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Ketone bodies are protective against oxidative stress in neocortical neurons

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Abstract

Ketone bodies (KB) have been shown to prevent neurodegeneration in models of Parkinson's and Alzheimer's diseases, but the mechanisms underlying these effects remain unclear. One possibility is that KB may exert antioxidant activity. In the current study, we explored the effects of KB on rat neocortical neurons exposed to hydrogen peroxide (H_2O_2) or diamide – a thiol oxidant and activator of mitochondrial permeability transition (mPT). We found that: (i) KB completely blocked large inward currents induced by either H_2O_2 or diamide; (ii) KB significantly decreased the number of propidium iodide-labeled cells in neocortical slices after exposure to H_2O_2 or diamide; (iii) KB significantly decreased reactive oxygen species (ROS) levels in dissociated neurons and in isolated neocortical mitochondria; (iv) the electrophysiological effects of KB in neurons exposed to H_2O_2 or diamide were mimicked by bongkrekic acid and cyclosporin A, known inhibitors of mPT, as well as by catalase and DL – dithiothreitol, known antioxidants; (v) diamide alone did not significantly alter basal ROS levels in neurons, supporting previous studies indicating that diamide-induced neuronal injury may be mediated by mPT opening; and (vi) KB significantly increased the threshold for calcium-induced mPT in isolated mitochondria. Taken together, our data suggest that KB may prevent mPT and oxidative injury in neocortical neurons, most likely by decreasing mitochondrial ROS production.

Keywords: ketone bodies, mitochondria., mitochondrial permeability transition, neocortex, oxidative stress.

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Normal cellular oxidative metabolism generates reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) radicals, which are scavenged by endogenous antioxidant systems (Droge 2002). However, when ROS levels exceed the antioxidant capacity of the cell, or when antioxidant systems become deficient – thereby resulting in oxidative stress, neuronal damage occurs (Halliwell 1992). Several neurodegenerative disorders are believed to arise in part through oxidative stress (Beal 1995; Simonian and Coyle 1996; Klein and Ackerman 2003), a phenomenon which is associated with excessive calcium influx, increased ROS production, and ultimately, mitochondrial permeability transition (mPT) (Maciel *et al.* 2001; Halestrap and Brennerb 2003).

Ketone bodies (KB) are important metabolic substrates, produced by the liver under conditions of fasting, caloric restriction, and intake of high-fat and low-carbohydrate diets such as the ketogenic diet (Huttenlocher 1976; Likhodii *et al.* 2003; Skrha *et al.* 2005; Denny *et al.* 2006). The major KB are D-β-hydroxybutyrate (BHB), acetoacetate (ACA), and

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Abbreviations used: ACA, acetoacetate; ANT, adenine nucleotide translocator; BHB, β-hydroxybutyrate; BKA, bongkrekic acid; CsA, cyclosporin A; DCF, 2′,7′-dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; KB, ketone bodies; mPT, mitochondrial permeability transition; NADH, reduced nicotinamide adenine dinucleotide; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; RMP, resting membrane potential; ROS, reactive oxygen species.

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acetone. Neurons switch from glucose to KB as the major energy source under these conditions, and it is also well known that suckling mammals rely primarily on KB for energy utilization (Veech *et al.* 2001). Interestingly, KB themselves have been shown to protect against acute insults such as hypoxia-ischemia in brain (Dardzinski *et al.* 2000; Suzuki *et al.* 2001; Smith *et al.* 2005), and have also been found to be neuroprotective in models of Parkinson's disease and Alzheimer's disease (Kashiwaya *et al.* 2000; Tieu *et al.* 2003). Despite a growing amount of literature supporting the protective effects of KB, the underlying mechanisms remain unclear.

Prior studies have suggested that the therapeutic benefits of KB in heart may be related to diminished free radical production within mitochondria – a consequence of oxidation of Co-enzyme Q and NADH, a situation which favors the reduction of glutathione and elimination of H_2O_2 by glutathione peroxidases (Veech *et al.* 2001). In rats fed a high-fat ketogenic diet, elevated glutathione peroxidase activity was detected in hippocampus (Ziegler *et al.* 2003), and increased mitochondrial uncoupling protein levels and activity – which results in a decrease in ROS production – was recently reported (Sullivan *et al.* 2004a). From these lines of evidence, it is possible that KB are not only substrates for fuel, but may also exert a cellular protective effect via an antioxidant mechanism. In the present study, we sought cellular, electrophysiological and biochemical evidence that KB could protect against acute oxidative injury induced by H_2O_2 or diamide – a thiol oxidant and activator of mPT – in neocortical neurons from rat sensorimotor neocortex. We also tested the hypothesis that KB can alter mitochondrial ROS production and the threshold for mPT in isolated cortical mitochondria.

Materials and methods

All protocols were approved by the Barrow Neurological Institute and the University of Kentucky Institutional Animal Care and Use Committees. All drugs and chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA), unless stated otherwise. Only the physiological isomer of β-hydroxybutyrate – i.e., R(-)BHB or D(-)BHB – was used in all experiments.

Electrophysiology

Acute brain slices (400 μm) and dissociated neurons were prepared from fronto-parietal cortex of brains from Sprague-Dawley rats (P21-P28 and P14-P23, respectively). Slices were transferred to physiological saline (in mmol/L: 124 NaCl, 1.3 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, and 10 D-glucose; pH: 7.4) bubbled with carbogen (95% O_2 : 5% CO_2). Acute neocortical slices were submerged in a recording chamber and infused continuously with physiological saline (32°C) at a rate of 2–3 mL/min. Layer V pyramidal neurons were visualized under infrared illumination using a Zeiss Axioskop 2 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). Recording electrodes (4–8 MΩ) were backfilled with a solution (in mmol/L): 140 K^+-gluconate, 10 HEPES, 2 $MgCl_2$, 1 $CaCl_2$, 0.2 EGTA, and 2 K_2ATP, pH 7.25. Whole-cell potentials were corrected *post hoc* for junction potentials. All experiments were performed using whole-cell configuration of the patch-clamp technique with a Multiclamp 700A amplifier or Axopatch 200B and pClamp V9.2 (Axon Instruments, Union City, CA, USA).

