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Quinone-Induced Protein Handling Changes: Implications for Major Protein Handling Systems in Quinone-Mediated Toxicity

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Abstract

Para-quinones such as 1,4-benzoquinone (BQ) and menadione (MD) and ortho-quinones including the oxidation products of catecholamines, are derived from xenobiotics as well as endogenous molecules. The effects of quinones on major protein handling systems in cells; the 20/26S proteasome, the ER stress response, autophagy, chaperone proteins and aggresome formation, have not been investigated in a systematic manner. Both BQ and aminochrome (AC) inhibited proteasomal activity and activated the ER stress response and autophagy in rat dopaminergic N27 cells. AC also induced aggresome formation while MD had little effect on any protein handling systems in N27 cells. The effect of NQO1 on quinone induced protein handling changes and toxicity was examined using N27 cells stably transfected with NQO1 to generate an isogenic NQO1-overexpressing line. NQO1 protected against BQ-induced apoptosis but led to a potentiation of AC- and MD-induced apoptosis. Modulation of quinone-induced apoptosis in N27 and NQO1-overexpressing cells correlated only with changes in the ER stress response and not with changes in other protein handling systems. These data suggested that NQO1 modulated the ER stress response to potentiate toxicity of AC and MD, but protected against BQ toxicity. We further demonstrated that NQO1 mediated reduction to unstable hydroquinones and subsequent redox cycling was important for the activation of the ER stress response and toxicity for both AC and MD. In summary, our data demonstrate that quinone-specific changes in protein handling are evident in N27 cells and the induction of the ER stress response is associated with quinone-mediated toxicity.

Keywords

Quinones; aminochrome; proteasomal inhibition; ER stress responses; autophagy; oxidative stress

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Conflict of interest

The authors declare that there are no conflicts of interest.

Introduction

Quinones can undergo one-electron enzymatic reduction by NADPH-cytochrome P450 and NADH-cytochrome b5 reductases to generate semiquinone radicals that can redox cycle leading to the formation of reactive oxygen species (ROS) including superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. Quinones are also electrophiles and cellular damage due to these species can result from reaction with nucleophilic amino groups on proteins or DNA. In addition, covalent binding of quinones with cellular thiols, such as GSH or cysteine residues on proteins may lead to depletion of cellular GSH levels and/or protein arylation. Previous studies have attributed quinone-induced toxicity to two major mechanisms: (1) ROS formation and (2) arylation with macromolecule targets including histone, tubulin, topoisomerase II, and DNA (Gurbani et al., 2012; Monks and Jones, 2002; Ross, 2000). Few studies have reported quinone-induced changes in major protein handling systems, which include the 20/26S proteasome, the ER stress response, autophagy, and aggresome formation. 1,4-Benzoquinone (BQ) was previously shown to induce the ER stress response (X. Wang et al., 2006) but whether this resulted from ROS production or arylation and the relationship to other protein handling changes was not determined.

Aminochrome (AC) is a reactive ortho-quinone metabolite derived from dopamine and has been used as a model neurotoxin to study Parkinson's disease in cell and animal models (Segura-Aguilar et al., 2014). The mechanism of AC induced neurotoxicity is manifested via one-electron redox cycling leading to oxidative stress and/or arylation with cellular nucleophiles, such as GSH and alpha-synuclein (Conway et al., 2001; Norris, 2005). Both AC and BQ have been shown to disrupt the microtubule network and the mechanism was implicated as formation of covalent adducts with reactive-SH groups of tubulin and subsequent perturbation of microtubule assembly (Das et al., 2010; Paris et al., 2010; Santa-María et al., 2005). This evidence suggested an important role for arylation in quinone-induced protein handling changes. More importantly, data from our own work and others has shown that AC could exert an inhibitory effect on proteasomal activity (Jinsmaa et al., 2011; Zafar, 2006; Zhou et al., 2010), and AC was able to induce a significant increase in autophagosome formation (Gurbani et al., 2012; Monks and Jones, 2002; Muñoz et al., 2012; Ross, 2000). Our previous work has also indicated a key role for the proteasome in the regulation of compensatory protein handling systems and the potential importance of proteasomal inhibition in the biogenesis of protein aggregates associated with Parkinson's disease (Xiong et al., 2013).

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) is generally considered to play a protective role in quinone-induced toxicity because it catalyzes two-electron reduction of quinones to less reactive hydroquinones thus decreasing the concentration of quinones available for one-electron reduction to semiquinones and arylation reactions (Ross, 2004; Ross and Siegel, 2004). However, hydroquinones generated by NQO1 may also be unstable to oxygen and can undergo autoxidation to produce ROS leading to toxicity (Paris et al., 2007; Ross, 2004; Watanabe et al., 2004).

Although the mechanisms of quinone toxicity have been extensively studied, the role of quinones in modulation of protein handling systems has not been investigated in detail. It is possible that redox active and electrophilic quinones may target the proteasome and result in the downstream modulation of other protein handling systems that could markedly amplify the consequences of a single quinone-induced arylation or oxidative event. In addition, quinones may have direct effects on other protein handling systems independent of any proteasomal effects. Proteasomal inhibition has been shown to perturb downstream compensatory protein handling changes (Choy et al., 2011; Xiong et al., 2013). We performed this study to determine whether quinones induce changes in major protein handling systems either independently or downstream of proteasomal inhibition and to define the role of NQO1 in modulating these changes.

