

Acute oxidative stress and systemic Nrf2 activation by the ketogenic diet

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ABSTRACT

The mechanisms underlying the efficacy of the ketogenic diet (KD) remain unknown. Recently, we showed that the KD increased glutathione (GSH) biosynthesis. Since the NF E2-related factor 2 (Nrf2) transcription factor is a primary responder to cellular stress and can upregulate GSH biosynthesis, we asked whether the KD activates the Nrf2 pathway. Here we report that rats consuming a KD show acute production of H₂O₂ from hippocampal mitochondria, which decreases below control levels by 3 weeks, suggestive of an adaptive response. 4-Hydroxy-2-nonenal (4-HNE), an electrophilic lipid peroxidation end product known to activate the Nrf2 detoxification pathway, was also acutely increased by the KD. Nrf2 nuclear accumulation was evident in both the hippocampus and liver, and the Nrf2 target, NAD(P)H:quinone oxidoreductase (NQO1), exhibited increased activity in both the hippocampus and liver after 3 weeks. We also found chronic depletion of liver tissue GSH, while liver mitochondrial antioxidant capacity was preserved. These data suggest that the KD initially produces mild oxidative and electrophilic stress, which may systemically activate the Nrf2 pathway via redox signaling, leading to chronic cellular adaptation, induction of protective proteins, and improvement of the mitochondrial redox state.

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Introduction

The ketogenic diet (KD) has been successfully utilized since the 1920s as a treatment for intractable seizures in children. The diet consists of a regimen of high-fat and low-carbohydrate, most often in a 4:1 ratio, fat to nonfat. Despite its lengthy history, the mechanisms by which it confers an anticonvulsant effect remain unknown. It has been noted in the clinic, as well as in animal studies, that there is a lag between initiation of the diet and decrease in seizure frequency (Uhlemann and Neims, 1972; Appleton and DeVivo, 1974; Rho et al., 1999; Kossoff et al., 2008). To date, the mechanisms underlying this phenomenon are poorly understood. Recent evidence suggests that chronic consumption of a KD may alter mitochondrial function by chronically decreasing production of reactive oxygen species (ROS), increasing the expression of uncoupling proteins, promoting mitochondrial biogenesis, and stimulating glutathione (GSH) biosynthesis (Sullivan et al., 2004; Bough et al., 2006; Jarrett et al., 2008), all of which can be components of a cellular stress response (Shi et al., 1994; Tian et al., 1997; Sagara et al., 1998; Woods et al., 1999).

The primary transcription factor responsible for initiating the response to oxidative stress is NF E2-related factor 2 (Nrf2). Nrf2 is a

member of the cap 'n' collar family of basic leucine zipper proteins. Under normal conditions, Nrf2 is sequestered in the cytosol through its interaction with an inhibitory binding partner, Keap1. At steady state, Keap1 acts as an adaptor for an E3 ubiquitin ligase, thereby targeting Nrf2 to the proteasome for degradation (Kobayashi et al., 2004). Oxidants and electrophiles can alter the binding interaction between Keap1 and Nrf2, allowing Nrf2 to dissociate and translocate into the nucleus where it binds DNA at a consensus sequence known as the antioxidant or electrophilic response element (ARE/EpRE) (Rushmore et al., 1991; Venugopal and Jaiswal, 1996). In this way, Nrf2 regulates transcription of a large group of detoxification genes, among which are the subunits of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH biosynthesis, which we previously found to be upregulated in the hippocampus of rats fed a KD for 3 weeks (Jarrett et al., 2008). The goal of this study was to determine whether this cellular detoxification pathway was activated by the KD, as the Nrf2 pathway is a primary mechanism by which to induce GSH biosynthesis. Here we report that the KD produced a time-dependent change in hippocampal mitochondrial H₂O₂ production, increases in 4-hydroxy-2-nonenal (4-HNE) levels, and Nrf2 activation, which may play a role in the protection afforded by the KD. Furthermore, in addition to the brain, we examined the possibility that the same pathways may be activated in the liver, due to the systemic effects of a dietary treatment. We found increased nuclear Nrf2 protein in the liver, as well as increased Nrf2 targets NAD(P)H:quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1). Since the liver is known to supply extrahepatic tissues with GSH, we examined the

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effects of the KD on liver GSH levels. A profound depletion of liver tissue GSH, accompanied by improved mitochondrial antioxidant capacity, was observed.

