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## Effects of a dietary ketone ester on hippocampal glycolytic and TCA cycle intermediates and amino acids in a 3xTgAD mouse model of Alzheimer's disease

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### Abstract

In patients with Alzheimer's disease (AD) and in a triple transgenic (3xTgAD) mouse model of AD low glucose metabolism in the brain precedes loss of memory and cognitive decline. The metabolism of ketones in the brain by-passes glycolysis and therefore may correct several deficiencies that are associated with glucose hypometabolism. A dietary supplement composed of an ester of D- $\beta$ -hydroxybutyrate and R-1,3 butane diol referred to as ketone ester (KE) was incorporated into a rodent diet and fed to 3xTgAD mice for 8 months. At 16.5 months of age animals were euthanized and brains dissected. Analyses were carried out on the hippocampus and frontal cortex for glycolytic and TCA (Tricarboxylic Acid) cycle intermediates, amino acids, oxidized lipids and proteins, and enzymes. There were higher concentrations of D- $\beta$ -hydroxybutyrate in the hippocampus of KE-fed mice where there were also higher concentrations of TCA cycle and glycolytic intermediates and the energy-linked biomarker, n-acetyl aspartate compared to controls. In the hippocampi of control-fed animals the free mitochondrial [NAD<sup>+</sup>]/[NADH] ratio were highly oxidized, whereas, in KE-fed animals the mitochondria were reduced. Also, the levels of oxidized protein and lipids were lower and the energy of ATP hydrolysis was greater compared to controls. 3xTgAD mice maintained on a KE-supplemented diet had higher concentrations of glycolytic and TCA cycle metabolites, a more reduced mitochondrial redox potential, and lower amounts of oxidized lipids and proteins in their hippocampi compared to

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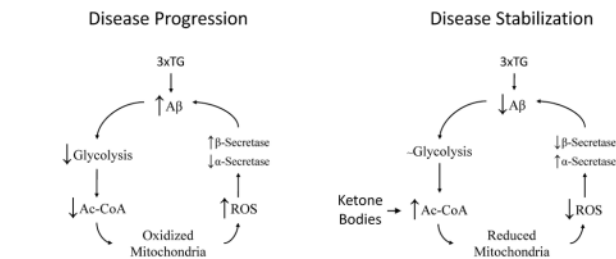
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controls. The KE offers a potential therapy to counter fundamental metabolic deficits common to patients and transgenic models.

## Graphical Abstract

We hypothesized that a dietary ketone supplement (an ester of D- $\beta$ -hydroxybutyrate and R-1,3 butanediol) would elevate blood ketones, overcome brain glucose hypometabolism, rescue mitochondrial function, and reduce beta-amyloid production in a triple transgenic (3xTG AD) mouse model of Alzheimer's disease (AD). This study is the first endeavor to comprehensively quantify neurochemical deficits in the 3xTG model and offers a novel approach for treating metabolic deficits in AD.



## Keywords

ketone bodies; hippocampus; cellular energetics; mitochondria; glycolysis

## Introduction

A preponderance of research carried out over the past several decades in both human subjects with Alzheimer's disease (AD) (Cunnane *et al.* 2011) and in animal models of AD (Ding *et al.* 2013a, Yao *et al.* 2009, Ding *et al.* 2013b) have demonstrated that well in advance of demonstrable cognitive decline and memory defects brain glucose hypometabolism is often evident. Early reports estimated that glucose utilization decreased between 20–30% based on  $^{18}\text{F}$ -fluorodeoxyglucose uptake using PET imaging in patients with “senile dementia of the Alzheimer's type” (de Leon *et al.* 1983, Frackowiak *et al.* 1981). Although low glucose metabolism occurs differentially across the brain, it is primarily associated with regions that affect learning, memory, and behavior. A glucose-replacement therapy utilizing ketone bodies may recover energy deficiencies associated with low glucose metabolism and offers a potential therapeutic advantage for delaying and/or decreasing cognitive decline in pre-symptomatic individuals (Mosconi *et al.* 2008, Veech *et al.* 2001, Veech and King, 2016).

A ketone dietary supplement using an ester of  $\beta$ -hydroxybutyrate (KE) was fed to a triple transgenic AD mouse strain (3xTgAD) when animals were 8.5 months of age for an additional 8 months. These animals were subjected to a battery of memory and anxiety-related behavioral paradigms at 12 and 15 months and histological analysis at the termination of the study (Kashiwaya *et al.* 2013). As reported, KE-fed animals showed improvements in behavior and learning and had significantly lower amounts of amyloid-beta

(A $\beta$ ) and phosphorylated Tau (p-Tau) proteins in regions of the hippocampus. To assess the effects of the KE dietary intervention on cellular metabolism we now present quantitative determinations of the glycolytic and TCA cycle intermediates and amino acids utilizing stable isotope dilution mass spectrometry and signatures of oxidative stress (protein and lipid oxidation) in the same sets of animals. Since accumulation of A $\beta$  may, in part, be influenced by the cellular redox environment (the free [NAD<sup>+</sup>]/[NADH] and [NADP<sup>+</sup>]/[NADPH] ratios) as the reduced forms of these couples restore the reductive capacity of cellular antioxidants (Kwak *et al.* 2011), we also determined the free [NAD<sup>+</sup>]/[NADH] and [NADP<sup>+</sup>]/[NADPH] ratios. The hippocampus and frontal cortex as regions that had relatively high and low accumulation A $\beta$  and p-Tau proteins were chosen for comparison in these analyses (Kashiwaya *et al.* 2013).

