Published in final edited form as:

Mol Cell Neurosci. 2018 January ; 86: 65–71. doi:10.1016/j.mcn.2017.11.014.

Peroxisomes contribute to oxidative stress in neurons during doxorubicin-based chemotherapy

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Abstract

Doxorubicin, a commonly used anti-neoplastic agent, causes severe neurotoxicity. Doxorubicin promotes thinning of the brain cortex and accelerates brain aging, leading to cognitive impairment. Oxidative stress induced by doxorubicin contributes to cellular damage. In addition to mitochondria, peroxisomes also generate reactive oxygen species (ROS) and promote cell senescence. Here, we investigated if doxorubicin affects peroxisomal homeostasis in neurons. We demonstrate that the number of peroxisomes is increased in doxorubicin-treated neurons and in the brains of mice which underwent doxorubicin-based chemotherapy. Pexophagy, the specific autophagy of peroxisomes, is downregulated in neurons, and peroxisomes produce more ROS. 2-hydroxypropyl- β -cyclodextrin (HP β CD), an activator of the transcription factor TFEB, which regulates expression of genes involved in autophagy and lysosome function, mitigates damage of pexophagy and decreases ROS production induced by doxorubicin. We conclude that peroxisome-associated oxidative stress induced by doxorubicin may contribute to neurotoxicity, cognitive

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Disclosure statement

The authors declare that there is no conflict of interest.

dysfunction, and accelerated brain aging in cancer patients and survivors. Peroxisomes might be a valuable new target for mitigating neuronal damage caused by chemotherapy drugs and for slowing down brain aging in general.

Keywords

Doxorubicin; Chemotherapy; Brain aging; Peroxisomes

1. Introduction

“Chemobrain” is a common side effect of cancer and its treatments. It is associated with multiple symptoms such as foggy thinking, memory problems, and a reduced capacity to process information (Wefel et al., 2015). Cognitive impairment appears in up to approximately 75% of people who undergo chemotherapy (Ahles et al., 2012; Schagen and Wefel, 2013). It can resolve after treatment ends, but often persists for decades or longer post-treatment. Anti-neoplastic agents such as doxorubicin, cisplatin, 5-fluorouracil, methotrexate, and other anti-neoplastic agents are known to trigger cognitive dysfunction (Soussain et al., 2009; Tannock et al., 2004; Vardy and Tannock, 2007). Even though there is a high frequency of cognitive impairment reported in patients and cancer survivors, there is a lack of effective treatments for cognitive dysfunction nearly all cancer types.

Research has shown that anti-cancer drugs or their metabolites may change or damage neuronal homeostasis, sometimes permanently, by numerous mechanisms. Anti-neoplastic agents may directly disturb synaptic structure by altering its biochemistry and, therefore, may affect neuronal activity (Ahles and Saykin, 2007; Chatton et al., 2001; Cole et al., 2013; Drachtman et al., 2002; Gozdz et al., 2003; Mayford et al., 2012; Mihara et al., 2011; Rzeski et al., 2004; Vijayanathan et al., 2011). Some other drugs damage mitochondria (e.g., paclitaxel and vincristine), leading to energy dyshomeostasis, production of reactive oxygen species (ROS), and oxidative stress (Broyl et al., 2010; Flatters and Bennett, 2006; Rzeski et al., 2004; Vijayanathan et al., 2011). Chemotherapy regimens based on the topoisomerase I poisons alter transcription in neurons (Mabb et al., 2016; Rossi et al., 2016). Overall, some or even all anti-cancer drugs may accelerate neuronal aging, leading to cognitive impairments and to increasing the risk for neurodegenerative diseases (Kesler, 2014; Kesler and Blayney, 2016; Kesler et al., 2015).