Materials and methods

Reagents

All reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Animals and diet protocol

Animal housing and all animal studies were conducted in compliance with University of Colorado Denver IACUC procedures and protocols. Adolescent male Sprague-Dawley rats (P28) were housed under a 12-h light/dark cycle with water *ad libitum*. Rats were fasted 8–10 h before initiation of KD or control diet (F3666 or F3517, respectively; Bio-Serv, Frenchtown, NJ). Diets were fed isocalorically, calorie-restricted to 90% of the recommended daily requirement, and maintained for 1 day, 3 days, 1 week, or 3 weeks. The KD was composed of 78% fat and 0.76% carbohydrate, while the control diet was 5% fat and 65% carbohydrate. Diets were matched for vitamins and minerals. KD-fed rats weighed significantly less than controls by the end of the 3-week time period (146.05 ± 4.37 g in KD vs. 201.33 ± 12.44 g in controls, values represent mean \pm SEM, $n=6$ rats per group, two-tailed *t*-test revealed $***p \leq 0.001$). This weight difference is consistent with animal studies, as well as children on the KD (Likhodii et al., 2002; Thio et al., 2006; Neal et al., 2008).

Mitochondrial isolation

Hippocampal mitochondria were isolated via a Percoll density gradient centrifugation procedure as previously described (Castello et al., 2007). Briefly, hippocampi were homogenized with a Dounce tissue grinder (Wheaton, Millville, NJ) in isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris-HCl, 1 mM EDTA, pH 7.4) and then diluted 1:1 in 24% Percoll. Homogenates were centrifuged at $30,700 \times g$ at 4°C for 10 minutes. The sediment was subjected to Percoll density gradient (19% on 40%) centrifugation at $30,700 \times g$ at 4°C for 10 minutes. The material located at the interface of the lowest two layers was slowly diluted 1:4 with mitochondrial isolation buffer containing 1 mg/ml bovine serum albumin and centrifuged at $6700 \times g$ at 4°C for 10 minutes to obtain final pellets consisting of respiring mitochondria. The final pellet was resuspended in fresh isolation buffer and used immediately.

Measurement of hydrogen peroxide (H_2O_2) production

H_2O_2 production in pure mitochondria was measured by fluorometric detection using the horseradish peroxidase-linked Amplex UltraRed assay (Molecular Probes, Carlsbad, CA) as previously described (Castello et al., 2007). Briefly, cellular fractions (10 μg) were added to a 96-well plate containing 100 μl of reaction buffer containing 0.1 U/ml horseradish peroxidase, 50 μM Amplex UltraRed, and 10 mM succinate. Resorufin fluorescence was monitored on a microplate reader equipped for excitation in the range of 530–560 nm and emission detection at 590 nm. Production of fluorescence was monitored continuously for 30 minutes.

Preparation of nuclear fractions

Nuclear fractions were prepared using the Pierce NE-PER reagent kit (Pierce Biotechnology, Rockford, IL). Samples were prepared according to the protocol provided. All samples were stored at -80°C until use.

Western blot analysis

Tissue or subcellular fractions were run on 10% acrylamide gels for immunoblot analysis. Antibodies used were anti-Nrf2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin (1:500; Sigma, St. Louis, MO), anti-HO-1 (1:1000; Stressgen, Ann Arbor, MI) and goat anti-rabbit (1:20,000 or 1:25,000). Antibodies were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and bands were imaged on a Storm Optical Scanner (Molecular Dynamics Inc, Sunnyvale, CA).

NQO1 activity assay

NQO1 enzyme activity was measured as described by Dehn et al. (2006). Brain or liver tissue was homogenized and sonicated in cold sample buffer (25 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 μM FAD) and centrifuged at $13,000 \times g$ for 15 minutes at 4°C . The supernatant was transferred to a new tube and centrifuged at $16,000 \times g$ for 60 minutes at 4°C . The supernatant was collected and used in the assay. Protein concentrations were determined using the Bradford method. Absorbance was read for 1 minute on a spectrophotometer set at 600 nm. NQO1 activity was defined as the dicumarol-inhibitable reduction of 2,6-dichlorophenol-indophenol at 600 nm at 30°C .

HPLC analysis of 4-hydroxy-2-nonenal (4-HNE)

Measurement of 4-HNE was performed by HPLC with UV detection as previously described (Murphy et al., 2003), with minor modifications. Briefly, tissue was sonicated in 40% acetonitrile with 100 μg of BHT. Samples were incubated at room temperature for 30 minutes, vortexing every 10 minutes to facilitate extraction of 4-HNE. About 4 N PCA was then added to each sample at a ratio of 1:40 and then mixed well. Samples were then centrifuged at $16,000 \times g$ for 15 minutes at 4°C . Supernatant was analyzed for levels of 4-HNE using a CoulArray system (Model 5600; ESA) equipped with a UV detector set at 221 nm and a 5 μM , 150×4.6 mm YMC ODS-A C18 column. The mobile phase was composed of 35% acetonitrile with 0.3% phosphoric acid, and the flow rate was set at 0.7 ml/min.