To prepare acutely isolated neurons, small pieces of gray matter were punctured from each slice and incubated for 20 min at 34°C in standard solution (in mmol/L: 150 NaCl, 3 KCl, 2 $CaCl_2$, 2$MgCl_2$, 10 HEPES, and 10 D-glucose) containing pronase (1 mg/6 mL; CalbioChem, La Jolla, CA, USA). After washing, tissue pieces were mechanically dissociated using Pasteur pipettes of progressively smaller bore diameter. Recording electrodes were backfilled with a solution (in mmol/L): 80 K^+-gluconate, 70 KCl, 5 $MgCl_2$, and 10 HEPES; pH 7.2 for voltage-clamp experiments, and 150 KCl, 4 $MgCl_2$, 0.1 EGTA, 4 ATP, and 10 HEPES for current-clamp studies. Amphotericin B (final concentration, 250 μg/mL) was added to the pipette solution before use to enable perforated-patch recording conditions. In voltage-clamp experiments, 70–75% series resistance compensation was made (holding potential, V_H = −60 mV). Currents were low-pass filtered at 1- or 2 kHz with a 4-pole Bessel filter.

Assessment of cell death

Cell death was assessed in acute, non-fixed neocortical slices using the fluorescent indicator propidium iodide (PI; n = 8–30 slices for each experimental group). To replicate similar conditions as in our electrophysiological experiments, all slices were allowed to recover for at least 2 h in physiological saline (32°C) prior to sham treatment or 10 min of exposure to H_2O_2 or diamide, with and without KB. Slices were incubated with PI (20 μg/mL) for 30 min and then measurements were made with an Olympus fluorescence microscope (20X lens; Olympus America, Inc., Center Valley, PA, USA) connected to a digital camera 2 or 4 h of post – H_2O_2 and diamide perfusion. Cell counts were performed manually in layers IV–V of neocortex in rat frontal cortex area 1 or area 2 above the cingulum. Slices were then transferred to physiological saline containing digitonin (200 μmol/L) and PI. After another 20 min incubation period, PI-stained cells were counted once again in the same anatomic locus (Fig. 2a), and this served as a reference value corresponding to maximal cell death (Badaut *et al.* 2005).

Measurement of reactive oxygen species production

Acutely dissociated neurons were incubated for 60 min in standard solution containing 20 μmol/L 2′,7′-dichlorofluorescein diacetate, H_2-DCFDA (Molecular Probes, Eugene, OR, USA). Fluorescence was monitored with Axiovision 4.3 (Carl Zeiss Microimaging, Inc.) using an Axiovert 200 microscope equipped with an EXFO X-Cite 120 fluorescence monitoring system (Photonics Solutions Inc., Mississauga, Ontario, Canada). Images were recorded under phase contrast and fluorescence (exposure time, 100 ms) every minute. Neuronal fluorescence was normalized to background (i.e., cell-free area) fluorescence.

Methods for measuring mitochondria ROS were modified from previously described protocols (Sullivan *et al.* 2004a; Brown *et al.* 2004). Cortical tissue was prepared from Sprague-Dawley rats (~250 g) and then transferred to cold isolation buffer (215 mmol/L

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mannitol, 75 mmol/L sucrose, 0.1% BSA, 20 mmol/L HEPES, 1 mmol/L EGTA, pH = 7.2). Dissected cortical tissue was subjected to multiple rounds of centrifugation (see Brown *et al.* 2004). Synaptoneurosomes were burst in a chilled nitrogen cell disruptor, and then placed atop a discontinuous Percoll gradient. A BCA protein assay kit was used to determine protein concentration by measuring absorbance at 560 nm with a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooskin, VT, USA).

Mitochondrial ROS production was also assessed using H_2DCFDA. Isolated mitochondrial protein (100–150 μg) was incubated in a total volume of 200 μL respiration buffer (125 mmol/L KCl, 0.1% BSA, 20 mmol/L HEPES, 2 mmol/L $MgCl_2$, 2.5 mmol/L KH_2PO_4 at pH 7.2) at 37°C for 15 min in the presence of 10 μmol/L H_2DCFDA using pryuvate (5 mmol/L) and malate (2.5 mmol/L) as respiratory substrates. Relative amounts of ROS generation were detected using a thermostated BioTek Synergy flurorometric plate reader in the absence (basal ROS) or presence of 1 μmol/L oligomycin (to maximize membrane potential and ROS production), and the data expressed as % of control (i.e., no KB added) values. For all experiments, tissues from at least three animals were combined per sample, and all experiments were replicated three times.

Fluorescent spectrofluorophotometer assays

Fractions enriched in mitochondria (50 μg protein/mL) were placed in 2 mL of KCl respiration buffer within a temperature-controlled cuvette at 37°C with 100 nm CaG5 N (excitation 506 nm, emission 532 nm; to monitor extra-mitochondrial Ca^{2+}) and 100 nm TMRE (excitation 550 nm, emission 575 nm; to monitor changes in the mitochondrial membrane potential, $\Delta\Psi_m$) in a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Tokyo, Japan) as previously described (Brown *et al.* 2006). Each time-scan began with a baseline reading followed by addition of 5 mmol/L pyruvate and 2.5 mmol/L malate at 1 min, then 150 μmol/L ADP at 2 min, and 1 μmol/L oligomycin at 3 min. At 5 min, Ca^{2+} was added via a KD Scientific Model 310 Series infusion syringe pump (KD Scientific, Holliston, MA, USA) (80 nmol Ca^{2+}/mg protein per minute) until the mitochondria were no longer able to buffer the added Ca^{2+}. The chemical uncoupler, FCCP, was added toward the end of each run. The spectrofluorophotometer traces, representative of at least three separate independent experiments, were quantified by calculating the average baseline CaG5 N fluorescence readings one minute prior to the beginning of the Ca^{2+} infusion using the Shimadzu Hyper RF software (Shimadzu) and Microsoft Excel. The time-point at which the CaG5 N signal was 150% above the average baseline reading was considred the point at which mitochondria were overloaded and no longer capable of removing Ca^{2+} from the media, indicating onset of mPT.

