, CA). Data containing more than 2 treatment groups were analyzed with one-way ANOVA with Bonferroni post-test versus appropriate control group(s). Otherwise, data were analyzed using two-tailed Student's t-test, with F-test conducted to determine whether the two groups' variance differed significantly. Results are reported as group means $\pm$ standard error of the mean (SEM). P value <0.05 was considered statistically significant.

RESULTS

DCB230 cardiomyocyte toxicity is radical- and time-dependent

After 2 hours of DCB230 treatment, a significant decrease in HL-1 myocyte viability occurred only at the highest concentration of DCB230 (Fig. 1A). After 8 hours of DCB230 treatment, significant decreases in HL-1 myocyte viability was seen across the concentration range (Fig. 1A). Eight hour treatment of HL-1 myocytes with either silica particles or CuO-coated silica particles did not reduce cell viability (Fig. 1B).

Since DCB230 contains an EPFR that generates hydroxyl radical ($\text{OH}^\cdot$) in biological and other solutions (13, 21), while its synthesis precursors silica and silica CuO do not (13), α -tocopherol was used to determine whether DCB230's cytotoxicity was radical dependent. Treatment with 25 µg/ml or 100 µg/ml DCB230 significantly reduced HL-1 cell survival during an 8 hour incubation (Fig. 2A). However, co-incubation with the antioxidant α -tocopherol (100 µM) significantly reduced DCB230-mediated cell death (Fig. 2A). In rat

ALVM, 12 hour incubation with DCB230 (150 µg/ml) also produced significant cell death, which was significantly attenuated by co-incubation with α -tocopherol (50 µM) (Fig. 2B).

FRET microscopy was used to determine whether exposure to DCB230 produced mitochondrial depolarization. Two hour treatment with 100 µg/mL DCB230 decreased mitochondrial membrane potential (Fig. 3A-E). Controls stained with either mitochondria-specific MitoTracker Green (Fig. 3A) or TMRE (Fig. 3B), a cationic dye that localizes to the mitochondria in a membrane potential-dependent fashion, both showed a distinct reticular pattern indicative of mitochondria within cells. When green MitoTracker images from vehicle-treated cardiomyocytes were overlaid with red MitoTracker Green-to-TMRE FRET images, the resulting orange colored images showed excellent co-localization (Fig. 3C). The strong fluorescence from MitoTracker Green-to-TMRE FRET showed that TMRE and MitoTracker Green molecules were within 10 nm of each other (a requirement for FRET) and confirmed that TMRE staining was mitochondria-specific (22). Importantly, this indicated that vehicle-treated cardiomyocytes' mitochondria maintained membrane potential. In contrast, the overlaid MitoTracker Green/MitoTracker Green-to-TMRE FRET images from DCB230-treated cardiomyocytes showed mitochondrial membrane depolarization as mitochondria shifted from orange-red to yellow-green (Fig. 3D). These observations were confirmed by quantifying the FRET intensities in images of control and DCB230-treated myocytes (Fig. 3E). Some non-cellular objects stained with MitoTracker Green were also visualized and were likely to be DCB230 particle aggregates.

DCB230 cytotoxicity in cardiomyocytes mediated by apoptosis

To determine whether the observed mitochondrial depolarization initiated apoptotic signaling, caspase 3 and subsequent poly(ADP-ribose) polymerase 1 (PARP1) cleavage were assessed in adherent HL-1 cells after 8 hours of exposure to DCB230. Protein levels of the pro, or inactive caspase 3 was significantly decreased at all concentrations of DCB230 (Fig. 4A). These were accompanied by significant increases in the levels of the cleaved, or active form of the protein (Fig. 4B). Similar to caspase 3, PARP1 was cleaved (activated) after 8 hours of treatment with DCB230 (Fig. 4C-D).

Early DCB230-induced apoptotic signaling is intrinsic

To assess apoptotic signaling upstream of caspase 3, we next examined caspase 9 activation. Cleavage of initiator caspases, such as caspase 9, allows for activation of downstream effector caspases such as caspase 3. After 8 hours of treatment with DCB230 there were marked decreases in the levels of the pro, or inactive form of caspase 9 at all concentrations of DCB230 treatment (Fig. 5A). The levels of pro-caspase 9 were also significantly decreased after 2 hours treatment with the 100 and 200 µg/ml concentrations of DCB230 (Fig. 5B). In contrast, after 2 hours of treatment with DCB230, the levels of cleaved (active) caspase 3 were not different than control (Fig. 5C).

To determine whether DCB230 produced oxidative damage of DNA we measured the numbers of lesioned AP sites in the DNA of HL-1 cells treated with DCB230 or silica particles. After 1 or 2 hours of exposure, the number of AP sites in the DCB230 and silica treated cells were not significantly different (Fig. 6). However, after 8 hours of exposure, the

number of AP sites were significantly greater in DCB230- treated cells than in silica treated cells (Fig. 6).