Doxorubicin is an anti-cancer agent that is commonly used to treat breast, esophageal, liver and other cancers (Tacar et al., 2013). Anti-neoplastic properties of doxorubicin include DNA damage, interference with transcription, mitotoxicity, the formation of free radicals, and lipid oxidation. Doxorubicin poorly crosses the blood-brain barrier; nevertheless, it still penetrates the brain at doses sufficient to cause cytotoxicity, leading to thinning of the brain cortex and reduced brain connectivity (Kesler, 2014; Kesler and Blayney, 2016; Kesler et al., 2015; Lepage et al., 2014; McDonald et al., 2010; Piccirillo et al., 2015; Stouten-Kemperman et al., 2015; Wefel et al., 2015). Patients treated with doxorubicin have elevated molecular markers of senescence, including p16INK4a and ARF (Sanoff et al., 2014). Doxorubicin induces abnormal activity, and damages DNA, synapses, and neurites in

neurons (Conroy et al., 2013; Liu et al., 2014; Moruno-Manchon et al., 2016). Neuronal autophagy is impaired in doxorubicin-treated neurons (Moruno-Manchon et al., 2016). Autophagosomes and damaged mitochondria accumulate in neurons exposed to doxorubicin. Liposomal doxorubicin induces a buildup of lipofuscin in the mouse brain. Lipofuscin, a hallmark of aged neurons, consists of oxidized proteins and lipids, and its accumulation indicates that neuronal cells exposed to doxorubicin underwent severe oxidative stress (Moruno-Manchon et al., 2016).

It is believed that the production of ROS associated with the exposure to doxorubicin largely takes place in mitochondria (Min et al., 2015). There is, however, another organelle, which is also the key in maintaining the cellular reduction-oxidation homeostasis – the peroxisome (Fransen et al., 2012; Trompier et al., 2014). Peroxisomes are vesicular organelles that participate in many vital metabolic processes. They oxidize fats and amino acids and synthesize lipids and, importantly, catabolize ROS produced during oxidation reactions. Ineffective removal of ROS by peroxisomes is linked to aging (Lizard et al., 2012; Zhou et al., 2012). Senescent peroxisomes also accumulate underoxidized metabolites that induce organelle dysfunction and, as a result, lead to cellular stress and dysfunction (Baes and Aubourg, 2009; Manivannan et al., 2012). The importance of peroxisomes is also illustrated by a number of metabolic diseases that affect multiple organs, with the brain always being affected (Wanders et al., 1995). Evidence suggests that enhancing peroxisomal clearance may slow down aging and age-associated diseases (Manivannan et al., 2012; Nordgren et al., 2013). Doxorubicin may induce oxidative stress in neurons, at least in part, by damaging peroxisomes or inhibiting pexophagy, the specific autophagy of peroxisomes; therefore, pharmacologically targeting peroxisomes during chemotherapy might be neuroprotective.

The current study investigated if peroxisomes contribute to oxidative stress in neurons treated with doxorubicin. In cultured primary neurons exposed to doxorubicin, we observed more peroxisomes, compared to control neurons. We also discovered that, in mice, pegylated liposomal doxorubicin induced accumulation of oxidized lipids and peroxisomes. Pexophagy is downregulated in neurons, which confirmed our previous study on autophagy (Moruno-Manchon et al., 2016), which is impaired in neuronal cells treated with doxorubicin. Peroxisomes produce more ROS in doxorubicin-treated neurons. Interestingly, 2-hydroxypropyl- β -cyclodextrin (HP β CD), an activator of the transcription factor TFEB, which regulates the expression of genes involved in autophagy and lysosome function, alleviates pexophagy impairment and decreases ROS production induced by doxorubicin. Taken together, this suggests that unhealthy peroxisomes are a part of a pathogenic pathway induced in neurons by doxorubicin, and that normalizing peroxisomal homeostasis in neurons might be an attractive therapeutic approach.