HPLC analysis of GSH

GSH was measured by HPLC equipped with electrochemical detection as previously described (Liang and Patel, 2006). Analytes were detected using a CoulArray system (Model 5600; ESA) on two coulometric array cell modules, each containing four electrochemical sensors attached in series. Electrochemical detector potentials were 120/355/480/560/680/800 mV (vs Pd). Liver tissue was sonicated in 0.1 N perchloric acid (1:20, wt./vol.) before thawing to prevent artificial formation of thiol disulfide during sample preparation. The homogenates were centrifuged at $13,000 \times g$ at 4°C for 15 minutes. Aliquots of the supernatant were injected into the HPLC and separated on a 5- μm 4.6×150 mm YMC ODS-A column (Waters Com., Milford, MA). The mobile phase was composed of 125 mM sodium phosphate and 1% methanol, pH 2.8, and a flow rate of 0.7 ml/min was maintained. GSH peaks were verified by adding standard solutions of known concentrations to the samples.

HPLC analysis of CoASH

Reduced coenzyme A (CoASH) was measured by HPLC equipped with UV detection as previously described (Liang and Patel, 2006). Briefly, liver tissue was sonicated in 0.1 M sodium phosphate containing 25 mM *N*-ethylmaleimide (NEM) buffer (pH 7.4) before thawing. The tissue was acidified with equal volumes of 4% HClO_4 and centrifuged at $12,000 \times g$ at 4°C for 20 minutes. Aliquots of supernatant were separated on a Zorbax SB-C18 column using the following

mobile phases: (A) 10% methanol and 10 mM tetrabutylammonium hydrogen sulfate (pH 5.0) and (B) 85% methanol and 10 mM tetrabutylammonium hydrogen sulfate (pH 5.0). The gradient was 5 minutes at 100% A, 0% B, followed by a 30-minute linear gradient to 40% A and 60% B. Peak height was quantified by UV detection at 254 nm. The mixture was analyzed by HPLC using individual standard solutions of known concentrations.

Statistical analyses

All data are presented as mean \pm standard error of the mean. Statistical analysis was performed using GraphPad Prism 5.0 (San Diego, CA). Experimental groups were compared using a two-tailed *t*-test, one-way ANOVA with Bonferroni's post hoc test, or two-way ANOVA. A value of $p \leq 0.05$ was considered statistically significant.

Results

Evidence of acute oxidative stress in rats on a KD

Previously, we reported that rats fed a KD for 3 weeks exhibited increased hippocampal GSH biosynthesis (Jarrett et al., 2008). In an effort to determine the mechanism by which this may occur, we hypothesized a role for the Nrf2 pathway, as this transcription factor is the primary means of inducing transcription of both subunits of GCL, the rate-limiting enzyme in GSH biosynthesis (Wild et al., 1999). Since low levels of ROS, such as H_2O_2 , can activate the Nrf2 pathway, we asked whether the KD altered mitochondrial H_2O_2 production. A previous study reported uncoupled brain mitochondria and decreased mitochondrial ROS production in mice fed a KD for at least 1 week (Sullivan et al., 2004). We hypothesized that an increase in mitochondrial H_2O_2 production may be an early consequence of switching to a KD, thus activating the Nrf2 pathway and a chronic adaptive response. Rats were fed a control or KD for 1 day, 3 days, 1 week, or 3 weeks. At each time point, we isolated hippocampal mitochondria and assessed succinate-driven H_2O_2 production. As hypothesized, we observed that hippocampal mitochondria from KD-fed rats produced greater substrate-driven H_2O_2 compared to controls after only 1 day (Fig. 1). The time course of hippocampal mitochondrial H_2O_2 production revealed increased production in the KD group after 1 day, followed by a significant decrease after 3 weeks (Fig. 1). The latter is in agreement with a previous study in which ROS production was decreased in animals maintained on a KD for more than 1 week (Sullivan et al., 2004). Thus, we observed a time-dependent change in mitochondrial H_2O_2 production following initiation of the

KD: increased production at early time points followed by decreased production at chronic time points.

As further evidence of acute oxidative stress, we asked whether mitochondrial GSH was preferentially oxidized to GSSG following initiation of the KD, before increasing at chronic time points, which we have already reported (Jarrett et al., 2008). After 3 days on the KD, we found a significant increase in GSSG levels in hippocampal mitochondria compared to controls (0.0488 ± 0.004 nmol/mg protein in KD vs. 0.0390 ± 0.003 nmol/mg protein in control, values represent mean \pm SEM, $*p \leq 0.05$), while GSH levels remained unchanged. Increased GSSG levels suggest a more oxidized state compared to controls. These data, together with H_2O_2 production, suggest that an early low level of oxidative stress occurs in response to the KD.

