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Role of lycopene in mitochondrial protection during differential levels of oxidative stress in primary cortical neurons

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ABSTRACT

Reactive oxygen species (ROS) are a major contributor to intracellular organelle damage in neurons. ROS-induced mitochondrial dysfunction is highly associated with impaired energy metabolism that occurs during neurodegeneration. Therefore, the use of antioxidants may be beneficial in protecting the brain from injury caused by ROS. Lycopene is a carotenoid that exhibits neuroprotective properties via its antioxidant capacity. In this study, we hypothesize that treatment with lycopene attenuates hydrogen peroxide-induced oxidative stress in primary cortical neurons. We further tested if lycopene reverses mitochondrial dysfunction caused by ROS. Primary rat cortical neurons were treated with lycopene, hydrogen peroxide, or a combination of both. We used two different concentrations of hydrogen peroxide (25 and 100 μM) to examine a moderate or a high ROS challenge. Treatment with hydrogen peroxide significantly increased intracellular ROS, decreased mitochondrial membrane potential, and depleted neuronal ATP. When neurons were co-treated with lycopene and 25 μM hydrogen peroxide, lycopene attenuated hydrogen peroxide-mediated mitochondrial dysfunction. Interestingly, neuroprotective properties of lycopene were only found during exposure to 25 μM hydrogen peroxide, but not in a 100 μM treated group. We further found that lycopene prevents accumulation of the cleaved form of *B*-cell lymphoma-extra large, ΔN-Bcl-xL. Since ΔN-Bcl-xL causes loss of mitochondrial inner membrane potential which impairs energy metabolism and leads to neuronal death, prevention of ΔN-Bcl-xL accumulation may be an important mechanism explaining lycopene-mediated neuroprotection. Our study suggests that mitochondria are a key target during lycopene-mediated neuroprotection but that the efficacy of lycopene as a neuroprotectant may vary under different levels of ROS.

Introduction

The brain is a metabolically demanding organ. Because of its high oxygen requirements for oxidative phosphorylation, it is particularly vulnerable to damage caused by reactive oxygen species (ROS). Mitochondria are the central organelle that regulate both redox homeostasis and energy metabolism, and mitochondrial dysfunction is highly associated with neurodegenerative diseases like Alzheimer's and Parkinson's. Therefore, approaches that protect mitochondria by decreasing ROS are crucial to protecting the brain during these pathological processes. *B*-cell lymphoma-extra large (Bcl-xL) is a mitochondrial protein that exhibits neuroprotective properties. Bcl-xL enhances mitochondrial function by increasing the efficiency of ATP production [1–3], maintaining normal mitochondrial membrane potential [4,5], and regulating the mitochondrial population via controlling fission and fusion [6]. Interestingly, we recently reported that generation of ROS contributes to post-translational cleavage of Bcl-xL to ΔN-Bcl-xL [7]. Accumulation of ΔN-Bcl-xL is causative to abnormal mitochondrial channel activity, loss of mitochondrial inner membrane potential, ATP depletion, and ultimately neuronal death [5,8–10], and the application of an antioxidant has been shown to be protective against ROS-induced ΔN-Bcl-xL production and/or activity [7].

Lycopene is a non-provitamin A carotenoid with strong antioxidant properties [11,12]. It is found in red-colored fruits and vegetables such as tomatoes and watermelons. Lycopene is a highly unsaturated hydrocarbon with 8 isoprene units. Due to the presence of multiple double bonds, it is capable of forming various cis or trans configurations. The most common isomer found in food sources is the all trans form,

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however, it can be isomerized during metabolism [13–15]. These double bonds provide an oxygen-quenching ability [16], exhibiting protective effects against ROS-associated damage on various cells, including brain cells. Both oral and intraperitoneal administration of lycopene attenuated oxidative stress-induced injury against cerebral ischemia in rodents [17–19]. Treatment with lycopene also prevented ROS-induced amyloid beta accumulation in Alzheimer's models [20–22] and attenuated neurological deficit in Parkinson's disease models [23,24]. Higher serum lycopene decreases the risks of atherosclerosis and stroke [25–27], Alzheimer's disease [28,29], and Parkinson's disease [30] among human subjects. Greater lycopene levels in plasma are correlated with enhanced cognitive performance among non-demented elders [31]. Despite increasing reports that show neuroprotective properties of lycopene, effects of dietary lycopene on brain-associated diseases in human subjects are still controversial [26,32,33]. Lycopene may exhibit differential effects based on varying microenvironments in the brain. By investigating the cellular mechanisms underlying lycopene neuroprotection, we may be able to develop personalized dietary strategies in patients with neurodegenerative diseases.

In this study, we found that lycopene exhibits a dual effect on primary cortical neurons challenged with oxidative stress. Treatment with lycopene prevents loss of mitochondrial membrane potential and depletion of neuronal ATP during moderate hydrogen peroxide challenge. However, effects of lycopene were insignificant or potentially toxic during high hydrogen peroxide challenge. We suggest competing roles of lycopene depending on varying levels of oxidative stress.