2. Materials and methods

2.1. Chemicals and plasmids

(7S, 9S)-7-[2R, 4S, 5S, 6S)-4-Amino-5-hydroxy-6-methyloxan-2-yl] oxy-6, 9, 11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8, 10-dihydro-7H-tetracene-5, 12-dione (doxorubicin) was from Selleckchem (#S1208). 2-hydroxypropyl- β -cyclodextrin (HP β CD) was from Cayman Chemical Company (#128446-35-5). Antibodies against ACAA1–3

ketoacyl-CoA thiolase were from Sigma (#HPA007244; 1:200). Alexa 546 secondary antibodies were from Life Technologies (#A11010; 1:200). pGW1-Hyper-per, tagged with a peroxisome targeting signal (PTS), was cloned from pHyper-cyto (Evrogen, #FP941). pGW1-GFP-per was cloned from pGW1-GFP. pGW1-Dendra2-per was cloned from pGW1-Dendra2, which was described (Moruno Manchon et al., 2015; Tsvetkov et al., 2013). pGW1-Hyper-per, pGW1-GFP-per and pGW1-Dendra2-per contain the “per” motif. The “per” motif is the type 1 PTS, which is commonly used to target a fluorescent protein to peroxisomes (Goedhart et al., 2012). pTagBFP was from Evrogen (#FP172). pGW1-GFP was described (Arrasate and Finkbeiner, 2005). pGW1-mApple was described (Moruno-Manchon et al., 2017b).

2.2. Cell cultures

Cortices from rat embryos (E19) were dissected, digested with papain (Worthington Biomedical, #9001-73-4), resuspended in Opti-MEM (Life Technologies, #31985070) with 20 mM D-glucose (Biomatik, #A2420), and plated on 24-well tissue-culture plates (600,000 cells/well) coated with poly-D-lysine (EMD Millipore, #A-003-E) as described (Moruno-Manchon et al., 2017a; Moruno-Manchon et al., 2017b). Primary neurons were maintained in Neurobasal Medium (Life Technologies, #21103-049) supplemented with B-27 (Life Technologies, #17504044), GlutaMAX (Thermo Scientific, #35050061), and penicillin-streptomycin (Sigma, #P4333).

2.3. Mice

Female mice (Ncr background; Taconic Farms), 4–6 months of age, were kept under pathogen-free conditions in facilities approved by the American Association for Laboratory Animal Care. Mice were in-traperitoneally injected with pegylated liposomal doxorubicin (Janssen Products, #NDC 59676-0960-01). Mice were injected weekly (4 mg/kg/week in 150 µl of sterile phosphate-buffered saline (PBS)) for 8 weeks. These conditions recapitulate a dose schedule used in human patients (Vail et al., 2004). 24 h after the last injection, two mice were anesthetized with isoflourane. The chest cavity was opened, and 20 ml of PBS were perfused into the left ventricle of the heart to purge the animal of blood. The animals were then perfused with 30 ml of 2.0% EM grade paraformaldehyde + 2.5% EM grade glutaraldehyde + 0.13 M sucrose in 0.1 M cacodylate buffer, pH 7.4.

2.4. Electron microscopy

The frontal cortices were dissected from the fixed mouse brains, minced into 1-mm cubes, and fixed for additional 2 days at 4 °C. After primary fixation, the tissues were washed in 0.1 M cacodylate buffer and post-fixed with 1% cacodylate-buffered osmium tetroxide and 0.8% potassium ferricyanide for 90 min. The samples were washed several times in water, then dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr's Low Viscosity embedding medium. The samples were polymerized in a 62 °C oven for 3 days. Ultrathin sections were cut at 60 nm on a Leica U7 ultramicrotome (Leica, Deerfield, IL), stained with saturated aqueous uranyl acetate and counterstained with Reynold's lead citrate. The sections were examined in a Hitachi H7500 transmission electron microscope using an accelerating voltage of 80 kV, and images were captured via an AMT XR-16 digital camera and AMT Image Capture, v602.600.51 software.

2.5. Fluorescence microscopy and image analysis

Neurons were imaged in automated fashion with the EVOS microscopy system (Life Technologies). The plate was placed on the EVOS microscope stage. The microscope automatically positions the 20 × objective to the center of the first well of the 24-well tissue plate and collects fluorescence images with the RFP filter and the GFP filter. Thereafter, the microscope moves the stage to each adjacent field in the well until a representative image is obtained from each well.