Materials and Methods

Materials and Antibodies

1,4-benzoquinone (BQ), menadione (MD), duroquinone (DQ), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), NADH, dopamine hydrochloride, ATP disodium salt, digitonin, phenylmethylsulfonyl fluoride (PMSF), tunicamycin, mouse monoclonal anti- β -actin (A5441) and rabbit polyclonal anti-ubiquitin (U5379) antibodies were obtained from Sigma Chemical (St. Louis, MO, USA). Polyphenol oxidase (mushroom tyrosinase, LS003793) was purchased from Worthington Biochemical (Lakewood, NJ, USA). Dithiothreitol was purchased from Fisher Scientific (Pittsburgh, PA, USA). MTT [Thiazolyl blue] was obtained from Research Products International (Mount Prospect, IL, USA). MG132, purified human 20S proteasome (BML-PW8720), mouse monoclonal anti-Hsp27, anti-Hsp70 antibodies and rabbit polyclonal anti-Hsp90 α antibody were obtained from Enzo (Farmingdale, NY, USA). FITC / annexin V (640906) and annexin V binding buffer (422201) were purchased from BioLegend (San Diego, CA, USA). The fluorescently labeled proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC was purchased from Bachem (Torrance, CA, USA). Rabbit polyclonal anti-LC3 (NB100-2331) was obtained Novus (Littleton, CO, USA). Mouse monoclonal anti-NQO1 (A180) antibody, rabbit polyclonal anti-phospho-eIF2 α (Ser51, #9721), anti-eIF2 α (#9722) and anti-caspase-3 (#9662) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-caspase 12 (ab62484) and anti-NQO1 (ab34173) antibodies were obtained from Abcam (Cambridge, MA, USA).

Cell culture and treatment

N27 rat mesencephalic dopaminergic cells were obtained from Dr. Curt Freed, Department of Clinical Pharmacology and Toxicology, University of Colorado Anschutz Medical Campus, Aurora CO (Adams et al., 1996). N27 cells were cultured in RPMI-1640 medium (Cellgro, Manassas, VA, USA) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin (Cellgro, Manassas, VA, USA) in a humidified atmosphere of 5% CO₂ at 37°C. Stock solutions of BQ and MD (10mM) were prepared in ethanol and AC (1mM) was generated as described below. All stock solutions were used immediately after preparation.

Stable transfection of N27 cells with human wild-type (wt) hNQO1

Stable transfection of N27 cells was carried out as described previously (Zafar et al., 2006). Briefly, the CMV-driven mammalian expression vector pcDNA3 containing human NQO1*1 wild type cDNA was transfected by electroporation into N27 cells with neomycin as the selection marker. Clones were selected after 7 to 10 days and examined for both NQO1 enzymatic activity and NQO1 immunoreactivity. The NQO1-transfected (clone 4) cells are routinely examined for NQO1 expression by activity assay, immunoblot analysis and confocal microscopy.

Measurement of NQO1 activity

NQO1 activity was determined spectrophotometrically in cell lysates using the dicumarol-inhibitable reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm as described previously (Benson et al., 1980). Protein concentrations were determined using the method of Lowry (LOWRY et al., 1951).

Aminochrome preparation

Aminochrome was prepared by enzymatic reaction (300 μ l, room temperature) containing 3.3mM dopamine and 500 μ g tyrosinase in 25mM Tris-HCl buffer (pH 7.4). After 3min at room temperature the reaction mixture was centrifuged (13,000 rpm for 5 min at 4°C) through a 100-kDa molecular mass cutoff membrane filter (Millipore Corporation, Bedford, MA) to remove tyrosinase and prevent additional tyrosinase-catalyzed oxidative reactions. After centrifugation the filtrates were collected and stored on ice. To determine the concentration of aminochrome generated 5 μ l of the filtrate was added to 995 μ l of 25mM Tris-HCl buffer (pH 7.4) and the absorption was determined at 474nm. The concentration of aminochrome was calculated using a molar extinction coefficient of 3,058 at 474nm (Baez et al., 1997).

MTT growth inhibition assay

Quinone toxicity was initially determined using the MTT assay and confirmed using trypan blue and apoptosis/necrosis assays (below). For MTT assay, N27 cells were seeded at 2000 cells per well in 96-well plates (in triplicate). After 24h the cells were treated with BQ or MD in complete medium or with AC in serum-free medium for 24h. After drug treatments, the medium were removed and replaced with MTT (1mg/ml) containing complete medium. After 4h the MTT containing medium was removed and the MTT formazan product was extracted from cells with dimethyl sulfoxide. The optical density of the extract was determined at 550nm with a microplate reader. IC₅₀ values were defined as the concentration of quinone that resulted in a 50% reduction in cell density compared to untreated controls.

Trypan blue exclusion assay

Cells were seeded at 1.5×10^5 cells into 60mm $\times$ 15mm tissue culture dishes in 3ml of complete medium and allowed to attach and grow for 2 days. Following treatment with quinone for the indicated times, cell pellets containing both floating and attached cells were collected and then resuspended in 1ml PBS. To assess cell viability the cell suspension

(10 μ l) was stained with 10 μ l trypan blue dye (0.4% w/v in PBS). The number of trypan blue positive cells was determined using a Life Technologies Countess™ automated cell counter immediately after staining.