Materials and methods

Culture of primary cortical neurons

Primary rat cortical neurons were prepared from rat feti (Sprague-Dawley, day 18 of gestation; Harlan, Indianapolis, IN) as described previously [34]. Briefly, neurons (0.5×10^6 cells/ 35 mm plate) were grown in neurobasal medium supplemented with *B*-27, glutamine, and antibiotics (Thermo Fisher Scientific, Waltham, MA) for 12 days *in vitro* (DIV), and treated with hydrogen peroxide (25–200 μM), lycopene (0.1 μM), or a combination of both as described in relevant figure legends. *Lycopene treatment:* A solution of lycopene (Sigma-Aldrich, St. Louis, MO, USA) was freshly prepared in tetrahydrofuran (Sigma-Aldrich) [20], and added to the cell culture medium 20 min prior to hydrogen peroxide treatment. Tetrahydrofuran was used as the vehicle control in all experiments with lycopene. *Hydrogen peroxide treatment:* 25, 50, 100, and 200 μM of hydrogen peroxide (Sigma-Aldrich) were freshly prepared in sterile PBS and added to the cell culture medium. The vehicle control for hydrogen peroxide experiments was sterile PBS. All protocols were approved by the Institutional Animal Care Committee (IACUC) of University of Alabama, Tuscaloosa, AL.

Lactate dehydrogenase (LDH) assay

The level of cytotoxicity in primary neurons was assayed by measuring leakage of LDH using an *in vitro* toxicology assay kit (Sigma-Aldrich) as previously described [7]. In brief, the culture media and lysed cells were collected after treatment of neurons with lycopene and hydrogen peroxide for 24 h. The LDH assay mixture was added to each sample. After 20 min incubation, the reaction was terminated by adding 1 N HCl. LDH activity was spectrophotometrically measured with a Clariostar microplate reader (BMG Labtech, Cary, NC) with absorbance set at 490 nm. Data were calculated by finding the activity of LDH which leaked into the medium from damaged cells / total LDH activity in the culture.

Calcein-AM, and propidium iodide (PI) staining

Viable or dead cells were stained with Calcein-AM or PI as previously described [5]. After treatment of neurons with lycopene and hydrogen peroxide, 25 nM Calcein-AM, 0.5 μM PI, or 1 μg/ml Hoechst (Thermo Fisher Scientific, Waltham, MA) was added into the culture medium for 30 min at 37 °C in the dark. Micrographs were taken using a Zeiss Axiovert A1 microscope (Zeiss, Oberkochen, Germany) using a consistent exposure time. The number of PI positive neurons, Hoechst positive neurons, or calcein fluorescence density per cell was analyzed using AxioVision 4.9.

Measurement of ATP production

Neurons were seeded on 96 well plates (0.03×10^6 neurons/well) for DIV12. Neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25 or 100 μM), or a combination of both. Neuronal ATP production was measured using the ATPlite™ Luminescence Assay System (PerkinElmer, Waltham, MA, USA) as previously described [5]. In brief, the plates were washed with sterile PBS, and cells were lysed on the orbital shaker for 5 min. Cells were then incubated with substrate (luciferin) on the orbital shaker for 10 min. The reaction between ATP, luciferase, and luciferin produced bioluminescence. ATP-induced luminescence was measured with a fluorescence microplate reader (CLARIOstar, BMG Labtech).

2′,7′- dichlorodihydrofluorescein diacetate (H_2DCFDA) staining

Neurons were treated with 10 μM of DCF (Thermo Fisher Scientific, Waltham, MA) solution prepared in a light protected vessel, then incubated for 30 min at 37 °C in the dark [35], and then processed as previously described [7]. After incubation, neurons were carefully washed with pre-warmed HBSS. Intracellular fluorescence was measured using a fluorescence microplate reader (CLARIOstar, BMG Labtech) at excitation and emission wavelengths of 483 nm and 530 nm, respectively.

MitoSOX staining

Production of mitochondrial superoxide was analyzed using MitoSOX Red (Thermo Fisher Scientific, Waltham, MA) [7]. The MitoSOX Red is oxidized by superoxide in the mitochondria, emitting red fluorescence. After treatment of neurons as described in relevant figure legends, 1.25 μM of MitoSOX was added to the cell culture medium. Cultures were incubated for 30 min at 37 °C and washed twice with warm HBSS. Fluorescence intensity was analyzed using AxioVision 4.9.

Measurement of mitochondrial potential (Δψ)

Mitochondrial membrane potential (Δψ) was measured using the fluorescent lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM) (Thermo Fisher Scientific, Waltham, MA), which accumulates within mitochondria in a potential dependent manner [36,37]. Primary neurons were stained with 5 nM TMRM for 30 min at 37 °C in the dark. Images were taken using a Zeiss Axiovert A1 microscope using a consistent exposure time and TMRM fluorescence density was analyzed using AxioVision 4.9.