## Materials and Methods

### Animal procedures, diets, and tissue harvesting

All procedures were approved by the Animal Care and Use Committee of the National Institute on Aging. The initial generation and characterization of 3xTgAD mice have been reported previously Kashiwaya *et al.* (2013). The mice used in the present study were from a colony that had been backcrossed onto a C57BL/6 genetic background for 8 generations. As reported previously in Kashiwaya *et al.* (2013), there were between 11 and 15 male 3xTgAD mice housed in groups of 2 to 3 mice per cage under a standard 12-hour light and dark circadian cycle. When they were 8.5 months old a simple randomization procedure was used in assigning mice to each of the two diet groups, whereby animals were randomized so that littermates were distributed equally between the control-fed and ketone ester-fed diet groups (ketone ester, KE; or control, CON) of 15 mice per group. Also, the mean weight of animals from each diet group, KE-fed (37.1  $\pm$  1.8 g) and CON-fed (37.5  $\pm$  0.8 g) approximated the mean weight of the total sample (37.3  $\pm$  1.1g). Mice were not pair-fed but received a 4- to 5-g pellet (10.8–13.5 kcal) at approximately 06:00 hours each day. Body weight was measured periodically throughout the study. Mice in both dietary groups were similarly active and as noted in Kashiwaya *et al.* (2013) KE-fed animals were noticeably more active in the open field test indicating a sufficient calorie intake. The CON and KE diets have been fully described previously (Kashiwaya *et al.* 2013). Briefly, the CON and KE diets were produced in-house following a customized procedure which included mixing purified dietary macronutrients with additional nutrient and vitamin mixes blended with water to obtain a semi-solid pellet in accord with nutritional guidelines set forth by American Institute of Nutrition for rodent maintenance diets (AIN-93) (Kashiwaya *et al.*, 2013). The caloric content of both CON and KE diets was 2.7 kcal/g. The carbohydrate content of the CON and KE diets was 64.9 and 43.5 percent calories, respectively. The KE diet also included an additional 21.5% of calories in the form of the ketone ester. The control diet contained 137 g corn starch/1000 g diet and 0 g KE. The KE diet contained 85 g starch/1000 g and 125 g KE/1000 g diet. The KE was synthesized from D- $\beta$ -hydroxybutyrate and R-1,3-butanediol (Kashiwaya *et al.* 2013). Animals were fed ad libitum either the CON or KE diet beginning at 8.5 mo. of age for an additional 8 mo. Animals fed the KE diet maintained body weight and further details of these determinations have been published (Kashiwaya *et al.* 2013). (Kashiwaya, *et al.*, 2013). Mice were euthanized at 16.5 months of age and the cortex and

hippocampus were removed, half of the tissue was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis and other half processed for histological analysis as reported brains (Kashiwaya et al. 2013). Rapid freezing of the brain was the method chosen for tissue preservation as microwave fixation has been shown to alter citrate concentrations and effect ATP levels (Srivastava *et al.* 2012a). The frozen brain tissue was then used for protein and metabolite assays as described below.

## Materials

**Reagents and instrumentation**—Stable isotopically-labeled ( $^{13}\text{C}$ - and  $^2\text{H}$ -) organic acid standards and amino acids (lactate, pyruvate, citrate,  $\alpha$ -ketoglutarate, succinate, fumarate and malate) were procured from CDN Isotopes (Pointe-Claire Quebec). The  $^{13}\text{C}$ -labeled phosphoenol pyruvate was obtained from Cambridge Isotope Labs (Cambridge, MA) (Srivastava et al. 2012a). Labeled 24(S)-hydroxycholesterol (d6) was obtained from Avanti Polar Lipids (Alabaster, AL). Labeled dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3PG) and isocitrate were synthesized in house as previously described (Srivastava et al. 2012a). The  $^{13}\text{C}$ -acetyl-CoA and  $^{13}\text{C}$ -glucose-6-phosphate were obtained from Sigma-Aldrich (St. Louis, MO). Labeled 4-HNE was obtained from Cayman Chemical Company (Ann Arbor, MI). The N-methyl-N-(tert-butylmethylsilyl) trifluoroacetamide (MTBSTFA) with 1% tert-butyl dimethylchlorosilane (TBDMCS) reagent and the bis-trimethylfluoro methyl silyl (BSTFA) were purchased from Pierce (Rockford, IL). Samples were analyzed on either an Agilent 5973 quadrupole GC-MS (Agilent, Wilmington, DE) or on an Agilent capillary electrophoresis-ion trap mass spectrometer (Ultra) using an Agilent 1100 series binary pump with electrospray ionization (Agilent, Wilmington, DE) according to previously established procedures (Srivastava et al. 2012a, Kashiwaya *et al.* 2010).

**Blood glucose and ketone analysis**—Thawed mouse plasma was analyzed for glucose and  $\beta$ -hydroxybutyrate using glucose and ketone sticks and a Precision-Xtra meter (Abbott Labs, Abbott Park, IL, USA) according to previous methods (Kashiwaya et al. 2013).

**Perchloric acid (PCA) extraction**—Frozen brain tissue (20–40 mg) was extracted into a 3.6% PCA solution and neutralized with  $\text{KHCO}_3$  (3 M) according to previously described methods (Kashiwaya et al. 2013). Final volume of the extract was 100  $\mu\text{L}$ .