Apoptosis assay

Apoptosis was determined by flow cytometry using FITC-conjugated anti-annexin V and propidium iodide (PI) as previously described (Benz et al., 2006; Xiong et al., 2013). After 24h treatment with quinones, cell pellets including both the floating and attached cells were collected and gently resuspended in 500 μ l of annexin V binding buffer and incubated with anti-annexin V antibody (2 μ l) and PI (0.7 μ l of 100 μ g/ml stock) for 10 min at 37°C in the dark. Samples were kept on ice, and then analyzed using a BD Biosciences FACS Calibur Flow Cytometer (San Jose, CA, USA). The fluorescence was measured at 530nm (FL1, FITC) and PI at above 600nm (FL2), and the data was acquired and analyzed using Cellquest software (Becton-Dickenson, Mountainview, CA, USA).

Proteasomal activity assay

Proteasomal activity was determined by measuring the fluorescence of cleaved proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity) as described previously (Xiong et al., 2013). Briefly, purified human 20S proteasome (1.25 μ g) was incubated with vehicle or quinone in 100 μ l of proteasome activity assay buffer (50mM Tris-HCl, pH 7.5, 250mM sucrose, 1mM dithiothreitol, 5mM MgCl₂, 2mM ATP, 0.5mM EDTA and 0.025% (w/v) digitonin). Proteasome activity substrate (Suc-Leu-Leu-Val-Tyr-AMC, 200 μ M) was then added. After 30min at 37°C 40 μ l of the above reaction was transferred to a 96-well plate and the fluorescence of liberated 7-amino-4-methyl coumarin (Ex: 380nm; Em: 460nm) was measured in duplicate samples. To measure intracellular proteasomal activity, cells were treated with quinones for the indicated times, washed with PBS (10ml) three times, and then lysed with proteasomal activity assay buffer and centrifuged at 10,000g for 14min at 4°C. The supernatants were collected and protein concentrations were determined using the method of Lowry (LOWRY et al., 1951). To assay proteasome activity cell lysate (20 μ g) was added to 100 μ l of proteasome activity assay buffer followed by the addition of 100 μ M proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC. After 30min at 37°C the reactions were stopped by the addition of 2 volumes of ice-cold ethanol and 40 μ l samples were transferred to a 96-well plate and the fluorescence of liberated 7-amino-4-methylcoumarin (Ex: 380nm; Em: 460nm) was measured in duplicate samples.

Immunoblot analysis

Following treatment with quinones cells were washed with phosphate buffered saline (PBS) and then lysed with RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate) supplemented with protease inhibitors (Complete, Mini Protease Inhibitor Cocktail, Roche, Germany), PMSF (2mM) and phosphatase inhibitors (Sigma Chemical, USA). Cells were sonicated on ice and then centrifuged at 10,000g for 14 min at 4°C. The supernatants were collected and protein concentrations were measured using the method of Lowry (LOWRY et al., 1951). Proteins were diluted in 2X Laemmli SDS sample buffer and heated to 70°C for 5 min, and then separated on a 12% SDS-PAGE precast minigel (BioRad Laboratories, Hercules, CA, USA).

A 7.5% SDS-PAGE precast minigel was used to separate the high molecular weight polyubiquitinated proteins. Proteins were transferred to polyvinylidene fluoride membranes in 25mM Tris, 192mM glycine containing 20% (v/v) methanol at 4°C. Membranes were incubated in blocking buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.2% (v/v) Tween-20 (TBST) containing 5% (w/v) non-fat dry milk) for 1h, and then incubated with primary antibodies overnight at 4°C. Membranes were washed every 10min in TBST for 1h and then probed with horseradish peroxidase-conjugated goat anti-rabbit or horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson ImmunoResearch Labs, West Grove, PA, USA) for 30min at room temperature. Membranes were washed three times in TBST and protein bands were visualized using enhanced chemiluminescence (Thermo Scientific, USA).

Immunocytochemistry for aggresome-like inclusion bodies

Aggresome-like inclusion bodies were determined using immunostaining for ubiquitin-positive aggregates as previously described (Xiong et al., 2013). Sub-confluent N27 cells were grown on glass coverslips in 6-well plates and treated with quinones for the indicated times. Cells were washed three times with PBS and fixed in 3.7% (v/v) formaldehyde in PBS for 12min, and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10min. After extensively washing with PBS, cells were incubated in blocking buffer (RPMI-1640 containing 10% FBS) for 1h, and then incubated with anti-ubiquitin antibody (1:100) overnight at 4°C. Cells were then washed three times in TBST and incubated with Alexa Fluor 594 conjugated goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch Labs) containing DAPI (1µg/ml) for 30min. Coverslips were washed three times with TBST then rinsed in distilled water, inverted and mounted on glass slides using SuperMount (BioGenex, USA). Cells were viewed on a Nikon TE2000 microscope with a Nikon C1 confocal imaging system.