Finally, since the Nrf2 pathway can be activated by electrophiles in addition to ROS, we assessed the levels of 4-HNE, an electrophilic byproduct of lipid peroxidation. This is particularly important given the high-fat composition of a KD. Hippocampal homogenates were analyzed for 4-HNE levels at 3 days, 1 week, and 3 weeks after initiation of either a control or KD. 4-HNE levels were higher in KD hippocampi compared to controls at the 3-day time point, further suggesting that mild oxidative stress occurs shortly following initiation of a KD (Fig. 2).

Nrf2 pathway activation in hippocampus of KD-fed rats

Oxidative and electrophilic stresses are known to activate the Nrf2 pathway to mount an adaptive response (Numazawa et al., 2003; Levonen et al., 2004; Kobayashi et al., 2006; Satoh et al., 2006). Since we observed acute increases in H_2O_2 and 4-HNE, we asked whether consumption of a KD activates Nrf2 by assessing nuclear expression of the protein, which is indicative of nuclear translocation. We fractionated hippocampal tissue from rats fed control or KD for 3 days, 1 week, or 3 weeks and assessed nuclear levels of Nrf2 protein by Western blot analysis. Nuclear Nrf2 protein levels in KD-fed rat hippocampus were significantly greater than controls after 1 week on the KD and remained chronically elevated at 3 weeks (Fig. 3).

To further confirm activation of the Nrf2 pathway in the brains of KD-fed rats, we assessed activity of the Nrf2-dependent gene product, NQO1. NQO1 is an enzyme that detoxifies quinones by reducing them to their corresponding hydroquinones and is a prototypical Nrf2 target (Venugopal and Jaiswal, 1996). After 3 weeks on either diet, NQO1 activity was greater in the hippocampus of rats fed a KD compared to controls, further suggesting an activation of the Nrf2 detoxification pathway (Fig. 4).

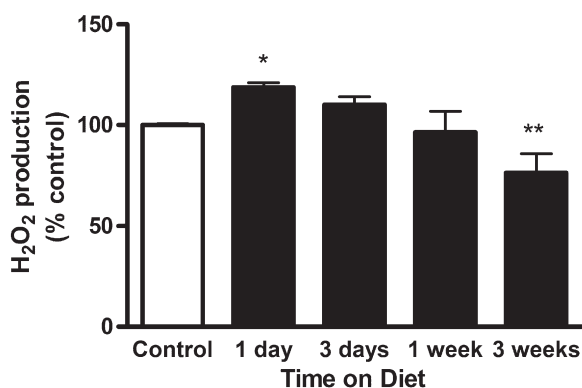


Fig. 1. Mitochondrial H_2O_2 production as a function of time on KD or control diet. Hippocampal mitochondria were isolated from rats fed a control or KD for 1 day, 3 days, 1 week, or 3 weeks. Substrate-driven H_2O_2 production was assessed using a fluorometric method. Bars represent mean \pm standard error of the mean. $*p \leq 0.05$, $**p \leq 0.01$ by one-way ANOVA.

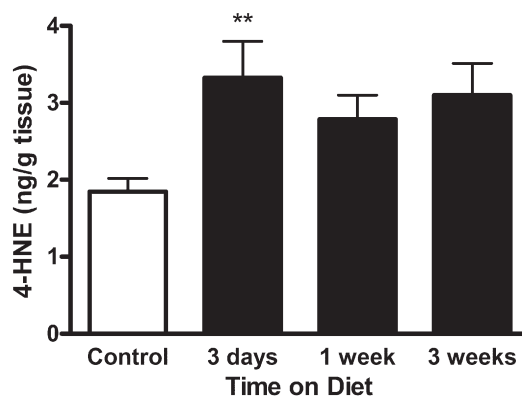


Fig. 2. Levels of 4-HNE in hippocampus of rats fed KD or control diet. 4-HNE levels were assessed in the hippocampus of rats fed a control or KD for 3 days, 1 week, or 3 weeks by HPLC with UV detection. Bars represent mean \pm standard error of the mean. $**p \leq 0.01$ by one-way ANOVA.