**Gas chromatography-mass spectrometry of organic acids**—Twenty microliters of the extract was taken for determination of lactate, pyruvate, citrate, isocitrate,  $\alpha$ -ketoglutarate, succinate, fumarate, malate,  $\beta$ -hydroxybutyrate, and acetoacetate and a commensurate amount of the  $\text{C}_{13}$ - or  $^2\text{H}$ - labeled internal standards (2 to 3 fold in excess of endogenous metabolite) were added for quantitative analysis by gas chromatography-mass spectrometry. The aqueous solution of the sample specimens was reduced to dryness under a stream of nitrogen (99.99%) and reacted with tertiary butyl dimethyl silyl chloride to form the silyl esters and ethers according to previously described methods (Srivastava et al. 2012a). One microliter of the derivatized sample was injected onto a 30 meter capillary gas chromatograph column and analyzed in the electron impact mode on a quadrupole mass spectrometer. Individual metabolites were quantified using the ratio of the area counts of the

most prominent ion fragments of the unlabeled compound referenced to the analogous ion of the labeled internal standards.

**Gas chromatography-mass spectrometry of phosphorylated glycolytic intermediates**—Twenty microliters of the extract was taken for determination of glucose-6-phosphate (G-6-P), dihydroacetone-phosphate (DHAP), 3-phosphoglycerate (3PG) and phosphoenol pyruvate (PEP). The phosphorylated compounds were analyzed as their tri-methylsilyl ether derivatives (TMS) using GC-MS in the electron impact mode and quantified using the  $^{13}\text{C}$ -labeled internal standards for each analyte according to previously published procedures (Srivastava et al. 2012a).

**Gas chromatography-mass spectrometry of glutamate, aspartate, N-acetylaspartate,  $\gamma$ -aminobutyric acid and 4-hydroxynoneal (4-HNE)**—Perchloric acid extracts of frozen brain were used to measure glutamate, aspartate, N-acetylaspartate,  $\gamma$ -aminobutyric acid (GABA) and 4-HNE. They were analyzed as the silyl ether derivatives, quantified using  $^{13}\text{C}$ -labeled standards for each analyte using gas chromatography-mass spectrometry as previously described (Pawlosky et al. 2010).

**Capillary electrophoresis-mass spectrometry of acetyl-CoA**—Determination of Acyl-CoA Compounds the brain tissue samples were extracted using a modified chloroform-methanol extraction procedure and analyzed by CE-MS according to the previously described method (Kashiwaya et al. 2010) with the addition of  $^{13}\text{C}$ -labeled acetyl CoA internal standards for quantification as described previously (Pawlosky et al. 2010).

**Gas chromatography-mass spectrometry of 24(S)-hydroxycholesterol**—For determining the concentration of 24(S)-hydroxycholesterol, to 200  $\mu\text{L}$  of thawed plasma was added to 6  $\mu\text{L}$  of 24(S)-hydroxycholesterol (d6) (52 ng/ $\mu\text{L}$ ) and extracted with a 3:4:2 water:chloroform:methanol solution. Samples were vortexed for 2 min and centrifuged at 4°C to allow the chloroform and water layers to separate. The chloroform layer was removed and 20  $\mu\text{L}$  of the solution was taken and evaporated under a stream of nitrogen for GC-MS analysis according to previously published procedures (Kemper et al. 2015) and quantified directly by comparing the peak area ratio of the pseudo-molecular ion of the labeled internal standard to that of the native compound.

**Protein and western analysis**—Whole cell lysate was prepared by adding 5 $\mu\text{L}$ /mg tissue of ice cold lysis buffer (20mM HEPES, 1% Triton x-100, 1% sodium deoxycolate, 1mM DTT, 1% SDS, 1X protease inhibitors (Thermo Scientific Pierce Protein Biology), 1 mM NaF, 5 mM EDTA, 10 mM  $\beta$ -glycerophosphate and 250  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ ) and sonicated 3 times for 5 seconds on ice, clarified by centrifugation at 15,000  $\times$  g for 12 minutes at 4°C and stored at -80°C until use. Protein concentration was measured using detergent compatible protein quantification kit (Biorad) and final loading samples were prepared at 4 $\mu\text{g}/\mu\text{L}$  protein in LDS Sample Buffer (Life Technologies) with 50 mM DTT (Life Technologies). Sixty  $\mu\text{g}$  of whole cell lysate were loaded into each well of a NuPAGE NOVEX 4–12% Bis-Tris gel with MES running buffer, transferred onto Immobilon-FL PVDF (Millipore). A solution of 5% milk in TBS + 0.1% Tween 20 was used to block and dilute primary and secondary antibodies (1:5000, LiCor Biosciences, Rabbit antibody

conjugated with IRdye 800, mouse antibody conjugated with IRdye 680), scans and quantifications were used using a LiCor Odyssey scanner. The primary antibodies included: ADAM10 (Rb 1:1000, Abcam ab1997), GFAP (Rb 1:50,000, Abcam ab7260), Actin (Ms 1:2000, Abcam ab3280), BACE1 (Rb 1:1000, Abcam ab2077).

