Mefloquine induces oxidative stress and neurodegeneration in primary rat cortical neurons

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A B S T R A C T

Mefloquine is an effective antimalarial that can cause adverse neurological events including headache, nausea, fatigue, insomnia, anxiety and depression. In this study, we examined the oxidative stress response in primary rat cortical neurons treated with mefloquine by quantifying oxidative stress markers glutathione (GSH) and F2-isoprostanes (F2-isoPs). Furthermore, we examined whether mefloquine induces synaptodendritic degeneration of primary rat cortical neurons. GSH was quantified in cortical neurons after 24-h treatment with mefloquine (0, 1, 5, 10 μM) using monochlorobimane. F2-isoPs were quantified in cortical neurons after 24-h treatment with mefloquine (0, 1, 5, 10 μM) using a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring. The concentration dependent decrease in GSH and the concomitant increase of F2-isoPs indicates the presence of oxidative stress in primary rat cortical neurons treated with mefloquine. Following a 24-h treatment with mefloquine, primary rat cortical neurons (0, 5, 10 μM) were fixed with 4% paraformaldehyde. Images from eight optical sections covering a distance of 2.88 μm on the z-axis were acquired using a confocal laser scanning unit. Traced images were analyzed with NeuroExplorer, a neurophysiological data analysis package. Mefloquine induces a concentration dependent decrease in the number of spines per neuron and the spine density, suggesting that mefloquine induced oxidative stress may be associated with the synaptodendritic degeneration. Together with previous work, there is strong evidence that a relationship exists between calcium homeostasis disruption, ER stress response, the oxidative stress response, and neurodegeneration. Understanding how oxidative stress alters the morphology of cortical neurons treated with mefloquine will provide further insight into the mechanism(s) related to clinically observed adverse neurological events.

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1. Introduction

Mefloquine (Lariam®) is a potent antimalarial effective against drug resistant Plasmodium falciparum. However, it is known to cause adverse neurological events during both prophylaxis and treatment. Mild symptoms include headache, nausea, fatigue, insomnia, anxiety and depression, while more severe events may include hallucinations and psychosis (Toovey, 2009). The observed clinical frequency of adverse incidents is known to be in direct proportion to dose, as prophylactic doses produce mild events in up to 25% of patients, increasing to nearly 90% at the treatment dose (Dow et al., 2005). Several studies have sought to determine the underlying mechanism leading to the adverse neurological events by examining the role of mefloquine in calcium homeostasis disruption, P-glycoprotein inhibition, cholinesterase inhibition, connexin blockage, adenosine 2A inhibition and ion channel disruption (Toovey, 2009). Still, no concrete connection exists between clinically observed adverse events and neuronal response to mefloquine.

While an oxidative stress response has been shown in cortical neurons and astrocytes treated with artemisinin derivatives (Schmuck et al., 2002), the role of oxidative stress in neurons treated with mefloquine has not yet been examined. Oxidative stress in neurons has been associated with insomnia (Sudati et al., 2009), anxiety, depression (Masood et al., 2008) and psychosis (Tsualchidu et al., 2008), which correspond to many of the clinically observed adverse events associated with mefloquine. Hence, identifying oxidative stress response in neurons treated...
with mefloquine could lead to potential therapeutic strategies to mitigate the adverse neurological events associated with mefloquine.

Oxidative stress is caused by increased production of free radicals from reactive oxygen that damage various components of the cell, including proteins, lipids and DNA, when not controlled by cellular antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase. Glutathione (GSH), a critical cellular antioxidant, is a common molecule measured to quantify oxidative stress in cells. At present, it is widely accepted that GSH acts not only as a cellular antioxidant, but also as a mediator of many other physiological reactions including metabolism of xenobiotics, thiol disulfide exchange reactions and cellular signaling (cell-cycle regulation, proliferation and apoptosis) (Mari et al., 2009). Levels of GSH can be determined by using monochlorobimane, a nonfluorescent cell membrane permeable dye that forms a fluorescent adduct with GSH in a reaction catalyzed by glutathione S-transferase (Neeley et al., 2005; Rice et al., 1986). In addition, lipid peroxidation products, such as F_2-isoprostanes (F_2-isopPs), can be measured to quantify cellular oxidative stress. F_2-isopPs are derived by the free radical peroxidation of arachidonic acid. Our methodology to measure F_2-isopPs using stable isotope dilution, negative ion chemical ionization chromatography/mass spectrometry with select ion monitoring (Milatovic and Ascher, 2009) allows the lower limit of detection to be in the picogram range. Therefore, F_2-isopPs can function as a sensitive and specific marker of oxidative stress in vivo.