Immunoblots

After treatment with lycopene and hydrogen peroxide for 24 h, neurons were scraped and lysed in 1X cell signaling buffer (Cell signaling Technology, Danvers, MA) and protein concentration was determined using BCA protein reagents (Thermo Fisher Scientific, Waltham, MA). Samples (50–100 μg of protein/lane) were separated on a 4–12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) and probed with anti-ΔN-Bcl-xL (1:10, Pacific Immunology, Ramona, CA), anti-active bax (1:100, Enzo Life science, Farmingdale, NY), and anti-beta actin (Sigma-Aldrich, 1:1000). Anti-ΔN-Bcl-xL was custom-produced (peptide sequence: CZ DSP AVN GAT GHS SSL D). Scanned images

were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Caspase 3 activity

Primary hippocampal neurons were lysed and then incubated with substrate solution containing Ac-DVD-AMC for 37 °C in the dark (Cell signaling Technology, Danvers, MA). After 30 min, relative fluorescence units produced by Ac-DEVD-AMC cleavage indicating caspase 3 activity were measured at 380 nm excitation and 420–460 nm emission using CLARIOstar (BMG labtech).

Statistical analysis

Data are reported as mean + SEM of at least three independent cultures with multiple independent experimental designs, such as independent neuronal isolation, independent performance dates, and independent plates within a culture using separately prepared reagents. All quantitative graphs were made from at least three independent neuronal isolation. Differences in means were tested using one-way ANOVA with Tukey's test. $P < 0.05$ was considered statistically significant. *P* values are provided in figure legends.

Results

Lycopene is neuroprotective during moderate hydrogen peroxide challenge

To analyze the neuroprotective properties of lycopene at varying levels of oxidative stress, we measured lactate dehydrogenase (LDH) release, a marker for loss of cell membrane integrity, in primary cortical neurons 24 h after treatment with lycopene (0.1 μM) and hydrogen peroxide at 5 different concentrations (0, 25, 50, 100, and 200 μM). Primary cortical neurons showed concentration-dependent toxicity with hydrogen peroxide treatment. Greater concentrations of hydrogen peroxide increased LDH leakage (Fig. 1A). Interestingly, we found that the effects of lycopene varied at different concentrations of hydrogen peroxide treatment. Lycopene (0.1–1 μM) significantly protected neurons co-treated with 25 μM hydrogen peroxide (Fig. 1A and Suppl S1). However, the protective effects of lycopene were diminished when neurons were treated with higher concentrations (50–100 μM) of hydrogen peroxide. Furthermore, when neurons were challenged with 200 μM hydrogen peroxide, lycopene treatment potentiated neurotoxicity (Fig. 1A). We have shown that neurons with LDH leakage below 150% of control group were responding to a pharmacological or nutritional treatment [5,7]. In this study, we chose 25 μM hydrogen peroxide as a 'moderate' insult and 100 μM as a 'severe' insult that causes irreversible damage to neurons as a comparison. We comparatively assessed lycopene's effect on these insults by measuring the treated population of viable and dead neurons using calcein and propidium iodide (PI), respectively. Additionally, primary cortical neurons were treated with 0.1 μM lycopene, hydrogen peroxide (25 or 100 μM), or a combination of both, and fluorescent micrographs were obtained after 24 h incubation. Cortical neurons treated with hydrogen peroxide (both 25 or 100 μM groups) showed significantly lowered calcein-positive signals, indicating a loss of viable neurons, and enhanced PI-positive signals, indicting an increased late apoptotic and necrotic population of neurons (Fig. 1B–D). PI negative and Hoechst positive cells were also used to verify the viable cell population (Supplement S2). Treatment with lycopene protected neurons against moderate-level hydrogen peroxide challenge (25 μM), but it was less effective or failed to rescue neurons during higher ROS insult (Fig. 1A-D).