Intracellular oxidative stress

Quinone-induced oxidative stress was determined using CellROX[®] Green reagent (Life Technologies, Carlsbad, CA USA) in combination with confocal microscopy. For these studies (sub-confluent) N27 and clone 4 cells were grown on glass coverslips in 6-well plates. After treatment with quinones the medium was removed and cells washed three times with PBS. The CellROX[®] Green fluorogenic probe (5µM in PBS) was added to cells for 30min at 37°C in the dark. Cells were washed three times with PBS then fixed in 3.7% (v/v) formaldehyde in PBS for 12min, and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10min. After extensively washing with PBS cells were incubated in blocking buffer (RPMI-1640 containing 10% FBS) for 1h, and then incubated with anti-NQO1 antibody (1:100) overnight at 4°C followed by Alexa Fluor 594 conjugated goat anti-rabbit IgG (1:1000) containing DAPI (1µg/ml) for 30min. Coverslips were washed three times with TBST then rinsed in distilled water, inverted and mounted on glass slides using SuperMount (BioGenex, USA). Cells were viewed on a Nikon TE2000 microscope with a Nikon C1 confocal imaging system.

Oxygen consumption assay

The ability of quinones to undergo redox cycling in N27 and clone 4 cells was determined using an airtight Clark electrode (Yellow Springs Instrument Company, Yellow Springs OH, USA). For these studies 3 million cells were suspended in 3ml cell culture medium at 37°C in the presence or absence of quinones and the rate of oxygen consumption was measured over 10min. Dissolved oxygen concentrations were adjusted for altitude (5280 feet) and temperature (37°C).

Statistical analysis

All experiments were repeated at least three times, and the values were expressed as means $\pm$ standard deviation (SD). The statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Tukey or Dunnett's multiple comparison tests.

Results

Inhibition of proteasomal activity by quinones

The ubiquitin-proteasome pathway is the major proteolytic system in all eukaryotic cells. Most substrates of this pathway are targeted for degradation by attachment of ubiquitin molecules and the resulting ubiquitinated proteins are then recognized and degraded by the 20/26S proteasome complex. The 20/26S proteasome contains three active sites (chymotrypsin-like, trypsin-like and caspase-like), however, it is the chymotrypsin-like site that contributes the most to the catalytic activity of the proteasome and is routinely used to measure proteasomal activity (Kisselev and Goldberg, 2001).

Since the 20/26S proteasome exists abundantly in the cytosol and is rich in cysteine and lysine residues, it is likely a good arylating target for quinones. We measured the chymotrypsin-like activity of purified human 20S proteasome, following treatment with BQ, MD and the dopamine oxidation product AC for 30min (Fig. 1). Results from these studies show that both BQ and AC, but not MD, induced significant proteasomal inhibition in a dose-dependent manner (Fig. 2A–C). Duroquinone (DQ) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) are two fully substituted quinone analogs of BQ and MD respectively (Fig. 1) which are incapable of arylation reactions due to structural constraints but can still redox cycle. DQ and DMNQ failed to exert any inhibitory effect on the proteasome (Fig. 2A,B). The results with DMNQ were to be expected given the inability of MD to exert any inhibitory effect (Fig. 2B), but the data obtained with DQ indicate a role for arylation in benzoquinone-induced proteasomal inhibition (Fig. 2A). Additionally, we found AC at an equivalent dose with BQ induced greater proteasomal inhibition (Fig. 2A and 2C). This effect may possibly be explained on the basis of the lowest unoccupied molecular orbital (LUMO) energy (E_{LUMO}) value (in eV). Decreasing the energy of the LUMO, enhances the ability of a molecule to accept an electron into the LUMO. Several studies have applied E_{LUMO} to predict a given molecule's electrochemical properties and reactivity with biological nucleophiles (LoPachin et al., 2007; 2009). We calculated the local absolute E_{LUMO} values of BQ, AC and MD with Spartan '08 v1.2 (Wavefunction, Inc.) as described previously (LoPachin et al., 2007). We found that the carbonyl moiety on AC may be more

likely to react with nucleophilic amino acids, as reflected by a greater absolute E_{LUMO} value, relative to the corresponding carbonyl sites on BQ and MD (Supplementary Figure 1). In addition, the calculation of electrophile softness which correlates most closely with reactivity with soft nucleophiles such as cysteine demonstrated that AC (0.602) was a softer electrophile than either BQ (0.524) or MD (0.498) which may also contribute to its greater reactivity.

The inhibitory effect of quinones on intracellular proteasomal activity was confirmed using dopaminergic N27 cells. As shown in Figure 2D and 2F, both BQ and AC induced dose-dependent proteasomal inhibition. Treatment with BQ (20 μ M) for 24 hours resulted in nearly 50% inhibition of proteasomal activity in N27 cells while AC (80 μ M) inhibited over 60% of proteasomal activity (Fig. 2D and F). It is noteworthy that AC did not inhibit proteasomal activity in N27 cells as markedly as the purified 20S proteasome (Fig. 2C and F) probably because in cells factors such as compound uptake and elimination as well as additional targets for alkylation may contribute to attenuate the level of proteasome inhibition.