To measure pexophagy, neuronal cultures 4–5 DIV were transfected with pGW1-Dendra2-per and pGW1-GFP (a marker of morphology). Photoswitching of Dendra2-per was performed as described (Moruno Manchon et al., 2015; Tsvetkov et al., 2013). The decays of photoswitched Dendra2 fluorescence were measured by measuring “red” fluorescence intensity over a region of interest corresponding to the neuronal soma and major neurites (fluorescence of GFP “green” molecules served as a guide for drawing the region of interest). The intensities were plotted against time and transformed into log values (Moruno Manchon et al., 2015; Tsvetkov et al., 2013).

To measure peroxisome-associated ROS, neuronal cultures 4–5 DIV were transfected with pGW1-Hyper-per and pGW1-mApple (a marker of morphology). The Hyper protein is a genetically encoded fluorescent sensor, which was developed by inserting a yellow fluorescent protein into the regulatory domain of *E. coli* protein OxyR (OxyR-RD) (Belousov et al., 2006; Choi et al., 2001). After HyPer is synthesized, it has two excitation peaks with maxima at 420 nm and 500 nm, and one emission peak with maximum at 520 nm. In an H₂O₂ environment, Hyper’s emission peak at 520 increases by up to 50% (Belousov et al., 2006). Fluorescence changes were measured by measuring a change in fluorescence intensity over a region of interest corresponding to the neuronal soma and major neurites (fluorescence of mApple “red” molecules served as a guide for drawing the region of interest).

Colocalization of GFP-per and thiolase fluorescence signals was measured with the Coloc_2 plugin form ImageJ/Fiji.

2.6. Ethics statement

Rats and mice were maintained in accordance with guidelines and regulations of the University of Texas, Houston, and the MD Anderson Cancer Center Institutional Animal Care and Use Committee (the protocol numbers #AWC-13–122, #AWC-16–0081, and #00001029-RN01). All experimental protocols were approved by the University of Texas McGovern Medical School at Houston, and by MD Anderson Cancer Center. The methods were carried out in accordance with the approved guidelines.

3. Results

3.1. Doxorubicin promotes accumulation of lipids and peroxisomes in cultured primary neurons and in mouse brains

Early studies demonstrated that doxorubicin induced lipid peroxidation and increased the number of peroxisomes in the heart of mice and rats (Myers et al., 1977; Zipper, 1997). We recently demonstrated that, in cultured primary neurons, doxorubicin promoted the accumulation of lipid droplets, which was not observed in control non-treated neurons (Moruno-Manchon et al., 2016). In mice which underwent a course of chemotherapy with liposomal doxorubicin, we also detected the accumulation of lipid droplets and lipofuscin, a mixture of oxidized lipids and proteins (Moruno-Manchon et al., 2016). Here, we investigated if doxorubicin has any effect on peroxisomes in cultured primary neurons and in mouse brains.

Primary cultured cortical neurons were treated either with a vehicle or with doxorubicin, and analyzed with electron microscopy. Neurons exposed to doxorubicin exhibited a clear lipid dyshomeostasis (Fig. 1A). Interestingly, neurons treated with the drug contained more peroxisomes, often in clusters, than control neurons (Fig. 1A, B). These data demonstrate that, similarly to cardiac muscle cells, doxorubicin unbalances lipid and peroxisomal homeostasis in neurons, possibly thereby contributing to cytotoxicity.

Next, mice were injected with pegylated liposomal doxorubicin, with a dose schedule used in human patients (Vail et al., 2004). After the last injection, mice were perfused, and their brains were analyzed by electron microscopy. We observed the accumulation of oxidized lipids, found in aged brains (Gilissen and Staneva-Dobrovski, 2013), demonstrating that the lipid metabolizing pathways are impaired by doxorubicin (Fig. 1C). Peroxisomes were also frequent and located near the clusters of lipid droplets (Fig. 1C), which suggests that the accumulation of lipids may be a result of dysfunctional peroxisomes (Lodhi and Semenkovich, 2014).