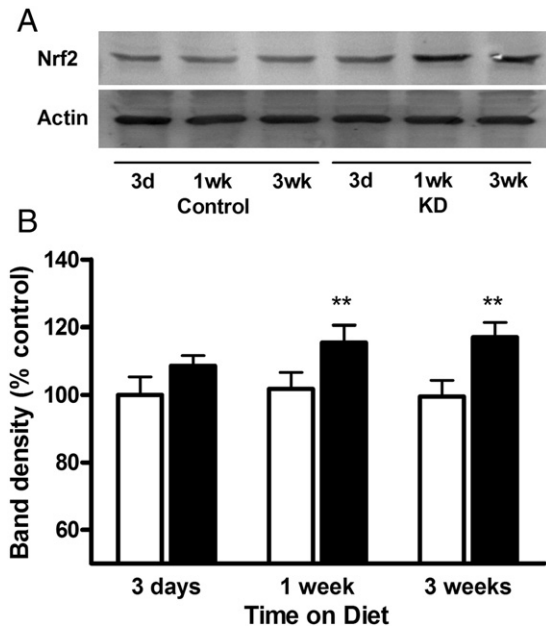


Fig. 3. Nrf2 nuclear expression in the hippocampus of rats fed KD or control diet. Nuclear fractions were prepared from rats fed a control or KD for 3 days, 1 week, or 3 weeks. (A) Western blot analysis of Nrf2 protein in nuclear fractions of rat hippocampus. Actin was used as a loading control. (B) Densitometry of Nrf2 protein expression exhibiting a time-dependent increase in the hippocampus of rats fed a KD (black bars) compared to control (open bars). Bars represent mean \pm standard error of the mean. $^{**}p \leq 0.01$.

Improved mitochondrial antioxidant capacity in the liver despite tissue GSH depletion

To determine whether the Nrf2 pathway was activated systemically by the KD or specific to the brain, we assessed key indices of this pathway in the livers of control and KD-fed rats. Interestingly, liver tissue GSH levels were significantly depleted in KD rats compared to controls at all time points examined (Fig. 5A). In previous work in the hippocampus, we found increased mitochondrial GSH and tissue CoASH but no significant changes in hippocampal tissue GSH (Jarrett et al., 2008). Therefore, to determine if liver mitochondrial fractions

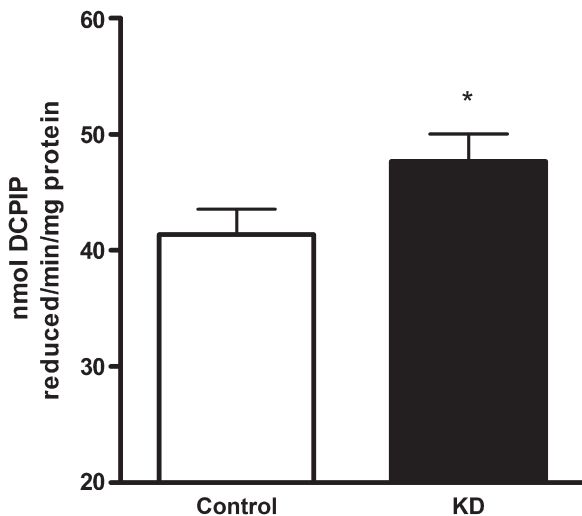


Fig. 4. Activity of the Nrf2 target, NQO1, in hippocampus of rats fed KD or control diet. NQO1 activity was assessed in the hippocampus of rats fed a control or KD for 3 weeks. Activity was assessed with a spectrophotometric assay. Bars represent mean \pm standard error of the mean. $^{*}p \leq 0.05$ by two-tailed *t*-test.

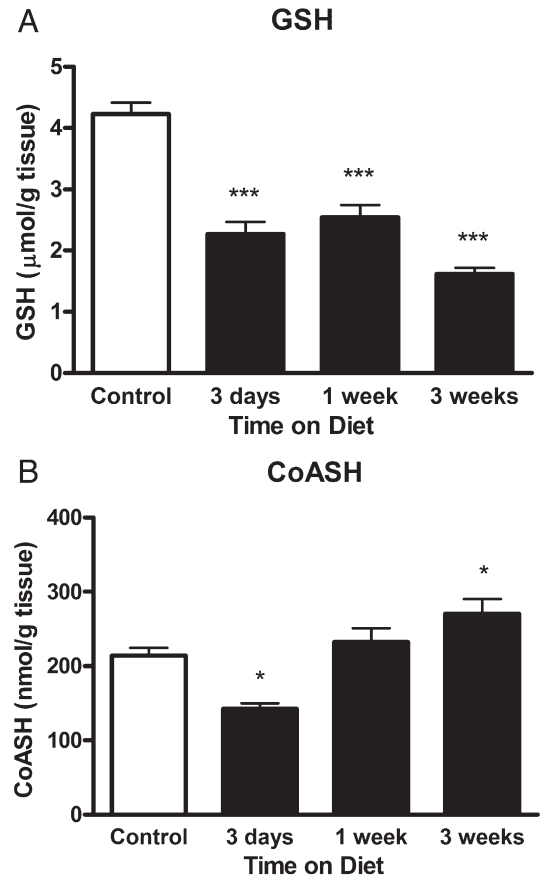


Fig. 5. GSH and CoASH levels in liver of rats fed KD or control diet. (A) Liver GSH levels in rats fed KD or control diets for 3 days, 1 week, and 3 weeks. (B) Liver CoASH levels in rats fed KD or control diets for 3 days, 1 week, and 3 weeks. Bars represent mean \pm standard error of the mean. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ by one-way ANOVA.