BQ induced proteasomal inhibition was tightly associated with induction of the ER stress response and autophagy, but had little effect on other major protein handling systems (heat shock proteins, HSPs and formation of aggresome-like inclusion bodies)

Previously, we have found proteasomal inhibition was able to induce downstream changes in other protein handling systems, including the ER stress response, autophagy, heat shock proteins (HSPs) and aggresome formation (Xiong et al., 2013). Since quinones including BQ and AC are able to inhibit proteasomal activity we conducted a detailed time-course study to characterize other biochemical changes induced by quinones in N27 cells and to dissociate any changes in protein handling from toxicity. Treatment of cells with BQ for 24h resulted in the loss of cell viability in a dose-dependent manner (Fig. 3A). A dose BQ (20 μ M) was sufficient to cause 50% of cell death at 24h. After a 10 min exposure to BQ (20 μ M) a rapid decrease (30%) in proteasomal activity was observed and the inhibitory effect was sustained up to 24h (Fig. 3B). Moreover, after treatment with BQ for 10min we were able to detect the accumulation of higher molecular weight polyubiquitinated proteins, an indicator of proteasomal inhibition (Fig. 3C), confirming proteasomal inhibition induced by BQ is an early event. No toxicity following BQ treatment could be detected at the early time points of 10min, 30min and 2h as indicated by trypan blue exclusion assays or by flow cytometry analysis of apoptosis/necrosis (data not shown) where marked proteasomal inhibition was observed (data not shown). MG132 was included as a positive control for proteasomal inhibition.

We then examined changes in other protein handling systems following treatment with BQ using phosphorylation of eIF2 α and LC3 II accumulation as biomarkers of the ER stress response and autophagy, respectively. As shown in Figure 3D, treatment with BQ induced expression of p-eIF2 α and LC3 II after 10min indicating that the ER stress response and autophagy were rapidly activated. However, we observed a subsequent decrease in p-eIF2 α expression at later time points following treatment with BQ and this may be due to the fact that BQ is highly reactive and can be rapidly detoxified as previously reported (X. Wang et

al., 2006). Interestingly, treatment with tunicamycin (Tn), a positive control for induction of ER stress in this study also induced LC3 II accumulation. Since phosphorylation of eIF2- $\pm$ has been shown to regulate autophagy formation (Kouroku et al., 2006) there may be crosstalk between the ER stress and autophagy pathways.

It is also noteworthy that after exposure to BQ for 2h the amount of LC3 II decreased while the polyubiquitinated proteins did not accumulate further (Fig. 3C and D). It is possible that the early induction of autophagy plays a role in the decreased accumulation of polyubiquitinated proteins observed at later time points. In these studies BQ was not able to induce the formation of aggresome-like inclusion bodies or cause significant induction of HSPs, including Hsp27, Hsp70 and Hsp90 (data not shown).

Dopamine derived AC triggered ER stress responses, induction of autophagy and formation of aggresome-like inclusion bodies in N27 cells

Because AC is unstable and readily reacts with protein thiols, including those in fetal bovine serum, we incubated N27 cells with AC in serum free medium as previously reported (Muñoz et al., 2012). No significant changes in protein handling systems and cell viability were observed in control experiments under serum-free conditions for 24h (data not shown). Treatment with AC led to dose-dependent cytotoxicity and exposure to a dose of 80 μ M of AC resulted in over 50% cell death (Fig. 4A). We then conducted the same time-course study used for BQ to examine the effect of AC on the activities of major protein handling systems and to dissociate protein handling changes from toxicity. As shown in Figure 4B, AC (80 μ M) inhibited proteasomal activity in a time-dependent manner and resulted in a significant decrease in activity at 2h. Accordingly, the accumulation of polyubiquitinated proteins was detected at 2h following treatment with AC (Fig. 4C). No toxicity could be detected at early time points of 10min, 30min and 2h as indicated by trypan blue exclusion assays or by flow cytometry analysis for apoptosis/necrosis (data not shown).

Treatment of N27 cells with AC did not cause HSPs induction (data not shown), however, rapid activation of the ER stress response and autophagy was observed. As shown in Figure 4D, p-eIF2 α and LC3 II were induced in a time-dependent manner following treatment with AC from 10min up to 24h and significant increases could be detected at 2h (supplementary Fig. 2). These results suggest that the activation of the ER stress response and autophagy were tightly associated with AC induced proteasomal inhibition. In addition, both perinuclear and more diffused aggresome-like inclusion bodies containing ubiquitin were found in N27 cells after prolonged 24h treatment with AC (Fig. 4E). To ensure ubiquitin containing aggresome-like inclusion bodies are distinct from LC3 containing autophagosomes, cells were double immunostained with anti-LC3 and anti-ubiquitin antibodies. No co-localization of ubiquitin and LC3-positive puncta could be detected (supplementary Fig. 3) demonstrating that the AC induced ubiquitin-positive, aggresome-like inclusion bodies were not autophagosomes. These studies demonstrated that AC is more reactive than BQ in terms of perturbing a greater number of intracellular protein handling systems and our results demonstrated for the first time that AC was able to trigger the ER stress response and aggresome formation.

Overexpression of NQO1 in N27 cells

To better define the role of NQO1 in modulating quinone induced protein handling changes and toxicity we generated a stably transfected N27 cell line (clone 4) that expresses high levels of human wild type NQO1 protein. As shown in Figures 5A and 5B parental N27 cells have low levels of NQO1 protein and activity (enzymatic activity of 259nmol DCPIP/min/mg protein), whereas clone 4 cells expressed much higher levels of NQO1 (1022nmol DCPIP/min/mg protein). In clone 4 cells overexpression and cytosolic localization of NQO1 was confirmed by immunostaining and confocal microscopy (Fig. 5C).