3.2. Doxorubicin impairs pexophagy, the specific autophagy of peroxisomes, in neurons

We demonstrated that neurons exposed to doxorubicin contained more peroxisomes. Since we recently showed that autophagy is impaired in doxorubicin-treated neurons (Moruno-Manchon et al., 2016), accumulating peroxisomes likely mean that their degradation is compromised. To confirm this hypothesis, we analyzed pexophagy in control neurons and in neurons treated with doxorubicin.

First, to target peroxisomes in neurons, we fuse GFP with a specific sequence of peroxisomal proteins, known as the “per” motif. Many of matrix proteins present in peroxisomes contain a C-terminal tripeptide, the type 1 PTS, or “per” motif (Agrawal et al., 2017; Effelsberg et al., 2016; Hofhuis et al., 2016; Wang et al., 2017). To characterize the “per” motif as a marker of peroxisomes, primary cortical neurons were transfected with GFP-per and TagBFP (a morphology marker). Neurons were fixed, permeabilized and stained with antibodies against ACAA1–3-ketoacyl-CoA thiolase, a peroxisomal enzyme. Colocalization of GFP-per and thiolase fluorescence signals indicates that the “per” motif targets peroxisomes in neurons (Fig. 2A, B).

Then, to measure the turnover of peroxisomes in live neurons, we used an optical pulse-chase method that combines a photoswitchable protein Dendra2 with automated imaging and longitudinal analysis. Irradiation with short-waved visible light causes this protein to undergo an irreversible conformational change and emit red fluorescence that can be tracked until the photoswitched molecules are cleared (Skibinski et al., 2017; Tsvetkov et al., 2013). Dendra2 was tagged with the “per” motif, Dendra2-per, and used to determine the half-life of the peroxisomal pool in neurons. Primary cultured cortical neurons were transfected with GFP (a morphology marker) and Dendra2-per, and photoswitched. Cells were then treated either with a vehicle or with doxorubicin, and tracked longitudinally (Fig. 2C, D). We discovered that neurons treated with a vehicle had the half-life of the peroxisomal pool being ~ 3 days, which is longer than the half-life of the peroxisomal pool in cell lines and the liver (1.5–2.2 days) (Fransen, 2014; Huybrechts et al., 2009; Leighton et al., 1969; Poole et al., 1969; Price et al., 1962). Neurons exposed to doxorubicin had the half-life of the photoswitched Dendra2-per fluorescence increased, indicating that pexophagy is impaired by doxorubicin. Taken together with our previous findings on downregulated autophagy in doxorubicin-treated neurons, our data demonstrate that neurons exposed to doxorubicin experience a systemic failure of the autophagic pathways, which results in neurotoxicity.

3.3. Peroxisomes generate ROS in neurons exposed to doxorubicin

The accumulation of oxidized lipids suggests that neurons endure substantial oxidative stress, which may at least partially come from ROS-producing dysfunctional or senescent peroxisomes, normally removed by pexophagy (Dunn et al., 2005). To investigate if peroxisomes produce more ROS, we cloned a fluorescent H₂O₂ sensor targeted to peroxisomes (Elsner et al., 2011). The Hyper (hydrogen peroxide) protein is a green fluorescent protein, which can be oxidized by H₂O₂, leading to a conformational and spectral change (Belousov et al., 2006). Cortical neurons were transfected with Hyper-per and with mApple (a morphology marker) and either treated with a vehicle or doxorubicin, and analyzed (Fig. 3A, B). Expectedly, based on changes in the fluorescent properties of Hyper-per, we discovered that peroxisomes produced more H₂O₂ in neurons treated with doxorubicin than in control neurons. We, therefore, concluded that doxorubicin promotes oxidative stress in neurons at least in part by reducing peroxisomal health and enhancing secretion of oxidants from peroxisomes.