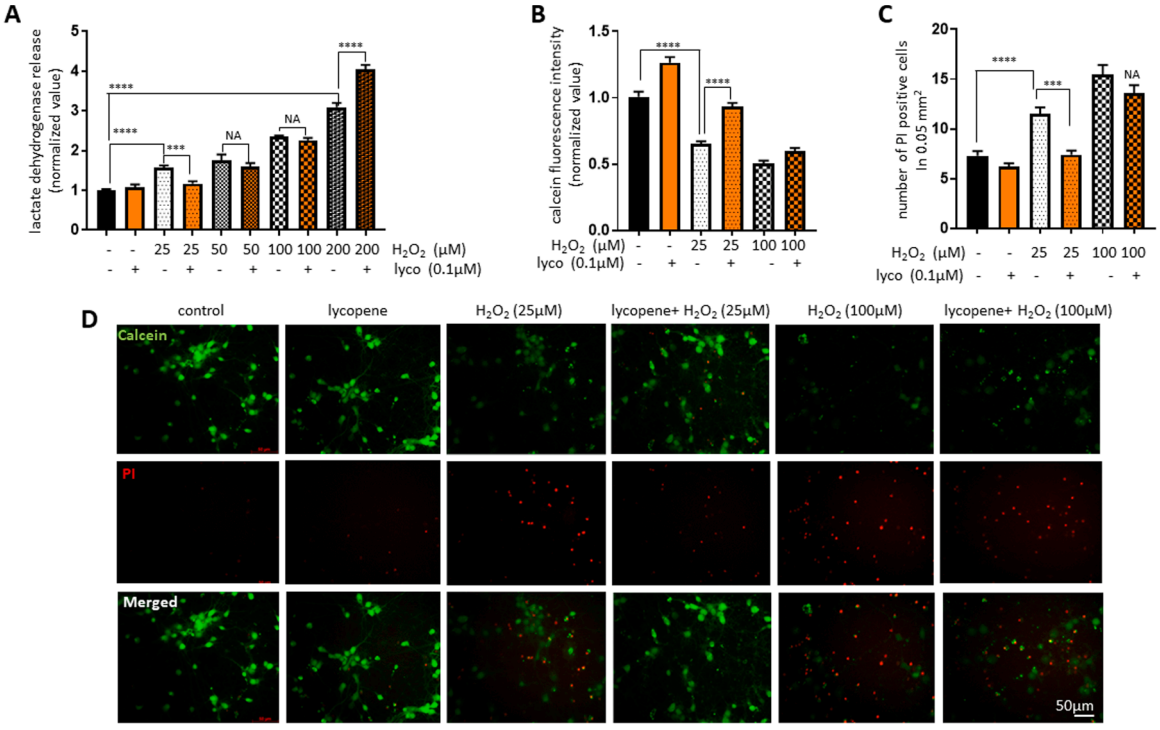

Fig. 1. Lycopene protects primary cortical neurons during moderate hydrogen peroxide challenge. Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25, 50, 100, and 200 μM), or a combination of both for 24 h. Quantified neuronal toxicity, viability and death were measured by lactate dehydrogenase (LDH) release ($n = 4$) (A), Calcein retention ($n = 30$) (B), and PI positive cells ($n = 45$) (C), respectively. Calcein-stained live cells and PI-stained death cells were imaged using a 40X fluorescence microscope (D). Lycopene protected neurons under 25 μM hydrogen peroxide challenge, but it failed to rescue neurons with 100 μM hydrogen peroxide treatment. Green: calcein; red: PI. Scale bar=50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, one-way ANOVA (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Lycopene prevents loss of ATP in primary cortical neurons exposed to moderate oxidative stress

To verify intracellular hydrogen peroxide production during extracellular hydrogen peroxide treatment, we measured 2′.7′-dichlorofluorescein (DCF) retention in neurons. Hydrogen peroxide oxidizes non-fluorescent DCFH to fluorescent DCF, and the fluorescent intensity of DCF confirms the presence of hydrogen peroxide. Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25 μM), or a combination of both, and then neurons were assayed following a 6 h incubation. Neurons treated with 25 μM hydrogen peroxide had significantly increased DCF-positive fluorescent signal, indicating greater levels of intracellular hydrogen peroxide, and treatment with lycopene attenuated hydrogen peroxide-induced DCF signal (Fig. 2A). We further measured mitochondrial ROS using mitoSOX, a fluorescent dye that detects mitochondrial superoxide. Hydrogen peroxide significantly increased the mitoSOX positive signal, and lycopene attenuated hydrogen peroxide-induced mitoSOX fluorescence, indicating a decrease in mitochondrial ROS (Fig. 2B). Next, we tested whether this prevention of mitochondrial ROS improved mitochondrial function in lycopene treated primary cortical neurons. We measured intensity of bioluminescence generated by ATP, luciferin, and luciferase reaction. Hydrogen peroxide significantly decreased ATP production in primary cortical neurons, but lycopene treatment prevented this hydrogen peroxide-mediated energy loss (Fig. 2C).

Lycopene fails to prevent neuronal energy loss when challenged with a high concentration of hydrogen peroxide

During screening, we observed that the efficacy of lycopene-mediated neuroprotection is influenced by the level of oxidative stress (Fig. 1A). Lycopene significantly protected neurons from a moderate level of hydrogen peroxide (25 μM) challenge, but it failed to rescue neurons under high concentrations (50–200 μM) of hydrogen peroxide (Fig. 1). Here, we tested if irreversible mitochondrial damage during high levels of oxidative stress is causative to failure of lycopene-mediated neuroprotection. Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (100 μM), or a combination of both, and then neurons were assayed after 6 h incubation. Neurons treated with 100 μM of hydrogen peroxide had significantly increased endogenous production of ROS in whole neurons (Fig. 3A) and mitochondria (Fig. 3B). In particular, 100 μM hydrogen peroxide significantly increased mitochondrial superoxide by greater than 2-fold compared to vehicle control (Fig. 3B), and this caused a greater than 70% depletion of ATP in primary cortical neurons (Fig. 3C).