Modulation of quinone-induced ER stress response by NQO1 and its relationship to toxicity

We first examined the mode of quinone toxicity by flow cytometry using annexin V and propidium iodide (PI) double staining as markers for apoptosis. As shown in Figure 6, treatment with BQ resulted in significant levels of apoptosis at 24h with more than 40% of cells staining positive for annexin V (Fig. 6A), while treatment with AC and MD induced apoptosis in about 30% of cells (Fig. 6B and 6C). These results indicate that N27 cells were sensitive to apoptotic cell death after treatment with quinones for 24h. A significant decrease in BQ induced apoptosis was detected at 24h in NQO1-overexpressing clone 4 cells suggesting that clone 4 cells are more resistant to BQ toxicity (Fig. 6A). In contrast, clone 4 cells were more sensitive to AC- and MD-induced toxicity as indicated by a significant increase in the percentage of apoptotic cells (Fig. 6B and 6C). These data suggest that overexpression of NQO1 protected against BQ induced toxicity but potentiated AC and MD toxicity.

To examine which protein handling system could be modulated by NQO1 and contributed to quinone toxicity we compared the activities of the major protein handling systems following treatment of N27 and clone 4 cells with quinones. No significant differences in quinone induced proteasomal inhibition, autophagy and aggresome formation could be observed between N27 and clone 4 cells (data not shown). However, our data revealed that the ER stress response was tightly associated with quinone toxicity. In these studies caspase 12 was used as a marker for ER stress-mediated apoptosis as reported previously (Lien et al., 2008; Yuan et al., 2000) and as shown in Figure 6D, treatment of clone 4 cells with BQ for 24h resulted in a decrease in p-eIF2 α and caspase12 cleavage (C-Caps 12) compared to N27 cells. In contrast, either AC or MD induced a further increase of p-eIF2 α and cleaved caspase12 in clone 4 cells at 24h (Fig. 6E and 6F). These data correlated with quinone-induced apoptosis in N27 and clone 4 cells (Fig. 6A–C) suggesting that NQO1 modulated the ER stress response to potentiate toxicity of AC and MD, but protect against BQ toxicity.

NQO1 increased quinone induced oxidative stress that may contribute to the activation of the ER stress response and toxicity

Since quinones are also capable of redox cycling and inducing oxidative stress we next examined the role of NQO1 in modulation of quinone induced oxidative stress. It is possible that NQO1 catalyzes two-electron reduction of quinones to unstable hydroquinones and subsequent redox cycling leads to increased oxidative stress and toxicity of AC and MD. To

assess the relative stability of hydroquinones generated by NQO1 the rates of oxygen consumption were measured in N27 and clone 4 cells immediately after treatment with quinones. In both N27 and clone 4 cells the rate of oxygen consumption after treatment with BQ did not change significantly suggesting that hydroquinone does not undergo significant oxidation in these cells. In contrast, the rates of oxygen consumption following treatment with the equivalent dose of AC or MD were greater in clone 4 cells compared to parental N27 cells (Fig. 7A) indicating that reduction of AC and MD by NQO1 generates redox active metabolites (leukoaminochrome and menadiol). These data demonstrated that NQO1 increased the rates of redox cycling of AC and MD, which may in turn, led to increased levels of oxidative stress.

To confirm that NQO1 contributed to the increased oxidative stress induced by quinones we combined immunostaining using anti-NQO1 antibody with the superoxide reactive fluorescent probe CellROX Green[®]. Following oxidation by superoxide CellROX Green forms a relatively photostable green fluorescent product that is stable after fixation with formaldehyde and suitable for confocal microscopy. Green fluorescent staining is normally observed in the nucleus because CellROX Green is an analog of dihydroethidium that intercalates into DNA. As shown in Figure 7B(a), no green fluorescence was observed in the absence of quinone treatment in both N27 and clone 4 cells. In addition, treatment of N27 or clone 4 cells with BQ (20 μ M) did not result in green fluorescent staining confirming that BQ did not induce significant oxidative stress in either N27 or clone 4 cells (Fig. 7B–b). However, treatment with AC (80 μ M) or MD (16 μ M) resulted in intense green fluorescence in the nucleus of both N27 and clone 4 cells indicating oxidative stress was induced by these quinones (Fig. 7B–c,d). Moreover, green fluorescent staining was greater in the nucleus of clone 4 cells following treatment with AC and MD further confirming a role for NQO1 in the production of redox active metabolites (Fig. 7B–c,d). In agreement with the oxygen consumption data these results demonstrated that NQO1 potentiated quinone induced oxidative stress which may contribute to the increased activation of the ER stress responses and subsequent toxicity in clone 4 cells (Fig. 6).

Discussion

In the present study, three representative quinones including the dopamine oxidation product AC, and the environmentally relevant quinones BQ and MD were compared to define the role of protein handling systems in quinone-mediated toxicity. Although AC has previously been shown to inhibit proteasomal activity in rabbit reticulocyte lysates (Zafar, 2006) and dopaminergic neuronal MN9D cell extracts (Jinsmaa et al., 2011; Zhou et al., 2010), our data demonstrated for the first time that AC and BQ were capable of proteasomal inhibition using purified human 20S proteasome. Quinone-induced proteasomal inhibition was confirmed in rat dopaminergic N27 cells.

Chemically, AC, BQ and MD are all capable of both redox cycling and arylation. To discriminate whether quinone-induced proteasomal inhibition was induced by ROS production or arylation, we used DQ the fully substituted quinone analog of BQ. Substituted analogs of AC were not synthetically accessible and MD itself did not inhibit the proteasome so our analysis was restricted to BQ. Exposure to DQ did not result in inhibition

of 20S proteasomal activity suggesting a major role of arylation in BQ-induced proteasomal inhibition. Interestingly, AC induced the most pronounced changes in the number of major protein handling systems affected. Conversely, MD had little effect on the ER stress response and autophagy (supplementary Fig. 4).