Results

KB protect against peroxide-induced electrophysiological changes

Layer V pyramidal neurons exposed to 2 mmol/L H_2O_2 exhibited a slow but steady hyperpolarization of their resting membrane potential (RMP): −3.1 ± 0.7 mV in slices (n = 26) and −3.5 ± 0.5 mV in dissociated neurons (n = 7) within 10 min (Figs 1a and b). After washout of H_2O_2, 20 of 26 cells in slices, and seven of seven dissociated neurons showed a rebound depolarization, hyperexcitability and loss of the pipette seal (Figs 1a and b). In voltage-clamp mode (V_H = −60 mV), 10 of 10 isolated cells exhibited a stable current level during H_2O_2 administration, which was interrupted by a large inward current (>1 nA) upon washout of H_2O_2 (Fig. 1b). These findings were similar to those described in thalamo-cortical slices during wash-out of H_2O_2 (Frantseva *et al.* 1998).

In contrast, co-application of 1 mmol/L BHB plus 1 mmol/L ACA and H_2O_2 blocked the robust H_2O_2-induced inward current in eight of ten dissociated neurons, consistent with what we observed in slices (14 of 17 cells) (Figs 1a and c), but did not block the membrane hyperpolarization induced by H_2O_2. This protective effect was seen for up to 30 min after H_2O_2 washout. KB alone exerted no effects on baseline current levels (n = 6; Fig. 1c) or membrane potential (data not shown). Further, the protective effects of KB were dose-dependent; BHB or ACA applied separately (3 mmol/L each) completely blocked the H_2O_2-induced inward current response (n = 7), whereas neither 1 mmol/L BHB nor ACA applied alone blocked this effect (n = 6) (Fig. 1d). These data indicate that – at blood concentrations similar to those achieved during clinical ketotic states (Hasselbalch *et al.* 1995; Thio *et al.* 2000) – KB can protect against acute electrophysiological changes induced by H_2O_2.

KB enhance neuronal viability against oxidative stress

Although our initial electrophysiological experiments suggested a functional protective effect of KB, it was unclear whether these substrates indeed prevented oxidative stress-induced cell death. Using a similar experimental paradigm and PI as a fluorescent marker of cell death, we assessed the effects of KB in neocortical slices exposed to 2 mmol/L H_2O_2. We found that KB significantly decreased neuronal death induced by H_2O_2 (Fig. 2a). In control slices subjected to sham perfusion with physiological saline, 23% ± 2.2 (n = 30) and 23% ± 1.5 (n = 23) (Fig. 2b) of cells demonstrated PI fluorescence at 2 and 4 h post-sham exposure, respectively. In contrast, slices exposed to 2 mmol/L H_2O_2 for 10 min demonstrated 64% ± 3.3 (n = 9) and 75% ± 4.2 (n = 8) cell death 2 and 4 h after exposure, respectively (Fig. 2b). Differences between control and peroxide-treated groups were statistically significant ($p < 0.001$). Co-application of H_2O_2 with KB resulted in a significant increase in cell viability. KB prevented H_2O_2-induced cell death (26% ± 2.6, n = 8 and 33% ± 4.5, n = 9 at 2 and 4 h, respectively) (Fig. 2b).

KB reduce reactive oxygen species in dissociated neurons and isolated mitochondria

Based on these initial findings, we hypothesized that KB might protect against H_2O_2-induced injury via a direct or

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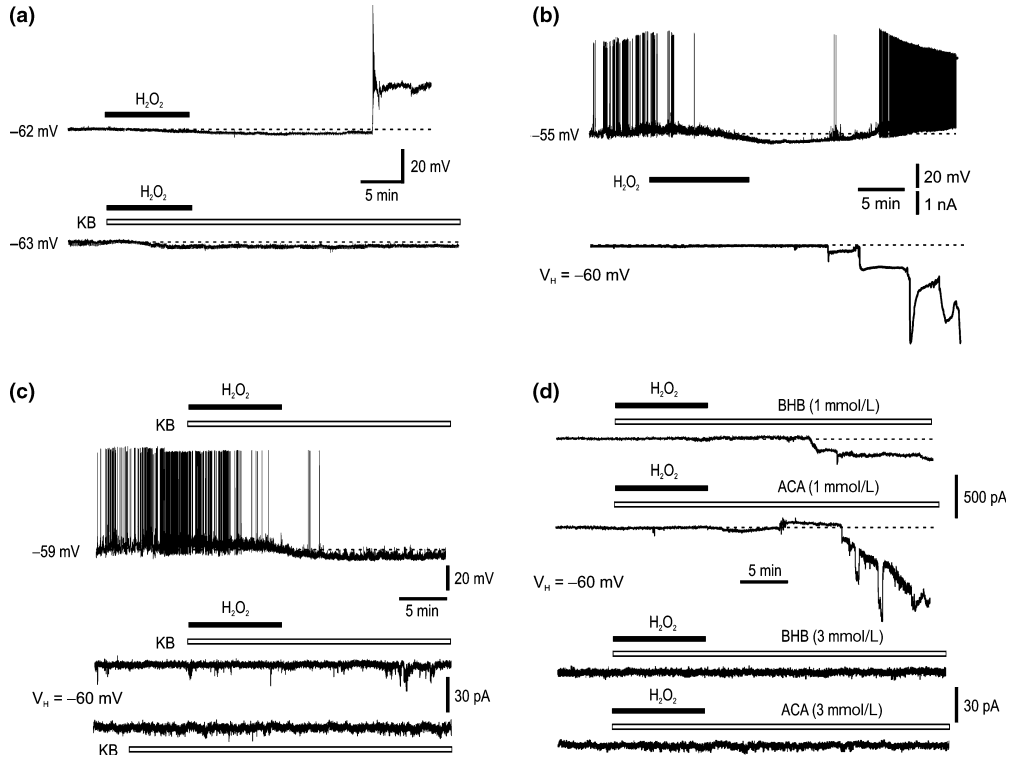