DISCUSSION

This study is the first to show that the EPFR DCB230 can produce apoptotic cell death in cultured mouse HL-1 cardiomyocytes. EPFR-mediated cell death was significantly reduced by co-treatment with the antioxidant α -tocopherol, indicating that oxidative stress played a prominent role. Moreover, treatment with amorphous silica particles or CuO/Si particles, both of which lack EPFRs, did not decrease the viability of HL-1 cells, indicating that the EPFRs and the hydroxyl radicals they generate, were the most likely cause of the myocyte destruction. Treatment with DCB230 also killed rat ALVM. Although we did not determine whether the ALVM were killed by an apoptotic mechanism, the DCB230-mediated death of these cells was similarly attenuated by treatment with α -tocopherol. Similarly, Kim and colleagues (23) showed that exposure to diesel exhaust particles, which contain EPFRs (10), induces apoptosis in neonatal rat cardiomyocytes.

Exposure to the bioavailable component of residual oil fly ash also increases the expression of apoptotic genes in neonatal rat cardiomyocytes (24). EPFRs are also cytotoxic to a number of different cell types. For example, DCB230 and another EPFR formed from the chemisorption of 3-monochlorophenol to CuO (MCP230), also produce oxidative stress and cytotoxicity in cultured human bronchial epithelial and human laryngeal cells (13-15). Although the degree of oxidative stress and cytotoxicity produced by the EPFRs in the human cells was greater, significant oxidative stress and cytotoxicity were also produced by control CuO/Si particles presumably due to the oxidant properties of Cu^{2+} (13-15). Why our HL-1 myocytes were not similarly killed by CuO/Si particles is not known, but may reflect the fact that our study used lower concentrations of particles, or that HL-1 cells and ALVM are more resistant to the oxidizing actions of CuO/Si. Alternatively, as described in the following sections, epithelial and phagocytic cells take up particles in to lysosomes where they can subsequently alter cell function. It is not known whether HL-1 cells and ALVM take up particles. Nonetheless, our data indicate that the EPFRs are responsible for the cytotoxic action of DCB230 on the HL-1 cardiomyocytes and ALVM.

In order to distinguish between the intrinsic or extrinsic apoptotic pathways in DCB230-mediated myocyte death, we examined the time course of mitochondrial depolarization and changes in the activation state of various downstream apoptotic signaling proteins. A key step in the intrinsic apoptotic pathway is mitochondrial membrane depolarization, followed by cytochrome C translocation from the mitochondria to the cytosol where it complexes with Apaf-1 to form the apoptosome, culminating in the recruitment and cleavage of caspase 9 (22), followed by activation of the executioner caspase, caspase 3 with subsequent activation of PARP-1. Consistent with an intrinsic mechanism, we found that after 2 hours of DCB230 treatment, depolarization of the mitochondrial membrane potential was temporally related to the activation of the initiator caspase 9, but not the downstream effector caspase 3, or significant cell death. However, activation of downstream caspase 3 and PARP1 was evident after 8 hours of treatment and was coincident with significant cell death. Similarly, DNA damage was only significantly increased after 8 hours of exposure. The lack of significant

DNA damage at the earlier time points (1-2 hours), suggests that oxidation of DNA did not initiate the intrinsic apoptotic pathway.

Although we have compelling evidence that DCB230-mediated oxidative stress plays a major role in killing cardiomyocytes, the mechanism(s) by which EPFRs initiate apoptosis has not been defined. In saline and biological solutions, DCB230 undergoes prolonged redox cycling producing significant amounts of OH^- (13). Interestingly, CuO/Si particles are poor generators of OH^- in aqueous solutions, thus possibly explaining the lack of toxicity against HL-1 cells (13). Contact between the EPFR particles and the cell membrane could lead to the oxidation of membrane lipids, since the highly reactive radicals generated by the EPFRs most likely not diffuse across the membrane (13). Lipid peroxidation leads to the formation of reactive aldehydes and isoprostanes. These products are often stable and are used as markers of oxidative stress. EPFRs increase 8-isoprostane levels in human tracheal and lung epithelial cells in vitro (14, 15). DCB230 exposure in vivo also increases plasma 8-isoprostane levels in rats (7) and in the lungs of neonatal rats (11). Oxidized lipids can initiate signaling pathways, induce oxidative stress and can directly target the mitochondria (25). High concentrations of oxidized lipids can also initiate apoptosis by intrinsic and extrinsic pathways.

Alternatively, the particles may directly damage the mitochondria after being phagocytosed by cells. Cormier and colleagues reported that EPFR-containing particles are taken up into lysosomes within BEAS-2B bronchiolar epithelial cells, macrophages and fibroblasts (12), whereupon the lysosomes migrated to the perinuclear region resulting in cytoplasmic lipid peroxidation. Lipid peroxidation in the perinuclear region was correlated with increased expression of mitochondrial superoxide dismutase. Other studies also report that particles are actively taken up by a variety of cell types. In fact, Li and colleagues (26) reported that ultrafine particles localized to the mitochondria in RAW 264.7 (mouse macrophage) and BEAS-2B cells where they cause disruption of the mitochondrial cristae. Whether cardiomyocytes take up particles is not known and future studies are needed to conclusively determine whether EPFRs enter myocytes.