Arylating quinones, such as BQ, have been shown to induce ER stress by activating the p-eIF2 α signaling pathway (X. Wang et al., 2006). Although the underlying mechanism remains unclear, it has been proposed that Michael adducts formed by quinones with ER protein disulfide isomerases (Lamé et al., 2003) disrupt the formation of disulfide bonds in the ER and thus cause accumulation of misfolded proteins and ER stress. Our findings suggest an additional possible explanation for a quinone-triggered ER stress response. We found BQ induced early proteasomal inhibition that was tightly associated with the rapid activation of the ER stress response in N27 cells. Since the ER stress response can be triggered by impairment of the ubiquitin-proteasome system (Choy et al., 2011; Fribley and C.-Y. Wang, 2006; Xiong et al., 2013) it is likely activated as a cellular defense mechanism. However, further aggravation of ER stress will trigger apoptosis. We have shown that the ER stress response could be modulated by NQO1 to potentiate the toxicity of AC and MD, or protect against BQ toxicity.

A neuroprotective role of NQO1 in AC toxicity has been proposed recently (Muñoz et al., 2012). This work used a cell model in which both vesicular monoamine transporter-2 (VMAT-2) and NQO1 were overexpressed. A lack of evidence using a NQO1 single-overexpression model makes it difficult to define the role of NQO1 alone in AC-mediated neurotoxicity. Therefore, we generated a NQO1-overexpressing dopaminergic cell line to better characterize AC induced changes, particularly with respect to protein handling, and further examined whether NQO1 modulated these changes and toxicity induced by AC. In contrast with the study of Munoz et al, we found that metabolism by NQO1 did not result in the detoxification of AC at least in N27 cells. Our data indicated that NQO1 mediated reduction of AC to an unstable hydroquinone and subsequent redox cycling was associated with activation of the ER stress response and subsequent toxicity. Although the potential importance of NQO1 in the modulation of redox cycling of leucoaminochrome (the hydroquinone form of AC) has been discussed previously (Graumann et al., 2002), we demonstrated for the first time that NQO1 mediated redox cycling with leucoaminochrome contributed to AC-induced toxicity. Since NQO1 was also found to increase the redox cycling and toxicity of MD, but not BQ, it appears that the reduction potential of a specific quinone/hydroquinone couple is tightly associated with the role of NQO1 in quinone-mediated toxicity.

Consistent with previous studies (Muñoz et al., 2012; Paris et al., 2011), we found AC activated autophagy. More intriguingly, we also found ubiquitin positive aggresome-like inclusion bodies in AC treated cells. Given autophagy and aggresome formation have been suggested as compensatory responses after proteasomal inhibition (Pandey et al., 2007; Rubinsztein, 2007; Xiong et al., 2013), and the potential importance of proteasomal inhibition in the biogenesis of protein aggregates in Parkinson's disease (Chung et al., 2003; McNaught et al., 2003; McNaught and Olanow, 2003; Olanow and McNaught, 2006), our

findings may provide a better understanding of accumulation of protein aggregates in Parkinson's disease associated with dopamine induced proteasomal inhibition.

In summary, our data have demonstrated (Fig. 8) that the nature and extent of protein handling changes were quinone-specific. AC induced changes in most of the major protein handling systems examined including proteasomal activity, the ER stress response, autophagy and formation of aggresome-like inclusion bodies. Both BQ and AC induced proteasomal inhibition that was closely associated temporally with induction of the ER stress response and autophagy. The effect of NQO1 on modulating protein handling changes and toxicity was also quinone-specific. While two-electron reduction of BQ protected against toxicity, the unstable hydroquinones generated from AC and MD underwent rapid autooxidation to produce ROS leading to an activated ER stress response and increased toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UPS	ubiquitin-proteasome system
HSPs	heat shock proteins
UPR	unfolded protein response
eIF2α	eukaryotic translation initiation factor 2 alpha
LC3	autophagy related microtubule-associated protein 1 light chain 3
NAD(P)H	quinone oxidoreductase 1 (NQO1)
BQ	1,4-benzoquinone
MD	menadione
AC	aminochrome

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Highlights

- 1,4-benzoquinone (BQ) and aminochrome (AC) inhibited proteasomal activity
- BQ and AC triggered the ER stress response and autophagy in dopaminergic N27 cells
- AC induced formation of aggresome-like inclusion bodies in N27 cells
- Only changes in the ER stress response correlated with changes in quinone toxicity
- Unstable hydroquinones contributed to quinone induced ER stress and toxicity for AC and menadione

Figure 1. Chemical structures of quinones utilized in this study

Figure 2. Inhibition of proteasomal activity by quinones

(A–C) The effect of quinones on purified 20S proteasomal activity. BQ and AC inhibited the chymotrypsin-like active site of purified human 20S proteasome in a dose-dependent manner after incubation for 30 min. MD had no effect on 20S proteasomal activity. DQ and DMNQ, the fully substituted analogs of BQ and MD respectively, did not inhibit 20S proteasomal activity. (D–F) The effect of quinones on 20/26S proteasomal activity in N27 cells. BQ and AC, but not MD, significantly inhibited the chymotrypsin-like active site of intracellular 20/26S proteasomal activity in N27 cells at 24h. These data are presented as

mean $\pm$ SD, (n=3); *p<0.05, **p<0.01 and ***p<0.001 are considered significant by one-way ANOVA using Dunnet's multiple comparison test.