showed improved mitochondrial antioxidant capacity in a manner similar to the brain, we measured CoASH levels at 3 days, 1 week, and 3 weeks following diet initiation. CoASH is primarily localized within the mitochondria, so the mitochondrial antioxidant capacity can be assessed without extensive sample preparation, thus decreasing the possibility of artificial oxidation occurring while processing the tissue. CoASH was significantly depleted after 3 days on a KD compared to control but was significantly increased after 3 weeks on a KD (Fig. 5B), despite the chronic depletion of tissue GSH. These data suggest that acute mitochondrial oxidative stress occurs in the liver, much like the brain, and that the mitochondrial antioxidant capacity improves above control levels by 3 weeks on a KD.

Systemic activation of the Nrf2 pathway by the KD

To determine whether the Nrf2 pathway was also activated in the liver, we examined Nrf2 accumulation in the liver. Nuclear fractions were prepared from liver tissue of rats fed control or KD for 3 days, 1 week, and 3 weeks. Nrf2 protein increased significantly throughout the time course, implying Nrf2 was activated and translocated to the nucleus (Fig. 6). In addition, we assessed two Nrf2 targets, HO-1 and NQO1. HO-1 is highly inducible in the liver and a regularly utilized marker of Nrf2 activation. After 3 weeks on a KD, HO-1 protein levels were significantly greater in KD-fed rats compared to controls (Fig. 7A). Similarly, NQO1 activity was significantly increased in liver of KD-fed rats compared to controls after 3 weeks (Fig. 7B). These data suggest that the Nrf2 pathway is systemically activated by the KD and not specific to the brain.

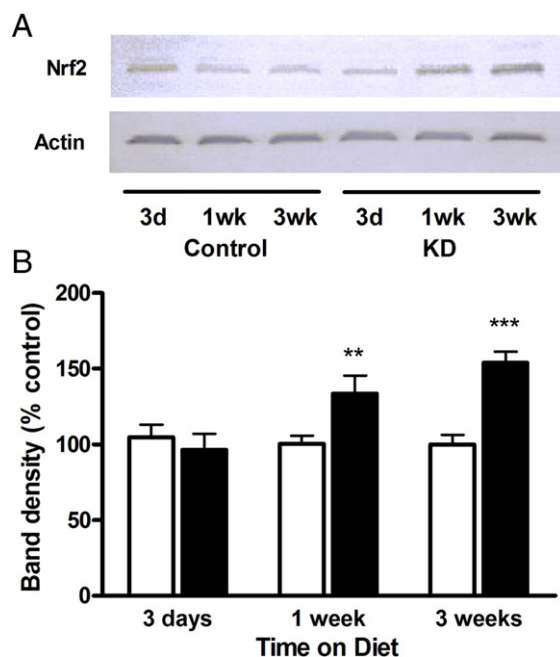


Fig. 6. Nrf2 nuclear expression in the liver of rats fed KD or control diet. Nuclear fractions were prepared from liver of rats fed a control or KD for 3 days, 1 week, or 3 weeks. (A) Western blot analysis of Nrf2 protein in nuclear fractions of rat liver. Actin was used as a loading control. (B) Densitometry of Nrf2 protein expression exhibiting a time-dependent increase in the liver of rats fed a KD (black bars) compared to control (open bars). Bars represent mean \pm standard error of the mean. ** $p \leq 0.01$, *** $p \leq 0.001$ by one-way ANOVA.

Discussion

We have previously shown that the KD increases glutathione biosynthesis (Jarrett et al., 2008). The rate-limiting enzyme in GSH biosynthesis is GCL, which is composed of two subunits, one catalytic (GCLC) and one modulatory (GCLM). Protein levels of both GCL subunits were increased in the hippocampus of rats fed a KD for 3 weeks, which is suggestive of adaptation to cellular stress (Shi et al., 1994; Tian et al., 1997). One of the primary means by which GSH biosynthesis is stimulated is through induction of the Nrf2 transcription factor pathway, as the promoters of both GclC and GclM contain consensus sequences to which Nrf2 can bind to initiate transcription (Moinova and Mulcahy, 1999; Wild et al., 1999). In response to cellular stress Nrf2 releases from its cytoplasmic inhibitory binding partner, Keap1, translocates to the nucleus, and activates transcription of protective target genes, such as GclC, GclM, Ho-1, and Nqo1, thus initiating an adaptive response.