Fig. 1 Ketone bodies protect against H_2O_2-induced injury in neocortical neurons. (a) Hyper-excitability following exposure to 2 mmol/L H_2O_2 in a neocortical slice, reversed by KB (BHB and ACA, 1 mmol/L each). (b, c) In acutely dissociated neocortical neurons, 2 mmol/L H_2O_2 provoked hyper-excitability and >1 nA inward current after washout in current- and voltage-clamp (V_H = −60 mV) modes, respectively. These effects were prevented by co-administration of KB; KB alone did not affect the baseline current trace. (d) Protection against H_2O_2-induced injury by KB was concentration-dependent. No effects on inward current were seen using either 1 mmol/L BHB or 1 mmol/L ACA alone (top), whereas 3 mmol/L concentrations were effective (bottom). The dotted horizontal line in this and following figures indicates the resting membrane potential (RMP) in mV (under current clamp mode), or current average for a 1 min period from the start of recording. Thick horizontal bar indicates drug infusion period.

indirect antioxidant mechanism. To demonstrate this, we first measured ROS levels in acutely dissociated neocortical neurons using 2′,7′-dichlorofluorescein diacetate (DCFDA), a cell-permeant indicator which is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of H_2O_2. In these experiments, fluorescence was quantified on an open scale with a value of 0 representing the absence of a perceivable signal. Cells exposed to 2 mmol/L H_2O_2 exhibited significantly higher DCF fluorescence 10 min after wash-out compared to controls exposed only to buffer ($p < 0.05$, $n = 7$). Ten mmol/L H_2O_2 resulted in yet a further increase in fluorescence ($n = 10$), and the addition of KB (BHB and ACA, 1 mmol/L each) largely prevented this change ($n = 8$) (Figs 3a and b). On a technical note, we were only able to observe DCF fluorescence changes using concentrations of $H_2O_2 \geq 2$ mmol/L, but this apparent disparity between electrophysiological and DCF imaging findings has previously been reported (Hoyt *et al.* 1997).

As mitochondria are the major source of ROS production, we asked whether KB can directly reduce ROS at the mitochondrial level. We found that both BHB and ACA (either alone or in combination) significantly reduced both basal and oligomycin-induced ROS production in isolated mitochondria ($p < 0.01$; Fig. 3c). The magnitude of these effects was similar in all treatment groups, suggesting that the concentrations used were saturating. These experiments strongly suggested that KB either directly inhibited ROS production and/or enhanced ROS degradation in neocortical pyramidal neurons. Although it remains unclear whether KB affect ROS production and/or degradation in neocortex, results from an earlier study suggest that KB may not alter antioxidant capacity in neocortex (Ziegler *et al.* 2003). However, in hippocampus, glutathione peroxidase levels are significantly elevated following a ketogenic diet (Ziegler *et al.* 2003).

KB suppress ROS-independent diamide-induced neuronal injury

To further evaluate the potential antioxidant activity of KB, we conducted electrophysiological experiments in which

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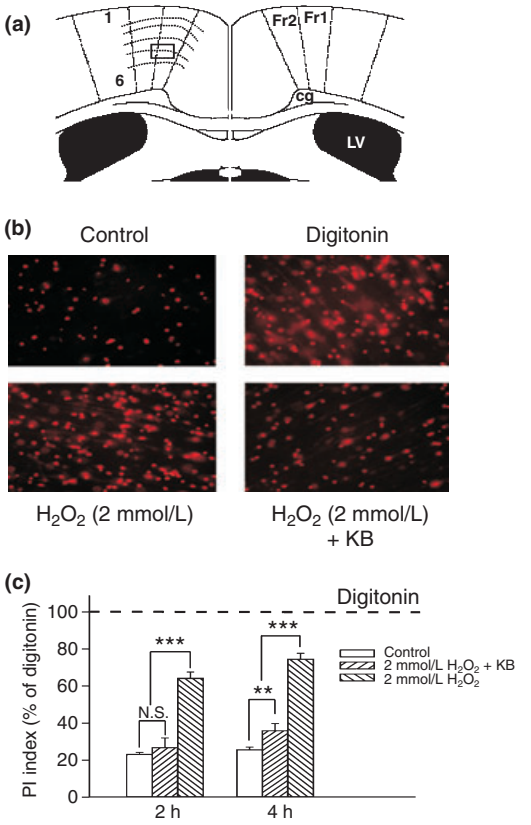

Fig. 2 Ketone bodies protect against H_2O_2-induced cell death measured by propidium iodide (PI) fluorescence. (a) Imaged area of PI staining (rectangular bar) within neocortex layer IV and V of rat cortex shown schematically. The vertical lines are orthogonal to neocortical layers 1 to 6 (Fr: rat frontal cortex; cg: cingulum; LV: lateral ventricle). (b) Representative images of neocortical slices 4 h after treatment with control, 2 mmol/L H_2O_2, 2 mmol/L H_2O_2 plus KB, and digitonin. (c) Summary data of cell death (PI index) in the three treatment groups compared to digitonin two and four hours after H_2O_2 exposure. Values represent group means ± SEM. one-way ANOVA followed by Tukey test; **, $p < 0.01$; ***, $p < 0.001$.