Lycopene prevents formation of ΔN-Bcl-xL to protect mitochondria during moderate hydrogen peroxide challenge

We previously showed that the anti-apoptotic protein Bcl-xL undergoes caspase 3-dependent proteolytic cleavage at the N-terminus to form ΔN-Bcl-xL during oxidative stress [5,7,38]. We have also reported a time course increase in ΔN-Bcl-xL 6 to 24 h after neurotoxic stimulation [5]. ΔN-Bcl-xL impairs mitochondrial function, lowers ATP production, and eventually leads to neuronal death [5,7,9]. Application of pharmacological inhibitors that directly block function of ΔN-Bcl-xL, or antioxidants that prevent production of ΔN-Bcl-xL, are shown to be neuroprotective via supporting mitochondrial function [5,7,9]. Here, we tested if neuroprotective properties of lycopene are associated with regulation of ΔN-Bcl-xL. Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25 μM), or a combination of both for 24 h, and protein levels of ΔN-Bcl-xL were quantified. Primary cortical neurons treated with lycopene prevented accumulation of ΔN-Bcl-xL under hydrogen peroxide challenge (Fig. 4A,B). We also found that treatment with lycopene may be effective to delay ΔN-Bcl-xL-mediated apoptosis. Lycopene prevented accumulation of the active form of Bax (Fig. 4C). Caspase 3 is the key enzyme that is responsible to form ΔN-Bcl-xL [5,39]. Hydrogen peroxide significantly increased caspase 3 activity, whereas treatment with lycopene prevented hydrogen peroxide-induced caspase 3 activity (Fig. 4D).

Endogenous production of ΔN-Bcl-xL during excitotoxicity and neurons that overexpress ΔN-Bcl-xL demonstrate loss of mitochondrial membrane potential which causes ROS generation and neuronal energy loss [5,7]. We therefore investigated if hydrogen peroxide-induced ΔN-Bcl-xL accumulation is associated with loss of mitochondrial membrane potential and if treatment with lycopene can reverse the effect of hydrogen peroxide. We applied tetramethylrhodamine (TMRM), a positively charged fluorescent probe that accumulates in the healthy negatively charged mitochondrial inner membrane. Mitochondria with intact membranes retain strong fluorescence. Primary cortical neurons were treated with lycopene, hydrogen peroxide, or a combination of both for 6 h. Neurons treated with 25 μM hydrogen peroxide showed significantly lowered TMRM-positive signal, however, lycopene treatment attenuated this hydrogen peroxide-mediated loss of mitochondrial membrane potential (Fig. 4E and F). Previously, we have reported that treatment with 10 nM ABT-737, a pharmacological inhibitor of Bcl-xL, prevents ΔN-Bcl-xL-mediated mitochondrial dysfunction during excitotoxicity. Similarly, primary hippocampal neurons treated with 10 nM ABT-737 were resistant to loss of mitochondrial membrane potential during oxidative stress (Suppl S3).

Lycopene fails to prevent formation of ΔN-Bcl-xL during high concentration hydrogen peroxide challenge

Primary cortical neurons treated with 100 μM hydrogen peroxide demonstrate severely altered redox homeostasis and energy metabolism which may indicate irreversible damage to mitochondria (Fig. 3). We further tested if ΔN-Bcl-xL contributes to impaired energy metabolism under severe oxidative stress. Primary cortical neurons treated with 100 μM hydrogen peroxide had increased protein levels of ΔN-Bcl-xL (Fig. 5A,B) and active Bax (Fig. C). Unlike the 25 μM treated group, lycopene co-treatment with 100 μM hydrogen peroxide did not reverse

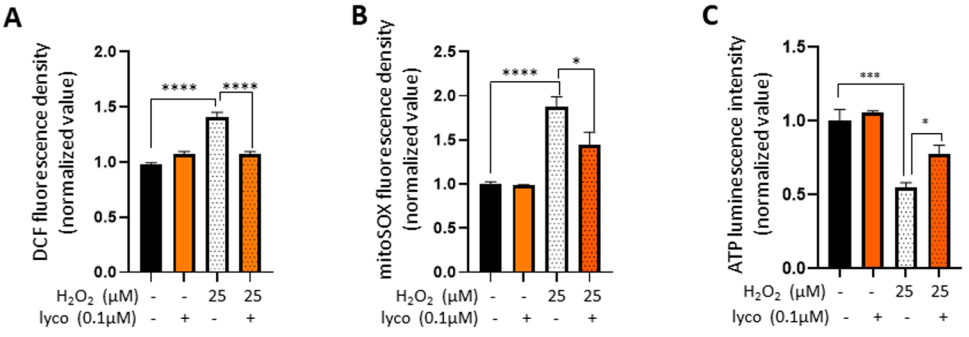

Fig. 2. *Lycopene prevents loss of ATP in primary cortical neurons during moderate hydrogen peroxide challenge.* Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25 μM) or a combination of both for 6 h. Intracellular hydrogen peroxide and superoxide levels were assayed by measuring 2′, 7′-dichlorofluorescein (DCF) (A) and mitoSOX fluorescence (B), respectively. C, intracellular ATP was assayed measuring bioluminescence. Treatment with lycopene alleviates oxidative stress and prevents neuronal energy loss. (DCF, $n = 4$; mitoSOX, $n = 6$; ATP, $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, one-way ANOVA.