Although it is evident from our work that EPFRs can initiate apoptosis in cardiomyocytes *in vitro*, a major question is whether environmental exposure to EPFRs can initiate apoptosis *in vivo*. There is accumulating evidence that fine and ultrafine particles can transit from the lung to the systemic circulation where they could have biological actions with organs throughout the body (3-5). While a number of studies have shown that inhalational exposure or intratracheal instillation of PM (including EPFRs) produces inflammation and oxidative stress in the myocardium and alters cardiac function, very few studies have examined whether these exposures produce apoptosis in the myocardium.

Claderon-Garciduenas and colleagues compared the hearts of dogs exposed to high levels of air pollution in Mexico City, with those of dogs living in less polluted neighboring cities (27). Compared to controls, the hearts from dogs exposed to Mexico City air pollutants contained apoptotic myocytes and endothelial cells as well as degranulated mast cells, and scattered foci of inflammatory infiltrates. Whether these apoptotic processes reflected an intrinsic or extrinsic pathway was not determined. A subsequent study from the same group

found increased apoptosis and expression of inflammatory genes in cardiac ventricles of Mexico City residents (28). In contrast, Kodavanti and colleagues noted that WKY rats exposed to oil combustion-derived PM 1 day per week for up to 16 weeks showed degranulated mast cells, chronic inflammation, fibrosis and multifocal degeneration, but no signs of apoptosis (29).

Here we showed for the first time, that PM, and in particular the EPFR DCB230, can directly induce oxidative stress in cultured cardiomyocytes, leading to mitochondrial depolarization, the initiation of an intrinsic apoptotic pathway, and cell death. These data suggest that upon entering systemic circulation and reaching the heart, EPFRs have the potential to directly induce apoptosis in cardiomyocytes, and may provide insight into the link between exposure to airborne particulates and cardiovascular disease and dysfunction.

ACKNOWLEDGEMENTS

The authors would also like to thank Dr. Charles Nichols for this assistance with the confocal microscopy and Dr. Martin Ronis for his insights and editorial suggestions.

FUNDING

This work was supported by the National Institutes of Health P42ES013648 and P30GM106392 to K.J.V.

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1). DCB230-mediated toxicity is radical- and time-dependent. (A) Viability of HL-1 cardiomyocytes after 2 or 8 hour treatment with DCB230 or vehicle (open bars) (LDH assay). (B) Viability of HL-1 cardiomyocytes treated 8 hours with vehicle (open bars), control silica particles or silica particles coated with only 5% CuO (LDH assay). n=3 * p<0.05 indicates significant difference from vehicle.

2).

The anti-oxidant, α -tocopherol (100 μ M) protects HL-1 cardiomyocytes from DCB230-mediated toxicity. A) Viability of HL-1 cardiomyocytes treated for 8 hours with vehicle, tocopherol (100 μ M), DCB230 or DCB230 and α -tocopherol (MTT assay). (B) Viability of isolated adult rat ventricular myocytes after treatment with vehicle, DCB230 or DCB230 and α -tocopherol (LDH assay). * $p < 0.05$ indicates significant difference between control and DCB230, # $p < 0.05$ indicates significant difference between DCB230 and DCB230 + tocopherol, $n = 6-8$ for (A) and 3 for (B).

3).

DCB230 induces mitochondrial membrane depolarization. Mitochondrial membrane potential was assessed using confocal fluorescence resonance energy transfer (FRET) microscopy with TMRE and MitoTracker Green after 2 hour treatments. Representative images of (A) MitoTracker Green only control (mitochondria-specific green fluorophore), (B) TMRE only control (membrane potential dependent red fluorophore), (C) vehicle, and (D) 100 µg/ml DCB230. (C) and (D) images are overlay composites of MitoTracker Green and MitoTracker Green-to-TMRE FRET. Mitochondrial membrane depolarization causes TMRE to diffuse out of the mitochondria, resulting in decreased red FRET signal and increased MitoTracker Green signal. Quantified FRET signal with background subtracted and normalized by cell count. n=3-4, p<0.05 indicates significant difference from vehicle.

4).

DCB230 cytotoxicity in HL-1 cardiomyocytes is mediated by apoptosis. Levels of late apoptotic cascade proteins (A) pro caspase 3, (B) cleaved caspase 3, (C) PARP1 (poly-ADP ribose polymerase 1 and (D) cleaved PARP1 were assessed by immunoblot and densitometry after 8 hour treatments as indicated. n=3, p<0.05 indicates significant difference from vehicle.

5).

Early DCB230-induced apoptotic signaling is intrinsic. Levels of intrinsic apoptotic pathway protein pro-caspase 9 after (A) 8 hour treatments or (B) 2 hour treatments as indicated. Levels of late apoptotic cascade proteins, (C) cleaved caspase 3 after 2 hour treatments as indicated. $n=3$, $p<0.05$ indicates significant difference from vehicle.

6).

DCB230 induces only late DNA damage in HL-1 cells. The number of apurinic/aprimidinic (AP) sites (a marker of DNA damage) was measured in HL-1 cells treated with DCB230 or silica for 1, 2, or 8 hours. DCB230 significantly increased the number of AP sites only after 8 hours of treatment. n=3. * p<0.05 indicates significant difference from silica control.