**Quantification of protein oxidation products**—Oxyblot Protein Oxidation Detection Kit (Millipore, USA) was used according to manufacturer's instructions. Fifteen  $\mu\text{g}$  of tissue lysate were used in each sample, half of the resulting derivitized/neutralized solution was used for downstream blotting. Briefly, NuPAGE Bis-Tris 4–12% Gels (Life Technologies) were run using MES running buffer (Life technologies), transferred onto Immobilon-FL PVDF (Millipore), incubated with fluorescent secondary antibody (Rabbit, LiCor Biosciences, 1:5000 in 1% BSA/PBST); blots were scanned using a LiCor Odyssey bed scanner (for additional details see Kemper *et al.* (2013)). As a control for auto-fluorescence of the derivitized protein, blots were first scanned without secondary antibody, no signal was observed in any of the blots without secondary antibody. Non-derivitized control had extremely low signal, which was used as background for quantification purposes.

**Calculations of the concentration of oxaloacetate, the  $\Delta G$  of ATP hydrolysis, the free  $[\text{NAD}^+]/[\text{NADH}]$  ratio, and the free  $[\text{NADP}^+]/[\text{NADPH}]$  ratio** (Sato *et al.* 1995, Bergman *et al.* 2010).

**Oxaloacetate by Malate Dehydrogenase** (Guynn *et al.* 1973)

$$K_{\text{MDH}} = 2.86 \times 10^{-12} \text{M} = \frac{[\text{Oxaloacetate}][\text{H}^+]}{[\text{Malate}]} \times \frac{[\text{NADH}]_{\text{Cytosolic}}}{[\text{NAD}^+]_{\text{Cytosolic}}}$$

$$[\text{Oxaloacetate}] = 2.86 \times 10^{-12} \text{M} \times \frac{[\text{Malate}]}{[\text{H}^+]} \times \frac{[\text{NAD}^+]_{\text{Cytosolic}}}{[\text{NADH}]_{\text{Cytosolic}}}$$

Where  $\text{pH} = 7.2$ ,  $[\text{H}^+] = 6.31 \times 10^{-8} \text{M}$

**$\Delta G$  of ATP hydrolysis** (Bergman *et al.* 2010, Williamson *et al.* 1967)

$$\Delta G = \Delta G^\circ + RT \left( \ln \left( \frac{[\text{NAD}^+]}{[\text{NADH}]} \right) \times \frac{10^{-7}}{10^{-\text{pH}}} \ln \left( \frac{[\text{Q}]}{[\text{QH}_2]} \right) \right)$$

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{[\text{ACAC}] \times [\text{H}^+]}{[\text{BHB}] \times k_{\text{BHBBDH}}}$$

$$\frac{[\text{Q}]}{[\text{QH}_2]} = \frac{[\text{Succinate}]}{[\text{Fumarate}] \times k_{\text{SDH}}}$$

$$\Delta G = \Delta G^\circ + RT \left( \ln \left( \frac{[\text{ACAC}] \times [\text{H}^+]}{[\text{BHB}] \times k_{\text{BHBBDH}}} \right) \times \frac{10^{-7}}{10^{-\text{pH}}} - \ln \left( \frac{[\text{Succinate}]}{[\text{Fumarate}] \times k_{\text{SDH}}} \right) \right)$$

Where  $k_{\text{BHBBDH}} = 4.93 \times 10^{-9} \text{M}$ ,  $k_{\text{SDH}} = 171.84$ ,  $\text{pH} = 7.2$ ,  $[\text{H}^+] = 6.31 \times 10^{-8} \text{M}$ ,  $\Delta G^\circ = -81.05 \text{kJ/mol}$

**Free  $[\text{NADP}^+]/[\text{NADPH}]$**  (Londesborough & Dalziel 1968)

$$\frac{[\alpha\text{KG}^{2-}] \times [\text{CO}_2]}{[\text{Isocit}^{3-}]} \times \frac{[\text{NADPH}]}{[\text{NADP}^+]} = K_{\text{ICDH}} = 1.17\text{M}$$

Thus

$$\frac{[\text{NADP}^+]}{[\text{NADPH}]} = \frac{[\alpha\text{KG}^{2-}] \times [\text{CO}_2]}{[\text{Isocit}^{3-}]} \times \frac{1}{K_{\text{ICDH}}}$$

Where  $[\text{CO}_2] = [\text{HCO}_3^-] \times [\text{H}^+]/(10^{-6.1}) = 1.986\text{mM}$  at pH 7.2 with  $[\text{HCO}_3^-] = 25\text{mM}$  via Henderson Hasselbach

**Free  $[\text{NAD}^+]/[\text{NADH}]$ -cytosolic (Williamson et al. 1967)**

$$\frac{[\text{Pyr}^-]}{[\text{Lac}^-]} \times \frac{[\text{NADH}]}{[\text{NAD}^+]} = K_{\text{LDH}} = 1.11 \times 10^{-4}$$

Rearranged

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{[\text{Pyr}^-]}{[\text{Lac}^-]} \times \frac{1}{K_{\text{LDH}}}$$

**Free  $[\text{NAD}^+]/[\text{NADH}]$ -mitochondrial (Williamson et al. 1967)**

$$K_{\beta\text{HBDH}} = \frac{[\beta\text{HB}][\text{NAD}^+]}{[\text{ACAC}][\text{NADH}][\text{H}^+]} = 4.93 \times 10^{-9}\text{M}$$

$$\frac{[\text{NAD}^+]}{[\text{NADH}]} = \frac{[\text{ACAC}][\text{H}^+]}{[\beta\text{HB}]} K_{\beta\text{HBDH}}$$

## Statistics

All data values are given as mean  $\pm$  SEM. Statistical differences were determined by Student's t test in GraphPad Prism 6. In all cases, statistical significance was set at  $P < 0.05$ .