Oxidative stress has been associated with alterations in neuronal morphology. Increased generation of markers of oxidative damage are seen in several models of synaptodendritic degeneration including innate immunity activation (Milatovic et al., 2003, 2004), excitotoxicity generated by kainic acid (KA) (Zaja-Milatovic et al., 2008), as well as neurotoxicities associated with manganese exposure (Zaja-Milatovic et al., 2009) and anticholinesterase agents (Gupta et al., 2007; Milatovic et al., 2009).

In this study, we examine the oxidative stress response in primary rat cortical neurons treated with mefloquine by quantifying GSH and F_2-isopPs. Furthermore, we examined whether mefloquine treatment induces synaptodendritic degeneration of primary rat cortical neurons. Quantification of oxidative stress and altered morphology in cortical neurons treated with mefloquine will provide further insight into the mechanism(s) related to clinically observed adverse neurological events.

2. Materials and methods

2.1. Materials

Mefloquine hydrochloride was purchased from Sigma (St. Louis, MO), as well as all other reagents, unless otherwise stated. Glass coverslips for cell culture were purchased from Carolina Biological Supply (Burlington, NC). All culture media and supplements were purchased from Invitrogen (Carlsbad, CA), except for Hyclone heat-inactivated fetal bovine serum, which were purchased from VWR (Suwanee, GA).

2.2. Primary rat cortical culture

All experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and were performed according to the Guidelines for Animal Experimentation as enunciated by Vanderbilt University. Primary rat cortical neuron cultures were prepared from rats as previously described (McLaughlin et al., 1998). Briefly, the brains of embryonic day 17 Harlan Sprague-Dawley rat embryos were removed and placed in a Petri dish filled with cold Hank’s balanced salt solution (HBSS). The cortices were dissected from the brain and transferred to another Petri dish containing HBSS, in order to remove blood vessels and meninges. The isolated cortices were then transferred to a Petri dish containing 0.6% (w/v) trypsin in HBSS for 30 min. The isolated cortices were rinsed twice in HBSS and dissociated mechanically with a glass Pasteur pipette. Dissociated cortical cells were plated onto poly-L-ornithine-coated glass coverslips in 6 well plates using a medium of glutamine free Dulbecco’s Modified Eagle Medium (DMEM)-Eagle’s salts (Invitrogen, Carlsbad, CA), supplemented with Ham’s F12 (VWR, Suwanee, GA), 10% heat-inactivated fetal bovine serum (FBS) (VWR, Suwanee, GA), and 200 IU/ml penicillin/streptomycin (Sigma, St. Louis, MO), at a density of 700,000 cells/well. After 2 days in vitro, non-neuronal cell division was halted by a 24-h exposure to 10 μM cytosine arabinoside (Sigma, St. Louis, MO). Cortical cultures were transferred to neurobasal media (Invitrogen, Carlsbad, CA), supplemented with B27 (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Sigma, St. Louis, MO). Cortical cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cortical cell culture media was changed every 2–3 days.

2.3. Cortical cell culture treatment with mefloquine

Cells were treated with mefloquine 10 days after isolation, prepared from a 100 mM stock solution in dissolved dimethyl sulfoxide (DMSO) with treatment buffer, for 24 h, at 37 °C in a humidified atmosphere of 5% CO_2 in air. Treatment buffer consisted of minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2× N2 media supplement (Invitrogen, Carlsbad, CA).

2.4. Cortical neuron viability

Control and treated cortical neuron viability was quantified by fluorescence activated cell sorting using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Eugene, OR) following 24-h treatment with mefloquine. The assay is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability—intracellular esterase activity and plasma membrane integrity. Following addition of 2 μl of 50 μM calcein AM working solution and 4 μl of the 2 mM ethidium homodimer-1 stock to each milliliter of cells (0.1–5 x 10^5 cells/ml) and 20 min incubation at room temperature, the stained cells were analyzed by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein (i.e., 530/30 bandpass) and red fluorescence emission for ethidium homodimer-1 (i.e., 610/20 bandpass).

Cell viability was also assessed by measurement of formazan production after the addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO). The number of surviving cells following 24-h treatment with mefloquine (0, 1, 5, 10, 50, 100 μM) was determined by measuring the optical density (OD) of the dissolved formazan product at A_590 nm after the addition of MTT for 1 h according to the manufacturer’s instructions. Untreated negative controls were run together with the treated cells, and plates with reagent only served as background controls. After background OD subtraction, the results were expressed as a percentage of the average negative control. Each experiment was performed in triplicate plates and repeated three times in cultured neurons.