3.4. 2-Hydroxypropyl- β -cyclodextrin mitigates the impairment of pexophagy and reduces ROS

2-Hydroxypropyl- β -cyclodextrin (HP β CD) is being actively investigated as a treatment for a cholesterol storage disorder, Niemann-Pick type C disease, which is characterized by the accumulation of cholesterol and glycolipids in lysosomes. HP β CD lowers cholesterol and activates TFEB and, as a result, promotes cytoprotection (Coisne et al., 2016; Moruno-Manchon et al., 2016). Intriguingly, HP β CD or its chemical relatives appear to be neuroprotective in models of neurodegeneration including Alzheimer's, Parkinson's, and Huntington's diseases (Del Toro et al., 2010; Kilpatrick et al., 2015; Yang et al., 2017; Yao et al., 2012). Methyl- β -cyclodextrin complexed with doxorubicin has been used in a mouse model of cancer (Mohammad et al., 2015). Tumor growth was reduced and survival

increased in mice treated with the methyl- β -cyclodextrin/doxorubicin complex, indicating that methyl- β -cyclodextrin does not protect cancer cells from doxorubicin.

We previously demonstrated that HP β CD mitigates damage of autophagy induced by doxorubicin, reduces the accumulation of lipid droplets, and increases neuronal survival (Moruno-Manchon et al., 2016). Here, we hypothesized that HP β CD may alleviate the impairment of neuronal pexophagy. An optical pulse-chase experiment was performed on primary cultured neurons treated with a vehicle, doxorubicin alone or co-treated with doxorubicin and HP β CD. HP β CD lowered the half-life of photoswitched Dendra2-per affected by doxorubicin, demonstrating that the clearance of peroxisomes is improved (Fig. 4A).

Since HP β CD improved pexophagy, we investigated if the levels of ROS are reduced in neurons co-treated with doxorubicin and HP β CD. Cultured primary neurons were transfected with Hyper-per and with mApple, then treated with a vehicle or with doxorubicin or with a combination of doxorubicin and HP β CD. As anticipated, neurons co-treated with HP β CD and doxorubicin exhibited less peroxisome-associated ROS production than neurons exposed to doxorubicin alone (Fig. 4B). Our data demonstrate that HP β CD improves pathology associated with doxorubicin treatment. Taken together, peroxisomes and pexophagy, therefore, might represent a novel target for mitigating neuronal damage caused by doxorubicin.

4. Discussion

In this study, we investigated if neuronal peroxisomes are affected by doxorubicin, a commonly used chemotherapy drug. We discovered that primary cultured neurons and brain samples from mice treated with doxorubicin contain lipid droplets and more peroxisomes, often in clusters. Pexophagy, the specific autophagy of peroxisomes, is downregulated in doxorubicin-treated neurons. These neurons exhibit elevated levels of ROS associated with peroxisomes. HP β CD, an activator of TFEB, a transcription factor that regulates autophagy and lysosome genes, alleviates pexophagic damage and decreases the levels of peroxisome-associated ROS. Our data suggest that peroxisomes and pexophagy may be targets for neuroprotective therapy development for cancer patients and survivors treated with doxorubicin-based chemotherapy.

Peroxisomes are present in all mammalian cell types excluding red blood cells. Peroxisomes metabolize various fatty acids via β -oxidation, degrade D-amino acids, and synthesize lipids. Many of these metabolic reactions result in the generation of ROS by-products. The significance of peroxisomes in neuronal maintenance and survival is demonstrated by a number of peroxisomal disorders, in which the brain always exhibits dramatic pathologic changes (Berger et al., 2016; Trompier et al., 2014). Only recently, peroxisomes have been recognized as important contributors to aging and age-associated neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Kou et al., 2011; Lizard et al., 2012; Trompier et al., 2014; Yakunin et al., 2010). Here, we demonstrate that peroxisomes are altered in neurons exposed to doxorubicin and abnormally generate ROS. Therefore, our

data have widespread ramifications for understanding how anti-neoplastic drugs affect the brain and for designing novel chemotherapeutic agents and neuroprotective strategies.