## Results

### Affect of diet on animal body weight

Animal body weights fluctuated during the study but appeared to reach a plateau for both dietary groups at about 12 months (Kashiwaya et al., 2013). KE-fed animals maintained weight throughout the study but weighed less than CON-fed mice. Mean body weights at 14 months of age were  $40.3 \pm 2.1$  and  $37.0 \pm 1.2$  g for the CON-fed and KE-fed mice, respectively. At the time of diet initiation the mean body weight for all animals was  $37.3 \pm$

– 1.1. At 16.5 months, mean body weight of mice in the KE-fed group was between 9–10% lower than that of controls (41.5 +/- 2.5 vs 37.1 +/- 0.5) (Kashiwaya et al., 2013).”

### **Tissue analysis, blood glucose and ketone levels and 24-s-cholesterol**

Altogether, 24 hippocampal and cortical tissue samples were available and evaluated in these analyses (KE diet = 12) (CON diet n=12). Because perchloric acid extraction destroys protein the samples were apportioned into groups, one for protein analysis (n = 5 or 6 for treatment and control subjects) and for analysis of glycolytic phosphorylated intermediates, TCA cycle acids, amino acids, acetyl-coA and 4-hydroxynonenal (4-HNE) (n = 5 or 6, in each treatment an control group).

### **Blood glucose, $\beta$ -hydroxybutyrate ( $\beta$ -HB), and 24-S-hydroxycholesterol concentrations**

There was no difference in the blood glucose concentrations between groups (CON: 155  $\pm$  8.5 and KE: 152  $\pm$  9.0 mg/dl). Blood  $\beta$ -HB concentrations were significantly greater in the KE-fed group (CON: 0.14 mM  $\pm$  0.01 vs KE: 0.72  $\pm$  0.13 mM; p<0.05). The Glucose Ketone Index (GKI) as described in Meidenbauer et al. is the ratio of the concentrations of blood glucose to that of blood  $\beta$ -hydroxybutyrate. The GKI for the CON-fed group was 72.0 +/- 25.1 and for the KE-fed group was 19.4 +/- 14.4, p < 0.001 and suggests that KE-fed mice are in a state of therapeutic ketosis (Meidenbauer *et al.* 2015). The plasma concentration of 24-S-hydroxycholesterol, a biomarker of brain cholesterol turnover and modulator of long term potentiation, was greater in the KE-fed mice, (CON: 0.135  $\pm$  0.016 vs KE: 0.212  $\pm$  0.018 nmol/mL; n = 8, p<0.05). The concentrations of 24-S-hydroxycholesterol from the combined brain regions (hippocampus and cortex) from KE-fed and CON-fed mice were analyzed. The concentrations of this metabolite in KE-fed mice compared with CON-fed animals were CON: 33.0 +/- 3.7 vs KE: 40.9 +/- 5.0 pMol/mg tissue, n=10, p <.06

### **Glycolytic intermediates**

In the cortex of the KE-fed mice the concentration of G-6-P was marginally lower (–10%) compared to that in controls (CON: 0.064  $\pm$  0.002 vs KE: 0.058  $\pm$  0.002  $\mu$ mol/g; p < 0.05) whereas the concentration of DHAP was notably lower (CON: 0.080  $\pm$  0.012 vs KE: 0.052  $\pm$  0.005  $\mu$ mol/g; p < 0.05) (Figure 1A). There were no differences in the concentrations of the other glycolytic intermediates, 3PG, PEP or pyruvate between the two groups (Figure 1A). Interestingly, the ratio of lactate to pyruvate was lower (p < 0.05) in the cortex of KE-fed mice (16.2  $\pm$  3.0) compared with mice maintained on the CON diet (25.7  $\pm$  3.2) and this appeared to be the result of the higher concentration of pyruvate in the KE-fed group compared to controls (CON: 0.057  $\pm$  0.014 vs KE: 0.087  $\pm$  0.015  $\mu$ mol/g) rather than lactate (CON: 1.35  $\pm$  0.20 vs KE: 1.31  $\pm$  0.10  $\mu$ mol/g) (see Table 1). In the hippocampi of control mice there were substantially lower concentrations of G-6-P, DHAP, 3PG and PEP compared to the concentrations of these metabolites in the cortex. In KE-fed mice the concentrations of these intermediates were similar in both the cortex and hippocampus (Figure 1A and B). Further, in the hippocampus of mice maintained on the CON versus KE diet the concentrations of the glycolytic intermediates were significantly lower G-6-P (–39%), DHAP (–29%), 3PG (–34%), PEP (–34%) and pyruvate (–34%) in mice maintained on the CON diet.



### TCA cycle intermediates

In the cortex of KE-fed mice only citrate was significantly higher (CON:  $0.089 \pm 0.010$  vs KE:  $1.14 \pm 0.008$   $\mu\text{mol/g}$ ;  $p < 0.05$ ). The other TCA cycle intermediates, isocitrate,  $\alpha$ -ketoglutarate, succinate, fumarate and malate were not different (Figure 1C). Notably, in the hippocampus of KE-fed mice the concentrations of citrate (+100%), isocitrate (+60%),  $\alpha$ -ketoglutarate (+112%), succinate (+80%), fumarate (+127%) and acetyl CoA (+103%) were all greater compared to animals on the CON diet (Figure 1D).