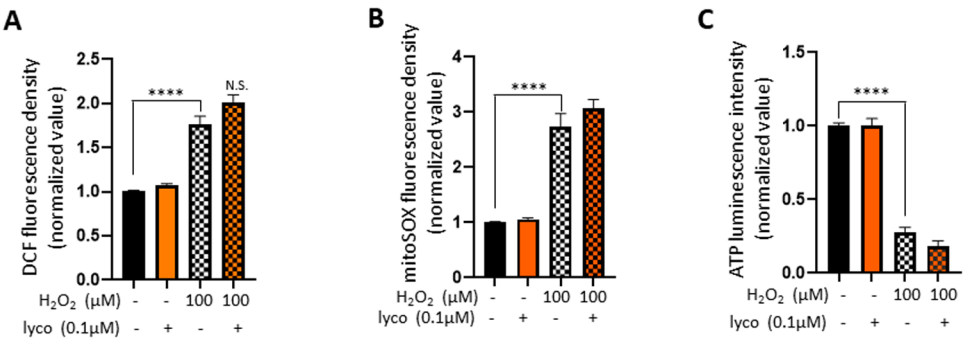

Fig. 3. *Lycopene fails to prevent neuronal ATP loss during a high concentration of hydrogen peroxide challenge.* Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (100 μM) or a combination of both for 6 h. Intracellular hydrogen peroxide (A), mitochondrial superoxide (B), and ATP level (C) were measured. Treatment with lycopene failed to reverse neuronal energy loss during severe oxidative stress (DCF, $n = 4$; mitoSOX, $n = 5$; ATP, $n = 4$). *P 0.05, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, one-way ANOVA.

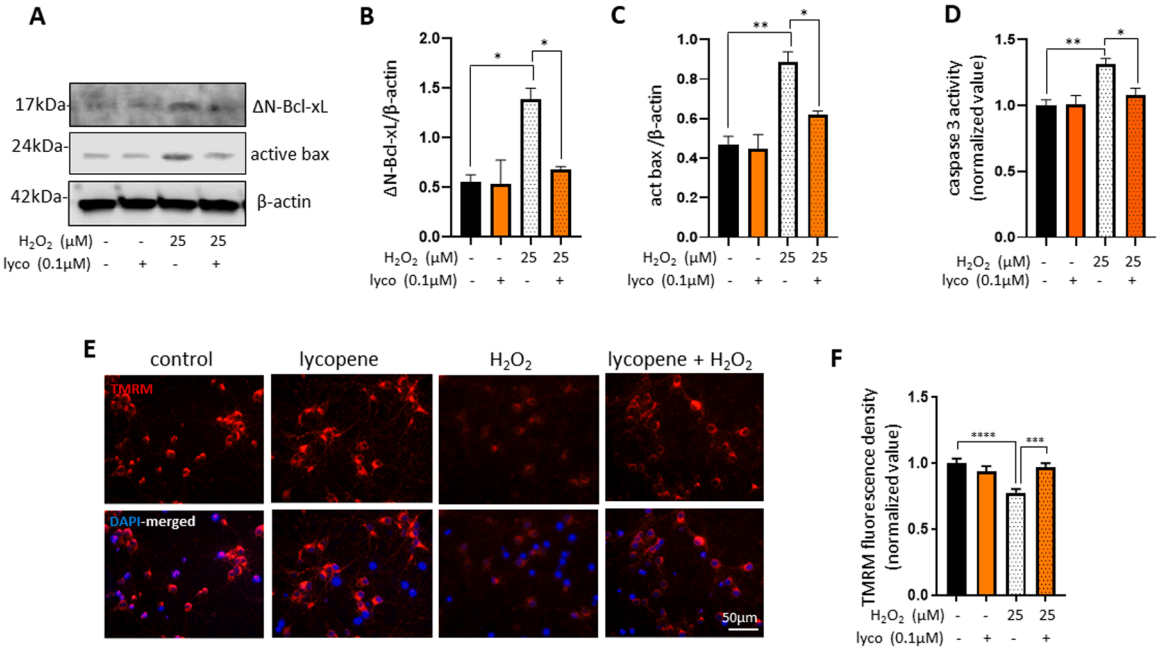

Fig. 4. *Lycopene prevents formation of ΔN-Bcl-xL protecting mitochondria during moderate hydrogen peroxide challenge.* Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (25 μM) or a combination of both for 24 h. A–C, Immunoblot data indicate that lycopene treatment prevents hydrogen peroxide-induced ΔN-Bcl-xL accumulation and Bax activation ($n = 3$). D, Lycopene treatment prevents hydrogen peroxide-induced caspase 3 activation ($n = 6$). After 6 h incubation, TMRM-stained neurons were imaged (E) and TMRM fluorescence intensity (F) was quantified ($n = 40$). Treatment with lycopene prevents loss of mitochondrial inner membrane potential against hydrogen peroxide challenge. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, one-way ANOVA.

this increase (Fig. 5A-C). Lycopene consistently failed to inhibit caspase 3 activity during 100 μM hydrogen peroxide treatment (Fig. 5D). Accumulation of ΔN-Bcl-xL caused loss of mitochondrial membrane potential in both the hydrogen peroxide treated group and the lycopene co-treated group (Fig. 5E and F), indicating impaired mitochondrial function.