Chemotherapy kills cancerous cells by multiple mechanisms, one of which is the generation of ROS. Is lowering ROS to protect the brain, therefore, an attractive therapeutic strategy? If the tumor itself is not located in the brain, then we believe so (Poljsak, 2011). Due to higher sensitivity of neurons to toxic agents compared with other tissue cells, research should focus on cytoprotective, neuron-specific anti-ROS pathways. Our current study is the first to demonstrate that neuronal ROS can be targeted and is a proof of concept. Our future studies will investigate what neuronal anti-oxidant pathways should be targeted.

Exposure to pesticides, herbicides, heavy metals and certain organic solvents dramatically increases the risk of developing Parkinson's disease (de Lau and Breteler, 2006). These chemicals primarily induce neurodegeneration of dopamine-producing neurons in the substantia nigra by interfering with the electron transport chain in mitochondria and generating ROS. Doxorubicin also inhibits the mitochondrial electron transport chain and induces oxidative stress, but its side effects do not include a higher risk for developing PD (de Oliveira and Niederer, 2016). Although it does not actively cross the blood-brain barrier, doxorubicin treatment is associated with greater disruption of brain connectivity and cognitive performance in patients. Doxorubicin induces greater reduction of cerebral metabolism in rats compared to chemotherapies that do actively cross the blood-brain barrier (Kesler and Blayney, 2016; Lim et al., 2016). Patients treated with doxorubicin also develop peripheral neuropathy affecting sensation and controlled movement (Anderson et al., 2015; Bandos et al., 2018). These injuries to the brain and peripheral nervous system may make the brain less resilient to the effects of aging (Kesler et al., 2015). In addition, patients treated with doxorubicin had elevated molecular markers of senescence such as p16INK4a and ARF in blood cells (Sanoff et al., 2014). Doxorubicin is highly toxic for the heart, causing cardiomyopathy in a dose-dependent manner, which is usually age-associated (Bristow, 1982; Gewirtz, 1999; Sishi et al., 2013; Tacar et al., 2013; Wallace, 2003). We recently demonstrated that doxorubicin ages the brain of mice exposed to doxorubicin. However, unlike the toxins that cause dopaminergic neurotoxicity and PD, doxorubicin appears to accelerate aging in general, impairing cognitive function, and the cardiovascular system as a few examples (Bielak-Zmijewska et al., 2014; Salas-Ramirez et al., 2015). A rotenone-based model of PD has been used for more than a decade (Johnson and Bobrovskaya, 2015); our studies suggest that doxorubicin can be used as a chemical model for accelerating aging.

In summary, our findings provide evidence that peroxisomes may play an important role in inducing oxidative stress in neurons during chemotherapy and underscore the importance of peroxisomal homeostasis in neuroprotection. Future studies in mice will determine if targeting peroxisomes alleviates neurotoxicity associated with doxorubicin-based chemotherapy treatment.

Acknowledgements

This work was supported by the University of Texas, McGovern Medical School at Houston, the Department of Neurobiology and Anatomy. This project was also supported by the Integrated Microscopy Core at Baylor College

of Medicine with funding from the National Institutes of Health (HD007495, DK56338, CA109298, and CA125123), the Dan L. Duncan Cancer Center, and the John S. Dunn Gulf Coast Consortium for Chemical Genomics, the Blanton-Davis Ovarian Cancer Research Program, and The Frank McGraw Memorial Chair in Cancer Research, the American Cancer Society Research Professor Award, and a grant from the National Institutes of Health (1R01NR014195 to JSW/SRK). Raquel Cornell, Sharon Gordon, Summer Hensley, and Martha Belmares provided administrative assistance.

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Fig. 1.

Doxorubicin induces accumulation of peroxisomes and lipid droplets in neurons and the brain of mice. (A) Electron micrographs of cultured cortical neurons treated with a vehicle (control, left panel) or 50 nM doxorubicin (Dox, right panel) overnight. M, mitochondria; L, lipid droplet; P, peroxisome. Bar, 250 nm. (B) Quantification of the area of peroxisomes relative to the area of cytoplasm in cultured neurons treated with a vehicle (control, cont) or 50nM doxorubicin (Dox) overnight. $^{**}p < 0.001$ (*t*-test). Data were pooled from twenty micrographs per group. (C) Electron micrographs of the frontal cortex of mouse brain exposed to pegylated liposomal doxorubicin. M, mitochondria; L, lipid droplet; Lp, lipofuscin; P, peroxisome. Bar, 250 nm.