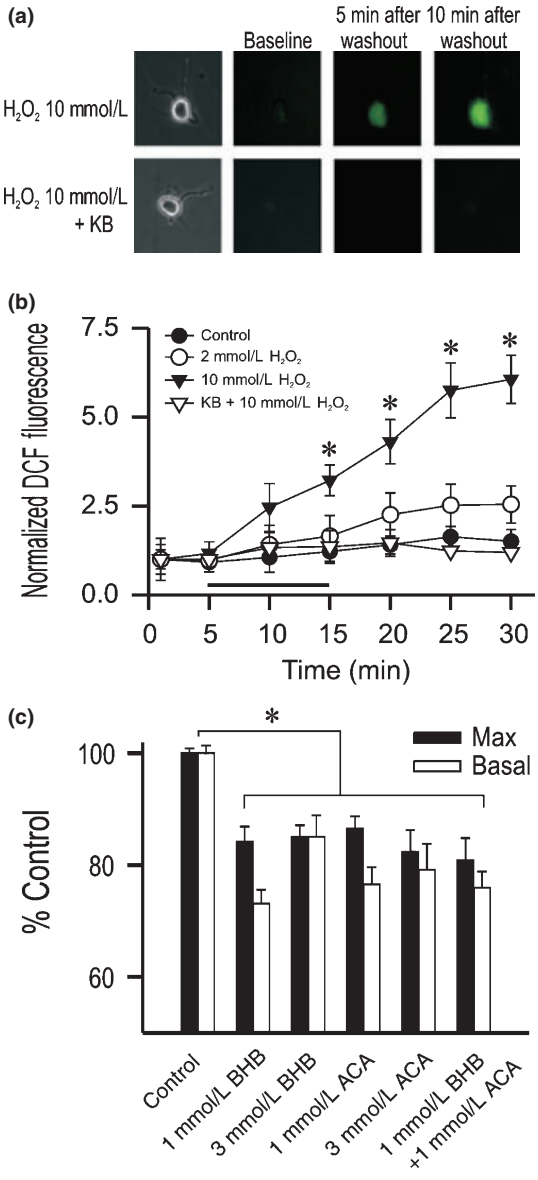

Fig. 3 Ketone bodies decrease reactive oxygen species (ROS) levels. (a) Baseline phase-contrast images of acutely dissociated neocortical neurons (left). H_2O_2 produced a significant increase in DCF signal after washout (top), whereas co-application with KB resulted in a barely perceivable DCF signal increase (bottom). (b) Summary of DCF signal changes. All groups displayed similar levels of fluorescence at baseline. In the control group, neurons were only exposed to the HEPES buffer but displayed a slight increase in DCF signal over time. H_2O_2 (2 mmol/L and 10 mmol/L) produced concentration-dependent increases in DCF levels that were largely blocked by KB. (c) Both basal and maximal (i.e., oligomycin-induced) ROS levels in isolated mitocondria were significantly decreased by KB compared to control levels of ROS (one-way ANOVA or student t-test; *, $p < 0.01$).

oxidative stress was induced by the thiol oxidant diamide. It is well known that diamide induces neuronal injury primarily by enhancing mPT opening either through glutathione depletion (Armstrong and Jones 2002) or oxidation of thiol groups on the adenine nucleotide translocator (ANT), a component of the mPT complex (McStay *et al.* 2002; Halestrap and Brennerb 2003). Endogenous free radical generation and cell death are a direct consequence of diamide's effects in directly oxidizing glutathione to glutathione disulfide.

Following a paradigm used for the H_2O_2 experiments, we found that a 10 min application of diamide (500 μmol/L and 1 mmol/L) induced a large inward current and irreversible hyperexcitability in isolated neocortical neurons, as well as in pyramidal neurons in neocortical slices ($n = 7$ and $n = 8$, respectively) within several minutes after washout (Figs 4a and b). And, as predicted, co-application of KB (BHB and ACA, 1 mmol/L each) and diamide blocked the inward current (seven of eight cells) and hyperexcitability (eight of

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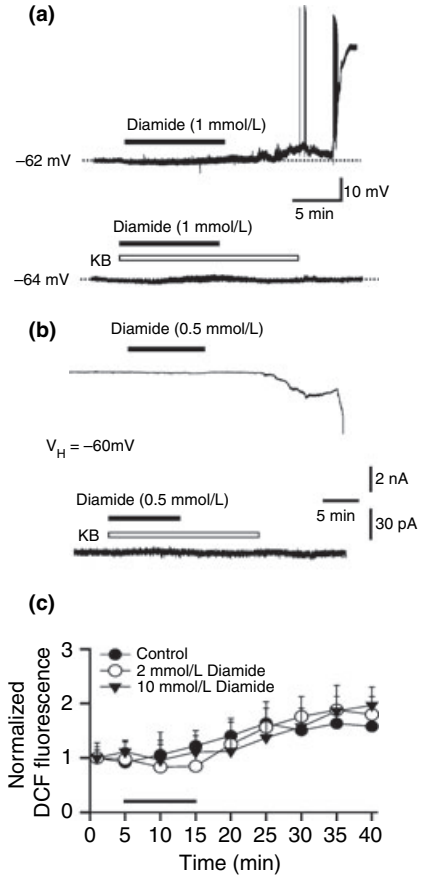

Fig. 4 Ketone bodies block diamide-induced oxidative stress independent of reactive oxygen species (ROS). (a) Hyper-excitability after 1 mmol/L diamide application in a neocortical slice, reversed by co-administration KB. (b) Acutely dissociated neurons expressed large (>1 nA) inward currents after washout of 500 μmol/L diamide, an effect which was reversed by KB. (c) No significance differences were seen between diamide and control groups in DCF fluorescence, indicating an absence of significant changes in ROS levels.

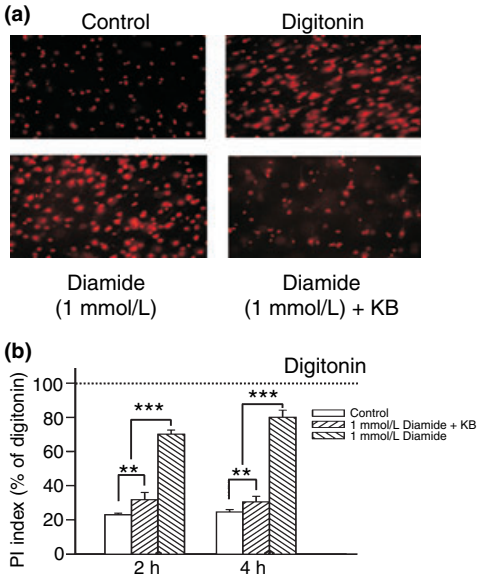

Fig. 5 Ketone bodies are protective against diamide-induced cell death, measured by propidium iodide (PI) fluorescence. (a) Representative images of neocortical slices 4 h after treatment with control, 1 mmol/L diamide, 1 mmol/L diamide plus KB, and digitonin. (b) Summary data of cell death (PI index) in the three treatment groups compared to digitonin two and four hours after diamide exposure. Values represent group means ± SEM. one-way ANOVA followed by Tukey test; **, $p < 0.01$; ***, $p < 0.001$; N.S., not significant.

nine cells) (Figs 4a and b). Interestingly, despite similar electrophysiological findings following H_2O_2 or diamide application, diamide (2 or 10 mmol/L) itself did not significantly change ROS levels as determined with DCF fluorescence in acutely isolated neurons ($n = 9$; Fig. 4c). These data suggested that diamide-induced acute oxidative injury may be mediated by activation of mPT, and not through an increase in ROS levels.