Discussion

In this study, we find that the mitochondrion is an important target during lycopene-mediated neuroprotection. Treatment with lycopene prevents production of intracellular ROS and inhibits the formation of ΔN-Bcl-xL in primary cortical neurons, and therefore lycopene attenuates the ΔN-Bcl-xL-induced loss of mitochondrial inner membrane potential and subsequent depletion of ATP. This prevention of mitochondrial damage results in neuroprotection during ROS challenge. We also suggest that the neuroprotective properties of lycopene vary depending on different levels of ROS (Fig. 6). In particular, in primary cortical neurons with mitochondria that are irreversibly damaged, lycopene is not effective in rescuing neurons against oxidative stress. Under a moderate level of oxidative stress, lycopene prevents ΔN-Bcl-xL formation and protects mitochondria.

Several studies have reported on the role of lycopene in regulating Bcl-2 family proteins in the brain. Lycopene upregulates Bcl-xL gene expression in the hippocampus of diabetic rats [40]. Lycopene treatment decreases the Bax/Bcl2 ratio in primary cerebrocortical neurons under ROS stress [41], and consistent data were found using primary neuronal culture and SH-SY5Y neuroblastoma cells under treatment with amyloid beta [20,42]. In this study, we investigated the role of lycopene in mitochondrial protection due to its primary role in regulating the abundance of ΔN-Bcl-xL. ΔN-Bcl-xL is found in the mitochondrial membrane [5] and causes abnormal mitochondrial channel activity [8, 10]. We have reported that ΔN-Bcl-xL-induced mitochondrial potential loss is prevented in neurons lacking the c-subunit of the F1Fo ATP synthase [5]. The *c*-subunit contributes to inefficient operation of the F1Fo ATP synthase and exhibits large, less-selective channel activity that resembles opening of the mitochondrial permeability transition pore [1,3,43–46]. Lycopene treatment may prevent opening of this mitochondrial death channel via preventing ΔN-Bcl-xL accumulation. In addition, ΔN-Bcl-xL may directly influence mitochondrial functions associated with mitochondrial membrane integrity. Our recent study showed that ΔN-Bcl-xL undergoes protein-protein interaction with

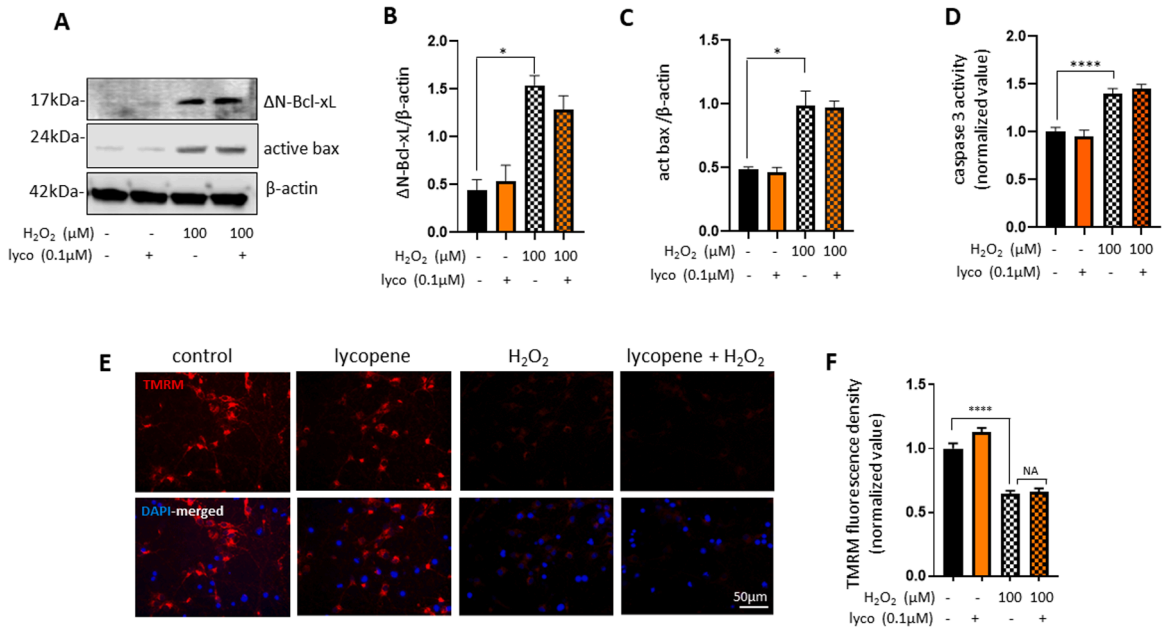

Fig. 5. *Lycopene fails to protect mitochondria under a high concentration of hydrogen peroxide.* Primary cortical neurons were treated with lycopene (0.1 μM), hydrogen peroxide (100 μM) or a combination of both for 24 h. A–C, Immunoblot data indicate that lycopene treatment failed to prevent hydrogen peroxide-induced ΔN-Bcl-xL accumulation and Bax activation ($n = 3$). D, Lycopene treatment failed to prevent hydrogen peroxide-induced caspase 3 activation ($n = 6$). After 6 h incubation, TMRM-stained neurons were imaged (E) and TMRM fluorescence intensity (F) was quantified ($n = 40$). Treatment with lycopene failed to prevent loss of mitochondrial inner membrane potential against hydrogen peroxide challenge. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, one-way ANOVA.