2.5. Cortical neuron cytotoxicity

Cytotoxicity, as indicated by cell membrane integrity, was assessed by measuring the activity of lactate dehydrogenase (LDH)
in the culture media by colorimetric detection of formazan, using a LDH diagnostic kit (Promega, Madison, WI, USA). Following 24-h treatment with mefloquine (0, 1, 5, 10, 50, 100 μM), the supernatant was transferred to a 96 well plate and incubated with the reaction mixture for 30 min at room temperature to develop color. The optical density was measured at 492 nm using a spectrophotometer (MRX, Dynatech Laboratories). Each experiment was performed in triplicate and repeated three times in different groups of neuronal cultures.

2.6. Glutathione response

GSH, a major antioxidant, was quantified in cortical neurons after 24-h treatment with mefloquine (0, 1, 5, 10 μM) using monochlorobimane (Molecular Probes, Eugene, OR), a fluorescent probe for GSH (Neely, Boutte, 2005). Cortical neurons were plated in culture medium in round bottom 96 well plates and treated with mefloquine. Following mefloquine treatment, the cortical neurons were rinsed twice with HBSS and treated with 40 μM mono-chlorobimane for 15 min in the incubator. Fluorescence was quantified with a fluorescence microplate reader.

2.7. F$_2$-isoPs production

F$_2$-isoPs, a product of arachidonic acid peroxidation, were quantified in cortical neurons after 24-h treatment with mefloquine (0, 1, 5, 10 μM) using a stable isotope dilution method with detection by gas chromatography/mass spectrometry and selective ion monitoring as previously described (Milatovic et al., 2009; Morrow and Roberts, 1991; Roberts and Morrow, 1994). Following mefloquine treatment, cortical neurons were sonicated, lipids chemically hydrolyzed using KOH and a stable isotope, 8-isoP$_{-}$d$_4$ internal standard added. Following extraction using C-18 and silica Sep-Pac cartridges, purification by thin layer chromatography, and conversion to O-methylxime pentafluorobenzyl ester trimethylsilyl derivatives, the compound was dissolved in undecane that is dried over a bed of calcium hydride. Negative ion chemical ionization mass spectrometry was performed with Hewlett-Packard HP5890A and Agilent 5973 instruments interfaced with monitoring ions for F$_2$-isoPs (m/z 569) and the internal standard (m/z 573). The ion source temperature was 250 ℃ and the electron energy was 70 eV.

2.8. Morphological changes in dendritic spines in cortical neurons

Following a 24-h treatment with mefloquine, cortical neurons (0, 5, 10 μM) were fixed with 4% paraformaldehyde and rinsed extensively in phosphate buffered saline (PBS). Cortical neurons were exposed for 2 h at room temperature to the drebrin (M2F6) mouse monoclonal antibody (Abcam Inc., Cambridge, MA) at a concentration of 1:200. Following the PBS rinses, coverslips were incubated for 1 h at room temperature with the secondary antibody, Cy2 (donkey anti-mouse, 1:100, in 1% normal donkey serum). Cy2 conjugates are excited at 492 nm and fluoresce (510 nm) in the green region of the visible spectrum. Coverslips rinsed with PBS were put on slides and mounted with anti-fade solution.

Images from eight optical sections covering a distance of 2.88 μm on the z-axis were acquired using a confocal laser scanning unit at an excitation wavelength of 482 nm. Each optical section was obtained by averaging four frames at a spatial sampling of 116 nm/pixel (512 × 512 pixels) through a 63× oil immersion objective. Confocal images of neurons were then transferred in Neurolucida v6.0 (MicroBrightField Inc., Williston, VT), a fully integrated software that enables control of microscopic hardware used for performing neuron tracing. Traced images were analyzed with NeuroExplorer (MicroBrightField Inc., Williston, VT), a neurophysiological data analysis package.

2.9. Data analysis

Measurements of MTT, LDH, GSH and F$_2$-isoPs, as well as experiments with neuronal morphology were conducted in duplicate or triplicate wells/experiment, and the mean from three to four independent experiments was used for statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons with statistical significance of p < 0.05. All analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Viability of primary rat cortical neurons treated with mefloquine

The effect of mefloquine on the viability of primary rat cortical neurons was investigated in order to determine concentrations adequate to elicit an oxidative stress response in cortical neuron cultures, but not to elicit appreciable cell death. As is illustrated in Fig. 1A, mefloquine causes a concentration dependent decrease in primary rat cortical neuron viability after 24-h treatment, with a LC$_{50}$ of 8.9 μM, which was consistent with MTT and LDH assay results (Fig. 1B and C). This result correlates well with that of previous experiments on NG108-15 (mouse neuroblastoma × rat glioma hybrid) cells (Dow, 2003) and primary neuronal cultures of fetal rat forebrains (Dow et al., 2003), where an LD$_{50}$ of 12 and 6.6 μM, respectively, were obtained.