To confirm whether KB were also neuroprotective against diamide-induced oxidative injury, further assessments of cell death were conducted in intact neocortical slices using PI as a marker. As expected, KB significantly decreased neuronal death induced by diamide (Fig. 5a). Slices exposed to 10 min of diamide demonstrated PI fluorescence in 71 ± 1.8% ($n = 8$) and 80 ± 2.7% ($n = 8$) of cells after 2 and 4 h, respectively (Fig. 5b). Differences between diamide and control groups were statistically significant ($p < 0.001$). Co-application of KB and diamide resulted in significant protection; after 2 and 4 h following diamide exposure, 32 ± 3.5% ($n = 8$) and 32 ± 4.8% ($n = 8$) of cells were positively labeled with PI (differences between diamide and KB plus diamide groups, $p < 0.001$) (Fig. 5b).

Antioxidants protect against H_2O_2- or diamide-induced neuronal injury

To evaluate the effects of known antioxidants in our models of oxidative stress, we tested the effects of catalase and DL-dithiothreitol (DTT, a disulfide reducing agent) in the presence of either H_2O_2 or diamide in acute neocortical slices. As expected, co-application of catalase (1000 U) and 2 mmol/L H_2O_2 fully blocked H_2O_2-induced neuronal excitability after washout in 10 of 11 cells (Fig. 6a). However, since catalase is extremely efficient at decomposing H_2O_2, it is possible that the cells may not have been fully exposed to 2 mmol/L H_2O_2. To address this issue, we applied catalase after the start of H_2O_2 infusion. Interestingly, catalase (1000 U) partially blocked H_2O_2-induced neuronal injury in five of ten cells (Fig. 6a). By contrast, 250 U catalase failed to exert neuroprotective effects in either the co-application group (six of seven cells) or the applied group of catalase after H_2O_2 infusion (seven of eight neurons) (Fig. 6b). DTT (0.5 mmol/L) fully blocked both H_2O_2- and diamide-induced neuronal injury (eight of ten

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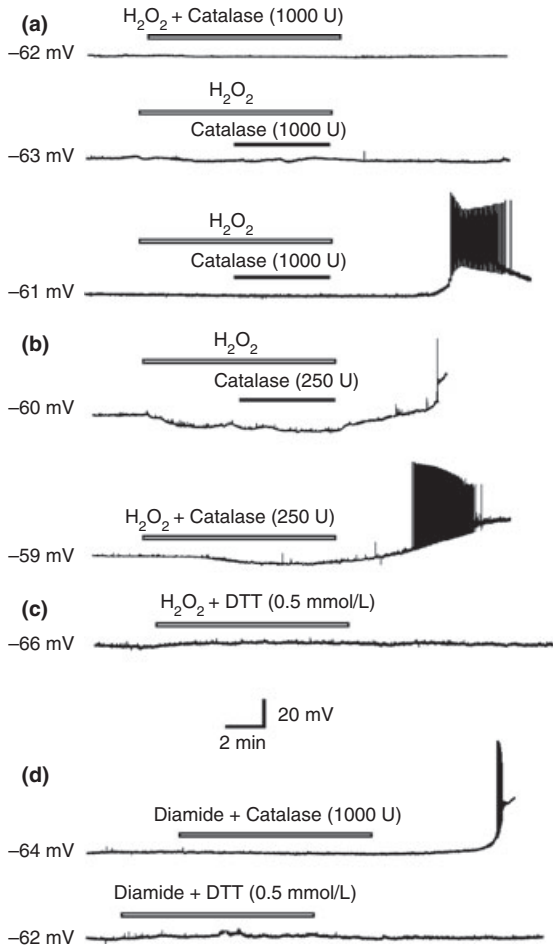

Fig. 6 The effects of antioxidants against H_2O_2- or diamide-induced neuronal injury. (a) Co-application of H_2O_2 and catalase (1000 U) fully inhibited H_2O_2-induced hyper-excitability after washout, whereas catalase applied after start of H_2O_2 infusion blocked neuronal injury in 50% of cells. (b) A lower concentration of catalase (250 U) did not rescue neurons from H_2O_2-induced oxidative injury. (c) DL-dithiothreitol (DTT) blocked 2 mmol/L H_2O_2-induced oxidative stress. (d) Co-application of diamide and catalase (1000 U) did not suppress diamide-induced hyper-excitability, whereas DTT alone blocked diamide-induced hyper-excitability.

cells and nine of eleven cells, respectively) (Figs 6c and d), whereas catalase (1000 U) did not block diamide-induced neuronal excitability (seven of eight cells) (Fig. 6d). These results indicate that diamide-induced neuronal injury seen in our model was not mediated by ROS.

Inhibitors of mPT mimic the effects of KB

Based on our diamide experiments, we hypothesized that blockers of mPT might mimic the electrophysiological effects of KB. Thus, we examined the effects of mPT inhibition on H_2O_2- and diamide-induced neuronal injury using two specific blockers of the putative mPT pore complex: cyclosporin A (CsA, which binds to mitochondrial cyclophilin D) and bongkrekic acid (BKA, an adenine nucleotide transporter [ANT] inhibitor; Biomol, Le Perray-en-Yveline, France). We found concentration-dependent effects of mPT blockers in both models of oxidative stress. Both 50 μmol/L BKA (n = 9) and CsA (50 and 100 μmol/L; n = 6) completely blocked diamide and H_2O_2-induced inward current (Figs 7a and b). By contrast, 25 μmol/L BKA showed only partial blockade in these two models (n = 10; Figs 7a and b), and 10 μmol/L CsA was ineffective (data not shown, n = 7). BKA by itself produced no changes in the magnitude of inward current (n = 6; Fig. 7b).