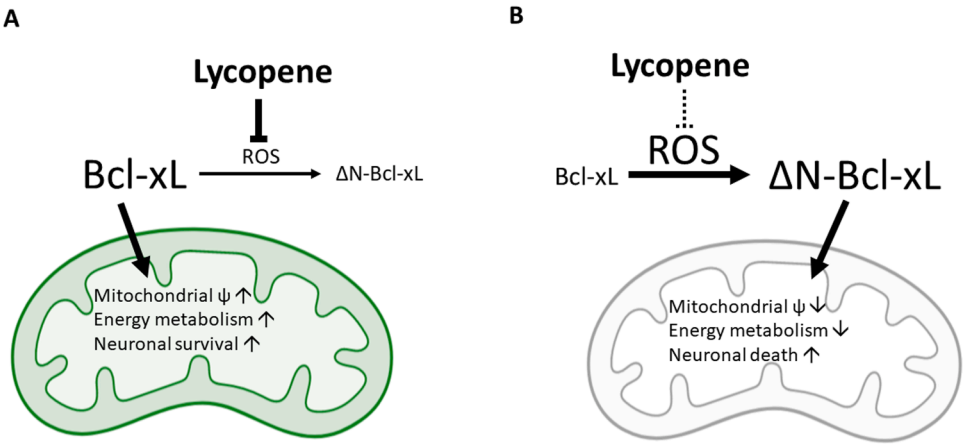

Fig. 6. *Schematic representation of lycopene-mediated neuroprotection against oxidative stress.* Oxidative stress causes conversion of Bcl-xL to form pro-death ΔN-Bcl-xL. A, During generation of oxidative stress in moderation, antioxidant lycopene blocks formation of ΔN-Bcl-xL preventing ΔN-Bcl-xL-mediated mitochondrial dysfunction rescuing neurons. B, However, during severe levels of oxidative stress, lycopene fails to prevent formation of ΔN-Bcl-xL. Accumulation of ΔN-Bcl-xL damages mitochondria impairing neuronal energy metabolism, ultimately leading to cell death. Illustration by BioRender.

Bcl-2-associated *X* protein (Bax) [7]. Bax undergoes oligomerization with other pro-apoptotic Bcl-2 proteins to make the mitochondrial membrane permeable. Due to the structural similarity between Bax and ΔN-Bcl-xL, which both contain Bcl-2 homolog (BH) 1, BH2, and BH3 domains, ΔN-Bcl-xL-Bax may mimic the effect of Bax oligomers in the mitochondrial membrane [7]. Indeed, primary hippocampal neurons overexpressing ΔN-Bcl-xL show significantly lowered mitochondrial inner membrane potential [5] and increased vulnerability to neurotoxicity. This study shows that preventing accumulation of ΔN-Bcl-xL using lycopene is effective in reversing ROS-induced loss of mitochondrial inner membrane potential.

Interestingly, our study shows that neuroprotective properties of lycopene are dependent on hydrogen peroxide concentration. Lycopene significantly lowers ROS levels, attenuates loss of mitochondrial membrane potential and ATP, and prevents ΔN-Bcl-xL accumulation in neurons under moderate levels of hydrogen peroxide treatment at 25 μM. All of these protective effects by lycopene were diminished under higher concentrations of hydrogen peroxide, and, in some cases, lycopene even potentiated neurotoxicity. Underlying mechanisms explaining these differential effects of lycopene are unknown. However, Lys16 of the Bcl-xL *N*-terminal has been identified as an ubiquitination site by several groups [47–49]. We speculate that Bcl-xL is highly influenced by ubiquitin-mediated degradation due to the presence of a Lys16 docking site, while ΔN-Bcl-xL is potentially resistant to proteolysis because of loss of the *N*-terminal which includes Lys16. When neurons are overwhelmed by ΔN-Bcl-xL during excessive ROS, it may cause a prolonged secondary effect favoring neuronal death due to failure of ΔN-Bcl-xL clearance.

Conclusions

In summary, this study suggests that mitochondria are an important target of lycopene-mediated neuroprotection. Lycopene attenuates ROS-induced ΔN-Bcl-xL accumulation, improves mitochondrial inner membrane potential, and prevents ATP loss. We also address the fact that the effectiveness of lycopene as a neuroprotectant may vary at different

levels of ROS. Translation of the current findings to *in vivo* models demonstrating these same effects during high and low ROS may be an important step in developing therapeutic strategies using lycopene.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Ethical approval

All animal protocols were approved by the Institutional Animal Care Committee (IACUC) of University of Alabama, Tuscaloosa, AL.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dscb.2021.100016.

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