### Amino acids

In the cortex of mice maintained on either diet, the concentrations of the amino acids, glutamate (CON:  $7.0 \pm 0.6$  vs KE:  $6.4 \pm 0.3$   $\mu\text{mol/g}$ ), aspartate (CON:  $3.9 \pm 0.2$  vs KE:  $4.1 \pm 0.8$   $\mu\text{mol/g}$ ) n-acetyl aspartate (CON:  $6.5 \pm 0.8$  vs KE:  $5.9 \pm 0.5$   $\mu\text{mol/g}$ ), GABA (CON:  $0.19 \pm 0.03$  vs KE:  $0.24 \pm 0.41$   $\mu\text{mol/g}$ ) were similar (Figure 2A). Conversely, in the hippocampus of mice fed the KE diet there were significantly higher concentrations of both aspartate (+63%, CON:  $2.13 \pm 0.22$  vs KE:  $3.48 \pm 0.52$   $\mu\text{mol/g}$ ;  $p < 0.05$ ) and n-acetyl aspartate (+59%, CON:  $8.42 \pm 0.44$  vs KE:  $13.01 \pm 1.08$   $\mu\text{mol/g}$ ;  $p < 0.05$ ) (Figure 2B). Low levels of n-acetyl-aspartate in the hippocampus have been linked to higher anxiety levels and depression in humans (Matthew S. J. et al., 2008). Therefore, we utilized previously determined data from the same 3xTgAD mice obtained from the anxiolytic paradigm phase I trial using the elevated platform plus sign-shaped maze described in Kashiwaya et al., 2013 and correlated the percent of time spent by individual mice in each of the two dietary groups from those outcomes to the concentrations hippocampal n-acetyl-aspartate (Figure 3). Animals that remained for a greater percentage of time in the open arm portions of the maze compared to the closed arm or center are presumed to exhibit lower general anxiety. The KE-fed mice spent significantly more time in the open arm portions of the maze compared with control diet-fed mice as reported (Kashiwaya et al., 2013). We now report strong positive correlations in the amount of hippocampal n-acetyl-aspartate to the percentage of time that individual animals remained in the open arm portion of the maze from KE-Fed ( $R = 0.862$ ) and control-fed ( $R = 0.789$ ) mice and conversely negative correlations in hippocampal n-acetyl-aspartate to the percent of time that mice remained in the closed arm and center portions of the maze (KE-fed,  $R = -0.769$ ) (control-fed,  $R = -0.805$ ). The ketone ester diet significantly increased the concentration of n-acetyl-aspartate in the hippocampus and KE-fed animals were much more likely to remain in the open arm portion of the maze (figure 3). The concentration of glutamate was similar in the hippocampi of mice maintained on either diet (CON:  $9.2 \pm 1.1$  vs KE:  $10.5 \pm 0.9$   $\mu\text{mol/g}$ ). Interestingly, hippocampal GABA was lower in the animals fed the KE diet compared to controls (CON:  $0.32 \pm 0.05$  vs KE:  $0.17 \pm 0.03$   $\mu\text{mol/g}$ ;  $p < 0.05$ ). Recent work has shown that an aberrant increase in GABA levels in AD-affected brain regions is associated with  $A\beta$  activation of astrocytes and other glial cells (Jo *et al.* 2014). In conjunction with the lower GABA levels observed in KE-fed mice we observed that the amount of the activated astrocyte marker glial fibrillary acid protein (GFAP) was significantly lower in hippocampal lysates ( $-27\%$ ,  $N=5-6$ ,  $p < 0.05$ ) from KE-fed animals compared to mice fed the CON mice (Figure 2C).

## Cytosolic and mitochondrial redox states, nicotinamide adenine dinucleotide pools, and the $\Delta G$ of ATP hydrolysis

The cytosolic free  $[NAD^+]/[NADH]$  ratio was determined from the ratio of the concentration of the glycolytic intermediates, lactate to that of pyruvate. Further details of these calculations are outlined in previous reports (Sato et al. 1995, Bergman et al. 2010, Kashiwaya et al. 2010). The concentrations of these intermediates in both the cortex and hippocampus were comparable to freeze-blown brain tissue which indicated tissue handling procedures preserved the sample integrity (Sato et al. 1995, Bergman et al. 2010, Kashiwaya et al. 2010). In the cortex and hippocampi of KE-fed mice the cytoplasm was significantly more oxidized (an approximate increase of 65% in free  $[NAD^+]/[NADH]$ ) compared to controls and the shift toward oxidation was primarily due to an increase in the concentration of pyruvate (Table 1). Notably, the cytosol redox potential in both the hippocampus and cortex was similar within each dietary group.

The mitochondrial free  $[NAD^+]/[NADH]$  ratios were determined using the ratio of the concentrations of  $\beta$ -hydroxybutyrate ( $\beta$ HB) to acetoacetate (AcAc), a near equilibrium reaction catalyzed by  $\beta$ -HB dehydrogenase (Bergman et al. 2010, Williamson et al. 1967). In the cortex of mice maintained on either diet the mitochondrial free  $[NAD^+]/[NADH]$  ratios were similar (Table 1). Moreover, KE-fed mice had similar redox states in the hippocampus and cortex. Notably, the mitochondrial  $[NAD^+]/[NADH]$  redox state in the hippocampus of mice fed the CON diet was highly oxidized (+127%) compared to KE-fed mice (Table 1).