Here we present novel data suggesting that consumption of a KD initially causes production of low levels of ROS, which may serve a redox signaling role, thereby activating one of the major cellular detoxification pathways, the Nrf2 pathway. Consistent with this, shortly following diet initiation, we observed increased mitochondrial H_2O_2 production, GSSG, and 4-HNE levels. In contrast, by 3 weeks on the diet, all indices decreased, concomitant with the previously reported increase in mitochondrial GSH (Jarrett et al., 2008). This time-dependent response to consumption of a KD suggests the activation of protective gene products as part of an adaptive response to cellular stress. Acute elevation of H_2O_2 and 4-HNE, together with our previous report of increased GSH biosynthesis, is suggestive of activation of the Nrf2 pathway. *In vitro* pretreatment with 4-HNE has been shown to protect against subsequent H_2O_2 -induced toxicity via a Nrf2-dependent mechanism and to be sufficient to induce Nrf2 dissociation from Keap1, thereby allowing nuclear translocation and target gene transcription (Numazawa et al., 2003; Chen et al., 2005). H_2O_2 itself has also been shown to increase DNA binding of Nrf2 to the

ARE (Wilson et al., 2005). In the KD, acute increases in 4-HNE and H_2O_2 may assist in activating the release of Nrf2 from Keap1 to initiate the detoxification response, but the question persists as to how this pathway remains activated if it is important for the chronic protection afforded by the KD.

While the functional significance of Nrf2 activation by the KD remains to be established, literature suggests that Nrf2 may play a role in seizure susceptibility in addition to its documented

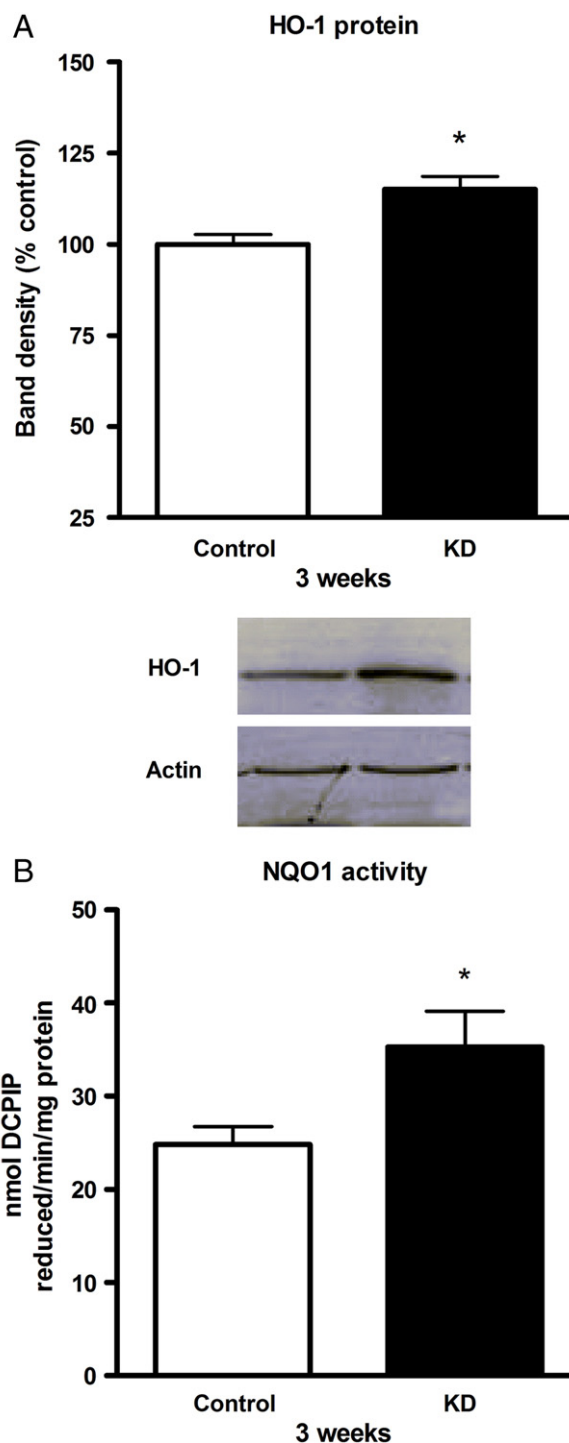


Fig. 7. Nrf2 target upregulation in liver of rats fed KD or control diet for 3 weeks. (A) HO-1 protein was assessed in liver of rats fed a control or KD for 3 weeks. Western blot densitometry analysis of HO-1 protein expression in liver, normalized to actin. (B) NQO1 activity was assessed in liver of rats fed a control or KD for 3 weeks. Bars represent mean \pm standard error of the mean. * $p \leq 0.05$ by two-tailed *t*-test.