3.2. Oxidative stress in cortical neurons treated with mefloquine

To investigate the presence of oxidative stress in cortical neurons treated with mefloquine, oxidative stress markers, GSH and F$_2$-isoPs, were quantified. Mefloquine treatment of cortical neurons is associated with a concentration dependent decrease in GSH, with an approximate 40% decrease near the LC$_{50}$ (Fig. 2A). At the same time, primary cortical neurons exposed to 10 μM of mefloquine exhibited a 40% increase in F$_2$-isoPs (Fig. 2B). F$_2$-isoP formation occurs in situ in the phospholipid bilayer and then subsequently released in free form. This creates two forms of F$_2$-isoPs, one that remains esterified in the membrane and a second that is hydrolyzed and released in free form. Since we have used base hydrolysis and thus quantified total F$_2$-isoPs formation, both free and esterified F$_2$-isoPs, exposure to even low concentrations of mefloquine (1 and 5 μM) induced significant increase in these markers of oxidative stress in primary rat cortical neurons (Fig. 2B). The concentration dependent decrease in GSH and the concomitant increase of F$_2$-isoPs, even at low mefloquine concentration, indicates the presence of significant (p < 0.05) oxidative stress in cortical neurons treated with mefloquine. Surprisingly, we noted a ceiling effect on mefloquine induced F$_2$-isoP formation in the presence of concentration dependent reduction in GSH by mefloquine. This suggests that other antioxidants may protect the neuron from oxidative stress induced by mefloquine.

3.3. Morphological structure of cortical neurons treated with mefloquine

To determine the effects of oxidative stress on the structure of cortical neurons, dendritic spines and dendritic spine density (spines/100 μm of dendrites) were quantified. Confocal images and corresponding tracings indicate a decrease in total dendritic length and dendritic spine number of cortical neurons treated with
mefloquine (Fig. 3). Furthermore, quantitative analyses of cortical neuron structural parameters show that mefloquine treatment is associated with a significant ($p < 0.05$) concentration dependent decrease in the total number of dendritic spines and dendritic spine density in cortical neurons (Fig. 4). Clearly, mefloquine induces a significant ($p < 0.05$) concentration dependent degeneration of dendritic spines, suggesting that mefloquine induced oxidative stress may play a role in the neurodegenerative process.

4. Discussion

While various studies have clearly demonstrated an oxidative stress response in cortical neurons and astrocytes treated with artemisinin derivatives (Schmuck et al., 2002), the role of oxidative stress in neurons treated with mefloquine has not been examined. Related efforts have implicated oxidative stress in many of the adverse neurological events associated with mefloquine prophylaxis and treatment, including insomnia (Sudati et al., 2009), anxiety, depression (Masood et al., 2008) and psychosis (Tsalu-chidu et al., 2008). Thus, quantification of oxidative stress and altered integrity in cortical neurons treated with mefloquine will provide further insight into the mechanism(s) related to clinically observed adverse neurological events.

Quantification of GSH is a standard measurement of the presence of oxidative stress in a cell. A decrease in GSH is characteristic of increased oxidative stress. However, quantification of $F_2$-isoPs, an arachidonic acid peroxidation product, provides a more sensitive and specific indicator of oxidative stress in a cell, in particular a neuron. Oxidative stress is caused by an increased production of reactive oxygen species (ROS) in the cell that overwhelms the mechanisms that maintain oxidative homeostasis. An increase in cytosolic calcium concentration can lead to the increased production of ROS via activation of permeability transition pores (Maciel et al., 2001) and stimulation...
of citric acid cycle dehydrogenases, including oxoglutarate dehydrogenase (Tretter and Adam-Vizi, 2004). In addition, increased cytosolic calcium activates various pathways via the stimulation of proteases, phospholipases and nucleases, which can lead to increased ROS and oxidative stress (Farooqui et al., 2006).