Fig. 2.

Neuronal pexophagy is downregulated by doxorubicin. (A) The “per” motif targets peroxisomes in neurons. Primary cortical neurons were transfected with TagBFP (BFP) and GFP-per, which contains the type 1 peroxisomal targeting sequence fused with GFP. Neurons were fixed and immunostained with antibodies against the peroxisomal enzyme thiolase. The merged image shows that GFP-per and thiolase colocalize. White arrows depict peroxisomes. Bar, 5 μ m. (B) The colocalization algorithms were run as the GFP-per signal against the thiolase signal (GFP-per vs thiolase), and the thiolase signal against the GFP-per (thiolase vs GFP-per). (C) Photoswitchable protein Dendra2 targeted to peroxisomes (Dendra2-per) as a surrogate for peroxisome turnover. Brief irradiation with short-wave-length visible light causes Dendra2 to undergo an irreversible conformational change and emit red fluorescence that can be tracked until targeted organelles (peroxisomes) are cleared. Image represents optical pulse-chase of a primary neuron expressing GFP (a morphology marker) and Dendra2 targeted to peroxisomes imaged with an automated microscope. Note the decay of red fluorescence over time. Bar, 10 μ m. (D) The half-life of “red” Dendra2-per from (C). Cortical primary neurons were transfected with GFP (a morphology marker) and Dendra2-per and treated with a vehicle (control, cont) or 10 nM doxorubicin (Dox). The change in red fluorescence intensity over time was used to calculate the half-life of “red” Dendra2-per in control and Dox-treated neurons. ** $p < 0.001$ (t -test). Data were pooled from three independent experiments.

Fig. 3.

Doxorubicin induces peroxisome-associated oxidative stress in neurons. (A) Hyper-per is used as a reporter of oxidative stress associated with peroxisomes. Primary cortical neurons were transfected with mApple (a morphology marker) and Hyper-per, and treated with a vehicle (control) or 50 nM doxorubicin (Dox) overnight. Neurons were then imaged. The fold change of the Hyper-per fluorescence intensity was calculated by dividing the green fluorescence intensity after the treatment by the green fluorescence intensity before the treatment. Bar, 10 μ m. (B) Fold change of Hyper-per fluorescence intensity from (A). Primary cortical neurons were transfected with mApple and Hyper-per, and imaged after 24 h. Neurons were treated with a vehicle (control, cont) or 50 nM doxorubicin (Dox) overnight. *** $p < 0.0001$ (t -test). Data were pooled from three independent experiments.

Fig. 4.

HPβCD mitigates damage of pexophagy and reduces ROS. (A) Cortical primary neurons were transfected with GFP (a morphology marker) and Dendra2-per and treated with a vehicle (control, cont), or with 50 nM doxorubicin alone (Dox) or in combination with 0.5 mM HPβCD. The decay of red fluorescence intensity over time was used to calculate the half-life of “red” Dendra2-per in neurons. ** $p < 0.001$, * $p < 0.05$ (t -test). Data were pooled from three independent experiments. (B) Fold change of the Hyper-per fluorescence intensity. Primary cortical neurons were transfected with mApple and Hyper-per, and imaged 24 h thereafter. Neurons were treated with a vehicle (control, cont), or with 50 nM doxorubicin alone (Dox) or in combination with 0.5 mM HPβCD overnight. Neurons were then imaged again. The fold change of the Hyper-per fluorescence intensity was calculated by dividing the green fluorescence intensity after the treatment by the green fluorescence intensity before the treatments. *** $p < 0.0001$, ** $p < 0.001$ (t -test). Data were pooled from three independent experiments.