neuroprotective effects. It has been shown that mice lacking Nrf2 are more sensitive to kainic acid (Kraft et al., 2006), and there is substantial evidence to support the idea that activation of Nrf2 is neuroprotective. *In vitro* studies have demonstrated that Nrf2 activation or overexpression protects neurons against toxicity from NMDA, glutamate, or H₂O₂ (Shih et al., 2003; Satoh et al., 2006). In addition, Nrf2-dependent protection against glutamate-mediated toxicity is dependent on GSH release from Nrf2-overexpressing glia, suggesting that increased GSH may play a central role in neuroprotection by Nrf2. Several groups have utilized the Nrf2 inducers *tert*-butylhydroquinone (tBHQ) and sulforaphane to demonstrate the protective effects of activating this pathway *in vivo*. Mice supplemented with tBHQ in their diets demonstrated attenuated toxicity to 3-nitropropionic acid, a mitochondrial complex II inhibitor (Shih et al., 2005b). This effect was lost when examined in Nrf2^{-/-} mice, suggesting that the observed neuroprotection was Nrf2-dependent. Rats pretreated with tBHQ before an ischemia–reperfusion injury demonstrated decreased cortical damage at both 24 hours and 1 month after injury (Shih et al., 2005a). Recently, the isothiocyanate sulforaphane, an extract of broccoli, has been found to potently activate Nrf2 *in vivo* and to protect postinjury loss of neurons in traumatic brain injury models (Zhao et al., 2006, 2007). These studies suggest that augmenting the Nrf2 pathway may prove beneficial as a new drug target.

The bulk of KD research has focused on the brain, as the diet's clinical application is primarily for the control of intractable epilepsies. This has left a void in the literature on the systemic effects of such a diet. One of the primary sources of brain GSH is export from the liver, which is consistent with our observation of depletion of liver GSH levels. This suggests that the liver may be exporting GSH to sustain GSH levels for other organs such as the brain. Even more striking was the finding that CoASH, a reduced mitochondrial thiol, was significantly increased in liver of KD-fed rats, suggesting a highly compartment-specific effect of the KD. These data suggest that mitochondria are specifically increasing their thiol pools and thereby maintaining a reduced state, despite ongoing GSH depletion in nonmitochondrial compartments, such as the cytosol. To our knowledge, the only other instance in which this has been reported is during fasting. It was found that during a 48-hour fast, hepatic GSH concentrations were depleted, while CoASH concentrations were increased (Jenniskens et al., 2002). This is particularly interesting given that the KD was initially designed to metabolically mimic the fasted state. With respect to Nrf2 activation in the liver, our results strikingly parallel those of acetaminophen toxicity studies in which liver GSH is depleted, concomitant with nuclear translocation of Nrf2 and increased transcription of Gclc and HO-1 (Goldring et al., 2004). Thus, the health effects of chronically depleting liver GSH need to be addressed in future studies of the KD.

While the KD is generally considered protective, here we show for the first time that this protection may be the result of redox signaling. This is not unreasonable given the extensive role of redox signaling as a preconditioning effect. In the traditional preconditioning paradigm, brief periods of sublethal stress lead to delayed protection against what would normally be a toxic insult. Importantly, it has been shown that preconditioning requires the production of ROS (Facundo et al., 2006) and that the ROS involved in this process are mitochondrially derived (Vanden Hoek et al., 1998). Our model fits within this paradigm as we see initial increases in mitochondrial H₂O₂ production, followed closely by nuclear translocation of the Nrf2 transcription factor. Furthermore, *in vitro* evidence suggests that treatment with sublethal concentrations of H₂O₂ is sufficient to increase GSH content (Seo et al., 2004). While H₂O₂ was initially recognized as a toxin, recent evidence suggests that at subtoxic levels, it can function as a redox signaling molecule.

One important question that remains to be addressed is whether these biochemical changes occur in an epileptic animal. Depletion of

brain GSH has been reported following kainic acid-induced status epilepticus (Liang and Patel, 2006). As we have previously reported KD-induced maintenance of brain tissue GSH in conjunction with increased mitochondrial GSH, it is important to establish whether the KD can reverse these deficits in an epileptic animal. In addition, the novel data that chronic consumption of a KD depletes liver GSH make it essential for the medical community to recognize the importance of systemic and brain GSH and how they are affected by the KD.

Whether activation of the Nrf2 pathway, and thus GSH biosynthesis, contributes to the anticonvulsant effects of the KD remains to be determined. We have identified systemic activation of a primary cellular detoxification pathway, presumably initiated and maintained by low levels of cellular stress. These findings can suggest strategies for turning this nonpharmacological dietary treatment into a pharmacological therapy for the treatment of medically refractory epilepsies.

Acknowledgments

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