Here we quantified oxidative stress with GSH and F2-isopPs in primary rat cortical neurons treated with mefloquine. Cortical neurons were treated with mefloquine concentrations up to 100 μM, as concentrations of mefloquine have been measured in the central nervous system at nearly 100 μM following therapeutic doses (Dow et al., 2005). The 24-h exposure time was selected based upon a peak in plasma concentrations from 6 to 24 h and an occurrence of adverse neurological events within 24 to 48 h after mefloquine administration (Dow, 2003). Cortical neurons treated with mefloquine for 24 h exhibit a concentration dependent decrease in GSH and an associated increase in F2-isopPs (Fig. 3), which, together, indicates the presence of oxidative stress. Cortical neurons exhibit a nearly 3-fold increase in LC50 when treated with the antioxidants N-acetylcysteine and α-tocopherol prior to mefloquine treatment when compared to cortical neurons treated with mefloquine alone (unpublished). These results are a further indication of the presence of and effect of oxidative stress on cortical neurons treated with mefloquine. Future studies will address the ability of antioxidants to attenuate mefloquine’s effects on the biochemical and morphological parameters described herein.

In addition, mefloquine induced degeneration of dendritic spines correlates well with the concentration dependent increase in oxidative stress, which suggests that oxidative stress is responsible for neurodegeneration in cortical neurons treated with mefloquine. Chloroquine, another antimalarial compound, has been associated with morphological alterations in peripheral nerves (Tegner et al., 1988). Data from our previous study with the mouse model of activated innate immunity revealed that coinciding with the peak in oxidative damage to cerebral neuronal membranes at 24 h post intracerebroventricular (ICV) injection of...
lippopolysaccharide (LPS, 5 μg/5 μl), a significant reduction in the spine density and dendritic length of pyramidal neurons occurred in the CA1 sector of hippocampus. Since LPS itself has no direct toxic effect on neurons, LPS-activated glial innate immune response leads to indirect neuronal oxidative damage and synaptodendritic degeneration exclusively through a CD14-dependent mechanism (Milatovic et al., 2003, 2004). Interestingly, both the dendritic spine density and dendritic length returned to near basal levels by 72 h post ICV LPS injection, again coinciding with resolution of oxidative damage in neurons (Milatovic et al., 2003, 2004). Therefore, neurons that have been structurally altered by toxic agents may recover on their own once the insult is removed which provides a link to the reversibility of the adverse neurological events associated with mefloquine.

Previous research indicates that mefloquine treatment disrupts calcium homeostasis and endoplasmic reticulum (ER) function in neurons (Dow, 2003; Dow et al., 2005, 2003). Though the precise mechanism by which mefloquine disrupts calcium homeostasis is unclear, it was suggested that mefloquine may cause the release of calcium from ER stores, which induces subsequent capacitative calcium entry (Caridha et al., 2008). Transcriptional profiles of primary neurons from fetal rat forebrains shows increased expression of ER chaperones and related transcription factors—Ddit3 (DNA-damage-inducible transcript 3) and Elf2ak3 (eukaryotic translation initiation factor 2-alpha kinase 3)—with mefloquine treatment, but not with hydrogen peroxide treatment (Dow, 2003; Dow et al., 2005, 2003). Therefore, oxidative stress alone is insufficient to induce expression of ER chaperones. Indeed, another study (Chen et al., 2008) indicates that oxidative stress in SH-SY5Y neuroblastoma cells and primary cerebellar granule neurons treated with ethanol is insufficient to induce expression of ER chaperones. The same study found that ethanol treatment enhances expression of ER chaperones following treatment with thapsigargin, a sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor that disrupts calcium homeostasis. Antioxidants were able to attenuate the ER stress, which suggests that ER stress response due to the disruption of calcium homeostasis is closely associated with the presence of oxidative stress (Chen et al., 2008).

In summary, the above results indicate that mefloquine induces a concentration dependent oxidative stress response in primary rat cortical neurons, which correlates well with concentration dependent dendritic spine degeneration observed in cortical neurons following treatment with mefloquine. This, therefore, suggests that oxidative stress is responsible for neurodegeneration in cortical neurons treated with mefloquine. Taken together with previous work (Caridha et al., 2008; Dow et al., 2005, 2003), it is clear that a relationship between calcium homeostasis disruption, ER stress response, oxidative stress response, and neurodegeneration exists. However, the precise relationship between the disruption of calcium homeostasis and increase in oxidative stress is unknown and merits further in vivo investigation. Understanding the precise cellular sequence of events that manifest the clinically observed symptoms could contribute to the development of strategies to mitigate the effects for individuals susceptible to mefloquine neurotoxicity.

Conflict of interest

None declared.

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