To provide further evidence that KB could inhibit mPT, we measured the thresholds for calcium-induced mPT in isolated mitochondria. CsA (1 μmol/L), BHB (3 mmol/L) and ACA (3 mmol/L) all significantly increased the threshold for calcium-induced mPT compared with the control group ($p < 0.05$; Figs 7c and d); 1 mmol/L BHB and 1 mmol/L ACA were also each capable of inhibiting mPT (Fig. 7d). However, CsA appeared to exert a greater inhibitory effect, as its calcium threshold was significantly higher than all other treatment groups ($p < 0.01$) (Figs 7c and d).

Discussion

The principal finding of this study is that BHB and ACA, at clinically relevant concentrations, protected neocortical neurons from oxidative injury induced by H_2O_2 and by the thiol oxidant diamide. Using these models of oxidative stress, we initially demonstrated that in both acutely dissociated neurons and in intact brain slices, KB prevented oxidative-stress induced hyperexcitability, reflected by rebound depolarization following washout and increased membrane conductance. These electrophysiological changes – while alone are not necessarily indicative of cell death – appeared to correlate with our results with propidium iodide, demonstrating that KB significantly reduced neuronal death in intact neocortical brain slices induced by H_2O_2 or diamide. Finally, our results indicated that KB can increase the threshold for mPT activation, most likely by decreasing mitochondrial ROS levels.

Oxidative stress is believed to represent an important pathogenic mechanism in seizure genesis (and possibly epileptogenesis). Earlier attempts to elucidate underlying mechanisms have relied on exogenous administration of H_2O_2 or diamide to mimic acute oxidative stress *in vitro* (Avshalumov and Rice 2002; Chang *et al.* 2003; Smith *et al.* 2003). Millimolar concentrations of H_2O_2 were shown to induce epileptiform activity in thalamocortical slices (Frantseva *et al.* 1998) and irreversible depolarization in cultured intestinal neurons (Vogalis and Harvey 2003). The current study demonstrates that KB prevent similar cellular electrophysiological changes (i.e., increased membrane excitability and conductance) induced by either model of oxidative stress in neocortical neurons.

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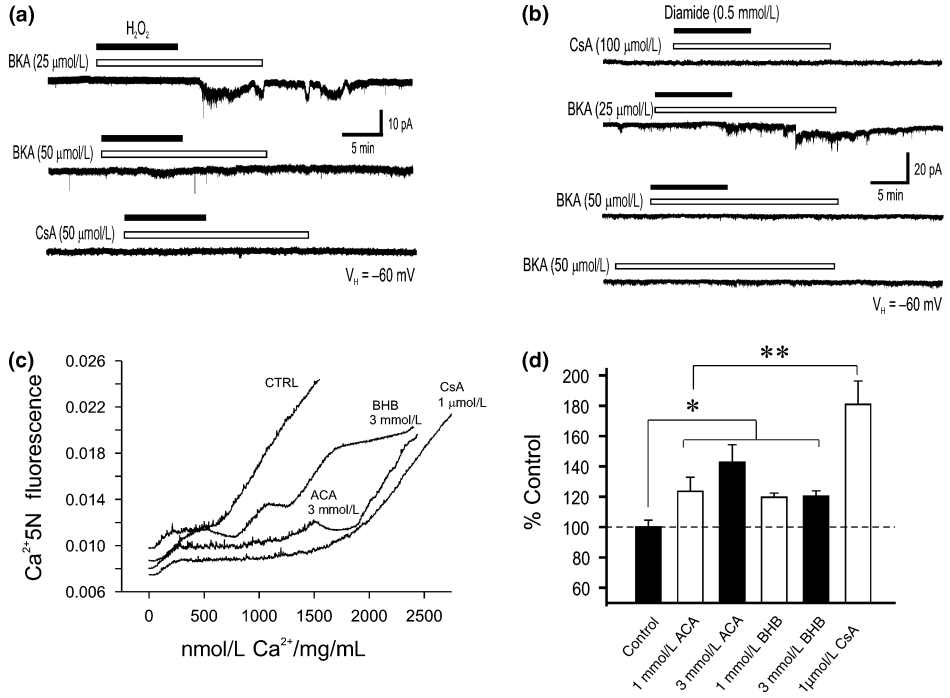

Fig. 7 Ketone bodies mimic the effects of mPT inhibitors. (a, b) H_2O_2- or diamide-induced inward current was reversed by mPT blockers in a dose-dependent manner. (c) Representative traces depicting thresholds for calcium-induced mPT in isolated neocortical mitochondria. BHB, ACA, and CsA all effectively raised the threshold for calcium-induced mPT. (d) Summary data demonstrating that KB significantly increase the thresholds for calcium-induced mPT. Cyclosporin A most potently increased the Ca^{2+} concentration necessary for mPT pore opening compared to all other groups. *, $p < 0.05$ or **, $p < 0.01$ by one-way ANOVA or t-test.

Underscoring the role of oxidative modulation of cellular homeostasis, enhanced antioxidant capacity has been shown to prevent neuronal hyperexcitability and damage (MacGregor *et al.* 1996; Medina *et al.* 2002). For example, hyperexcitability induced by NMDA receptor activation or by increased calcium influx through a non-specific cation channel can be blocked by application of ROS scavengers (Frantseva *et al.* 1998; Avshalumov and Rice 2002; Smith *et al.* 2003). Similarly, we have shown that the electrophysiological effects of KB in neurons exposed to H_2O_2 or diamide can be mimicked by known antioxidants such as catalase and DTT. While there is no reason to believe that KB – based on their structure – should act as antioxidants in the classical sense, our data support the hypothesis that KB reduce oxidative stress in neocortical neurons by decreasing ROS levels.