The free  $[NADP^+]/[NADPH]$  ratio was determined using the ratio of the concentrations of isocitrate to  $\alpha$ -ketoglutarate ( $\alpha$ KG) a reaction catalyzed by isocitrate dehydrogenase (Bergman et al. 2010, Veech *et al.* 1969). In the cortex of KE-fed mice, the free  $[NADP^+]/[NADPH]$  ratio was significantly reduced, signifying an increase in availability of NADPH (Table 1). Although, the hippocampal concentrations of isocitrate and  $\alpha$ KG were significantly greater in KE-fed mice, the free  $[NADP^+]/[NADPH]$  redox states were no different.

The energy associated with the  $\Delta G$  of ATP hydrolysis is the energy released through the cleavage of the terminal phosphate bond of ATP. This value, in kJ/mol, was determined using the difference in energy states between the mitochondrial free  $[NAD^+]/[NADH]$  ratio and the Q/QH<sub>2</sub> redox state (Bergman et al. 2010, Sato et al. 1995). The Q/QH<sub>2</sub> redox state was determined from the ratio of concentration of succinate to that of fumarate, a reaction catalyzed by succinate dehydrogenase (Bergman et al. 2010). Diet had no effect on the  $\Delta G$  of ATP hydrolysis in the cortex (CON:  $-62.3 \pm 0.5$  vs KE:  $-61.6 \pm 0.2$  kJ/mol) similar to observations in healthy brain tissue (Kashiwaya et al. 2010). In the hippocampus, the  $\Delta G$  of ATP hydrolysis in KE-fed mice was substantially more energetic compared to control-fed mice (CON:  $-59.3 \pm 0.4$  vs KE:  $-64.3 \pm 0.5$  kJ/mol;  $p < 0.05$ ). This notable increase in the energy of ATP hydrolysis associated with ketone metabolism in the compromised brain region is vital to restoring cellular work functions. This increase in the energy of hydrolysis was similar in magnitude to another instance where ketone body metabolism resulted in a more energetic  $\Delta G$  of ATP hydrolysis and restored work output in a model of the failing heart (Sato et al. 1995).

### Effects of ketone metabolism on lipid and protein oxidation

One consequence of the metabolism of ketones is the reduction of the redox potential of the NADP system (Veech et al. 1969) which controls the reduction of glutathione, which has been shown to be oxidized in this mouse strain (Ghosh *et al.* 2014, Veech 2004). We observed lower amounts of protein carbonylation (–19%,  $p < 0.05$ ) as determined by di-nitrophenyl-hydrazine (DNP) reactivity and 4-HNE (3 fold decrease,  $p < 0.05$ ) in the hippocampus of mice fed the KE diet compared to CON-fed mice (Figure 4). Protein carbonylation and lipid peroxidation result from the attack of hydroxyl radicals on proteins or membrane unsaturated fatty acids, respectively. Interestingly, mice maintained on either diet had similar levels of protein carbonylation and 4-HNE formation in the cortex which were also comparable to amounts observed in the hippocampus of KE-fed mice suggesting that ketone metabolism was highly effective in reducing free radical damage in regions prone to ROS injury.

### Effect of ketone metabolism on secretase protein levels

Hippocampal regions from these KE-fed mice were previously shown to have lower levels of A $\beta$  plaques (Kashiwaya et al. 2013). The lower amount of amyloid accumulation may, in part, be due to changes in the metabolism of the amyloid precursor protein (APP) through an alteration of the activity or amount of secretase enzymes, ADAM10 ( $\alpha$ -secretase) or BACE1 ( $\beta$ -secretase). Protein levels of these enzymes were determined using western blot of hippocampal lysates and found to be significantly altered, where ADAM10 was 21% higher ( $p < 0.05$ ), BACE1 was 23% lower ( $p < 0.05$ ) in KE-fed animals compared to controls. No significant changes were observed in cortex samples where the level of A $\beta$  plaque accumulation was no different (Kashiwaya et al., 2013).

## Discussion

PET studies using a radio-labeled glucose analogue (e.g. fDG) have consistently demonstrated a low rate of glucose metabolism (between 20–30% lower) in brain regions involved in processing memory (e.g. the hippocampus, posterior cingulate, temporal, and parietal lobes) in humans with AD (Kapogiannis & Mattson 2011, Cunnane et al. 2011). In a transgenic mouse model of AD (3xTgAD) Ding and coworkers observed a lower uptake of fDG and an elevation of the neuronal monocarboxylate transporter, MCT 2, in the aged hippocampus (Ding et al. 2013b). Further, Yao and coworkers found an increase in ketone metabolizing enzymes; succinyl-CoA:3-ketoacid CoA transferase (SCOT) and acetyl-CoA acetyltransferase 1 (ACAT1) expression in transgenic mice maintained on a diet intended to induce mild ketosis (Yao et al. 2009). Interestingly, using isotopically labeled  $\beta$ -hydroxybutyrate hibernating rodents have been shown to increase their brain utilization of ketones as a preferred energy substrate through an upregulation of MCT 1 which continues into the awakened state (Andrews *et al.* 2009). Insulin resistance, decreased cerebral blood flow, inhibition of GLUT transporters, inhibition of glycolytic enzymes and amyloid-stimulated PDH phosphorylation all contribute to decreased glucose metabolism in clinical AD and transgenic models (Prins 2008, Frackowiak et al. 1981, Cunnane et al. 2011, Kapogiannis & Mattson 2011). Since, our study did not include a wild type control group the concentrations of brain metabolites pertain only to the aged transgenic mouse model

used here. Furthermore, the cytosolic and mitochondrial redox potentials derived from these concentrations may not pertain to other mouse strains.