Figure 3. BQ inhibited proteasomal activity and further activated ER stress responses and autophagy

(A) Growth inhibition in N27 cells upon treatment with BQ. Cells were incubated with increasing concentration of BQ (0–80 μ M) for 24h and cell viability was assessed by the MTT assay. (B) The inhibition of proteasomal activity by BQ (20 μ M) occurred at early time points in N27 cells. (C) The representative immunoblot blot shows that as early as 10min BQ induced accumulation of higher molecular weight polyubiquitinated proteins. (D) Time course of the ER stress response and autophagy following treatment with BQ. Treatment (24h) with tunicamycin (Tn, 3 μ M) and MG132 (1 μ M) were included as positive controls. β -actin was included as a loading control. Values in (A) and (B) are presented as mean $\pm$ SD, (n=3); **p<0.01 is considered significant by one-way ANOVA using Dunnet's post test.

Figure 4. Dopamine derived AC triggered ER stress responses, turnover of autophagic flux and formation of aggresome-like inclusion bodies in N27 cells

(A) Growth inhibition in N27 cells upon treatment with AC. Cells were incubated with increasing concentration of AC (0–80 μM) for 24h and cell viability was assessed using the MTT assay. (B) AC (80 μM) inhibited proteasomal activity in a time-dependent manner in N27 cells. Values in (A) and (B) are expressed as mean ± SD of 3 determinations. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are considered significant by one-way ANOVA using Dunnett's post test. (C) The representative immunoblot blot shows AC induced the time-dependent accumulation of polyubiquitinated proteins. (D) Time course of the ER stress response and

autophagy following treatment with AC (80 μ M). Treatment (24h) with tunicamycin (Tn, 3 μ M) and MG132 (1 μ M) were included as positive controls. β -actin was included as a loading control. Untreated cells in complete medium is labeled as C(S+), or in serum free medium as C(S-). (E) AC induced formation of ubiquitin positive aggresome-like inclusion bodies in N27 cells. After exposure to AC (80 μ M) for 24h, cells were fixed and immunostained for ubiquitin (red) and nuclei (DAPI, blue). Note that aggregates of ubiquitinated proteins are observed near the perinuclear region (white arrows, bottom left and right).

Figure 5. Overexpression of NQO1 in N27 cells (clone 4 cells) after stable transfection with wild type human NQO1

(A) NQO1 overexpression in clone 4 cells was confirmed by immunoblot analysis (rhNQO1, 35ng purified recombinant human NQO1 standard). (B) Cytosolic NQO1 enzymatic activity was measured in parental N27 and clone 4 cells as described in *Materials and Methods*. (C) Confocal analysis using immunostaining for NQO1 (red) in N27 cells (left) and clone 4 cells (right) confirmed cytosolic overexpression of NQO1 in clone 4 cells. Values in (B) are presented as mean $\pm$ SD, (n=3); ***p<0.001 are considered significant by student t-test.

Figure 6. Overexpression of NQO1 in N27 cells modulated the ER stress response to protect against BQ induced toxicity while potentiated toxicity induced by AC and MD

(A) Overexpression of NQO1 protected N27 cells against BQ induced apoptosis. (B, C) Clone 4 cells were more susceptible to AC (80 μ M) and MD (16 μ M) induced apoptosis. Apoptosis was measured in N27 and clone 4 cells after treatment with the indicated quinones for 24h using annexin V/PI cell staining in combination with flow cytometry. These data are presented as mean $\pm$ SD, (n=3); ***p< 0.001 are considered significant by one-way ANOVA using Tukey's multiple comparison test. (D–F) Time course of the ER stress response and the ER stress mediated apoptosis pathway following treatment with BQ (20 μ M), AC (80 μ M) and MD (16 μ M). Note that cleaved caspase-12 (C-Caps 12) was activated indicating that quinones can induce ER stress mediated apoptosis.

Figure 7. Overexpression of NQO1 increased AC and MD but not BQ, induced oxidative stress in clone 4 cells

(A) NQO1 overexpression increased the rate of oxygen consumption following treatments with AC or MD in N27 cells. Oxygen consumption was measured in N27 cells and clone 4 cells in the absence (control) or presence of the indicated quinones. Measurements were made over 10min using a Clark electrode at 37°C. Overexpression of NQO1 in clone 4 cells resulted in significantly increased oxygen consumption after treatment with either AC or MD suggesting unstable hydroquinones of AC or MD were generated and undergo autoxidation. These data are presented as mean $\pm$ SD, (n=3); *p<0.05 and ***p<0.001 are

considered significant by one-way ANOVA using Tukey's post test, # $p < 0.05$ is significant comparing to untreated control. (B) Quinone-induced oxidative stress was measured using CellROX Green[®] reagent in combination with confocal microscopy. Note the intense green staining in the nucleus of clone 4 cells compared to N27 cells after treatment with AC and MD respectively, suggesting NQO1 increased the level of oxidative stress induced by AC and MD.

Figure 8. Schematic representation of quinone-mediated protein handling changes and toxicity