In the present study, KB prevented the increase in ROS levels following H_2O_2 application in acutely dissociated neurons and isolated mitochondria. However, it is not clear whether changes in ROS levels, reflected by DCF signal changes, are a result of KB directly inhibiting ROS production and/or enhancing ROS degradation. The interpretation that KB may decrease ROS production is supported by earlier studies demonstrating that ketone bodies improve mitochondrial respiration and ATP production, possibly through their higher inherent energy content relative to pyruvate, the normal mitochondrial fuel produced by glycolysis (Tieu *et al.* 2003; Masuda *et al.* 2005). Interestingly, we did not observe increased ROS levels following diamide treatment, indicating that the diamide-induced electrophysiological effects may in part be independent of ROS levels. These results support the notion that ROS scavengers do not block diamide-induced injury in *Aplysia* neurons (Chang *et al.* 2004). Collectively, our data suggest that KB may exert both ROS-dependent and independent effects on oxidative stress-induced hyperexcitability.

In previous studies, BHB protected cultured dopaminergic mesencephalic neurons against 1-methyl-4-phenylpyrinidium (MPP^+) and beta-amyloid peptide$_{1-42}$ toxicity by decreasing mitochondrial ROS generation (Kashiwaya *et al.* 2000). In contrast, another study suggested that BHB-derived NADH enhances mitochondrial ROS generation through accumulation of electrons upstream mediated by MPP^+ and rotenone-induced neurotoxicity (Tieu *et al.* 2003). This discrepancy can be explained in part by the authors' use of rotenone, which would result in such inhibition of complex I of the respiratory chain that no electrons could be accepted from NADH, and as such KB would be expected to increase both ROS and NADH levels. Further, there may be differences between intact normal

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mitochondria and mitochondria exposed to neurotoxicity (Armstrong and Jones 2002).

One major consequence of oxidative stress and cellular increases in calcium can be the onset of the catastrophic mPT, and it is well known that antioxidants can prevent mPT (Fiskum *et al.* 2004; Stavrovskaya and Kristal 2005). Indeed, in isolated mitochondria, Ca^{2+}-induced mPT was prevented by both KB, as well as the mPT inhibitors CsA and BKA. Furthermore, CsA and BKA prevented the electrophysiological changes induced by diamide and H_2O_2, similar to the protective effects seen with KB in these two models. Given these findings, it is possible that KB may interact with the mPT complex itself (Costantini *et al.* 2000; Clarke *et al.* 2002; Nakagawa *et al.* 2005). However, a more likely explanation is that the effects of KB on mPT may be a consequence of the aforementioned decrease in ROS levels, which would secondarily raise the threshold for mPT.

Our observation that KB directly increased the threshold for Ca^{2+}-induced mPT was somewhat surprising since an earlier study revealed that 1 mmol/L ACA actually slowly promoted activation of mPT in isolated rat liver mitochondria (Zago *et al.* 2000). In contrast to the study by Zago *et al.* (2000), we found that 1 and 3 mmol/L ACA significantly elevated the threshold for Ca^{2+}-induced mPT in isolated neocortical mitochondria; facilitation of mPT was never observed, even at lower ACA concentrations (100–300 μmol/L). Although the basis of this discrepancy remains unclear, tissue-specific differences in mitochondria may exist (Sullivan *et al*. 2004b). Interestingly, in our electrophysiological experiments, we found that neither 1 mmol/L ACA nor 1 mmol/L BHB alone, however, was sufficient to block H_2O_2- or diamide-induced hyperexcitability. On the other hand, higher concentrations of KB, either alone or in combination, afforded complete protection, results which were paralleled by a KB-induced decrease in ROS levels. These results suggest that KB may exert at least two specific effects on neocortical neurons: protection against ROS-induced cell membrane hyperexcitability as a result of decreased ROS levels, and inhibition of mPT that may also be ROS-dependent. While our data do not directly demonstrate that a reduction in ROS levels leads to an inhibition of mPT, it is reasonable to speculate that KB may act in this manner since oxidative stress has previously been shown to induce mPT (Sullivan *et al.* 2004b). KB may interact with the mPT complex itself, but there are no direct data to support this. Alternatively, KB may protect against oxidative injury through a different mechanism, perhaps through improved mitochondrial respiration and ATP production (Tieu *et al.* 2003; Masuda *et al.* 2005).

The induction of mPT is associated with cell death (Stavrovskaya and Kristal 2005). Given that KB prevented hyperexcitability in neurons and inhibited mPT in mitochondria, we hypothesized that KB would protect against oxidative stress-induced cell death. Indeed, application of either H_2O_2 or diamide caused extensive cell death and co-application of KB resulted in significant protection. These results are consistent with prior observations that KB play a critical role in suppressing neuronal apoptosis and necrosis through actions on pro-apoptotic factors caspase-3 and cytochrome C (Paulson *et al.* 1988; Noh *et al.* 2003). A possible interpretation of our results is that oxidative stress, in a feed-forward fashion, increases ROS levels, neuronal hyperexcitability, and both intracellular and mitochondrial calcium concentrations. These changes result in activation of mPT and ultimately cause cell death. KB exert neuroprotective effects, most likely by decreasing ROS levels, and thereby preventing the downstream effects of oxidative stress.

In conclusion, much of the evidence to date indicates a neuroprotective function for KB (Kashiwaya *et al.* 2000; Veech 2004). Calorie restriction (CR), which results in systemic ketosis, can extend life span in mammals through antioxidant effects (Sohal and Weindruch 1996); additional support is provided by a recent human study demonstrating that short-term CR accompanied by elevation of BHB levels diminished oxidative stress (Skrha *et al.* 2005). On the other hand, such effects may be mediated not by antioxidant activity, but by enhancement of nitric oxide levels (Nisoli *et al.* 2005). Although it remains unclear whether KB (generated in the context of CR) enhance longevity, our results are consistent with prior observations that KB are neuroprotective (Paulson *et al.* 1988; Noh *et al.* 2006). Ketosis is a metabolic adaptation seen with fasting, calorie restriction, and a high-fat anticonvulsant ketogenic diet. A mechanistic understanding of the neuroprotective role of KB may ultimately lead to a novel therapeutic approach toward the treatment of various neurological disorders associated with oxidative stress.

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