Low glucose metabolism results in the oxidation of NAD and NADP redox pools (Ghosh *et al.* 2012), and ultimately a decreased ability to perform essential functions while limiting the buildup of damaged biomolecules and organelles. To our knowledge this is the first study to examine the effects of a specific and direct elevation of circulating  $\beta$ -hydroxybutyrate on a range of neurochemicals in a mouse model of AD and the first fully detailed report to encompass a wide range of metabolites from both the glycolytic and TCA cycle pathways, some of which are used to determine the cellular energetic and redox potentials, and also derived neurotransmitters and amino acids, as well as measurements of correlative protein and lipid damage through their ROS products, from the hippocampus, a region, highly affected by accumulation of A $\beta$  and p-Tau as compared to the cortex using a KE diet which sustained high blood ketone levels. Here we report that a dietary KE corrects several key markers of an energy deficit, such as, reducing the mitochondrial free [NAD<sup>+</sup>]/[NADH] pool and increasing the  $\Delta G$  of ATP hydrolysis (Table 1) predominantly in the hippocampus. Many of the positive outcomes presented here have been associated with ketone metabolism in the hippocampus, where it was previously reported that hippocampal regions from the same KE-fed mice had lower numbers of immuno-reactive amyloid cells compared to control-fed animals (Kashiwaya *et al.*, 2013) Also, hippocampal regions from these same animals contained far greater numbers of immuno-reactive p-Tau cells compared to the cortex from mice on either diet (Kashiwaya *et al.*, 2013). The incorporation of a stable isotopically labeled ketone ester into a study design using this animal model of Alzheimer's disease would help clarify to what extent different affected brain regions utilize ketones to meet their energy demands and for synthetic purposes, such as the synthesis of amino acids and lipids. The tissue used in these analyses were taken from euthanized mice and was rapidly frozen diminishing the effects of anoxia as observed in the lactate to pyruvate ratio which accorded with freeze blown brain tissue, a procedure which preserves the *in vivo* state (Kashiwaya *et al.* 2010).

Although a number of studies have shown decreased uptake of fDG in AD patients or in animals models of AD there were no substantial differences in the concentration of G-6-P in the cortex in mice on either diet (CON: 0.064 or KE: 0.058  $\mu$ Mol/g tissue; Figure 1A). This concurs with a previous dietary intervention study in healthy rats where a KE-supplemented diet had no effect on the concentration of G-6-P in whole brains (Kashiwaya *et al.* 2010). In contrast, we show that higher concentrations of  $\beta$ -HB were observed in the hippocampus of KE-fed 3xTgAD mice (Table 1), there were also elevations in several glycolytic intermediates including G-6-P, DHAP, 3PG, PEP, pyruvate and calculated oxaloacetate (Figure 1B, D). The higher levels of  $\beta$ -HB in this region may be due to increased transport via the MCT2, which has been shown to increase in the hippocampus of older 3XTgAD mice (Ding *et al.* 2013a) and the elevated blood ketones in mice on the KE diet. There were also significantly elevated concentrations of the TCA cycle metabolites, citrate, isocitrate,  $\alpha$ -ketoglutarate, succinate and fumarate and acetyl-coA in the hippocampus of KE-fed mice (Figure 1D). The increase concentrations of TCA cycle intermediates observed in the hippocampus of KE-fed mice may be due to an increase in mitochondrial function or possibly to an increase in mitochondrial biogenesis. Previously, we observed that after 30

days pair-feeding a liquid KE-diet to C57BL/6J mice that animals had increased numbers of mitochondria and higher levels of mitochondrial biogenesis-regulating proteins in the interscapular brown adipose tissue compared with controls (Srivastava, S. et al., FASB J. 2012). Elevations in the concentrations of hippocampal glycolytic intermediates may have resulted from either a dis-inhibition of glycolysis or potentially through an increased flow of carbon substrates and reducing equivalents from the mitochondrial metabolism of ketones into the cytosol via malate or citrate transport toward anapleuristic pathways. This latter suggestion is supported by the observation of the relative oxidation of the cytosolic free  $[NAD^+]/[NADH]$  pool in KE-fed mice (Table 1), indicating a reversal of NAD-linked cytosolic enzymes such as Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Additionally, the increase in the apparent concentration of cytosolic oxaloacetate in the hippocampus of KE-fed mice (+53%) is consistent with the hypothesis that citrate and malate are being metabolized in the cytosol thereby increasing the levels of oxaloacetate, a product of both ATP-citrate lyase and malate dehydrogenase. Oxaloacetate is then utilized by phospho-enol-pyruvate carboxykinase (PEPCK) to produce phosphoenol-pyruvate (PEP) which was 50% greater in KE-fed mice followed by increases in 3PG, DHAP and G-6-P (Figure 1B). These cytosolic metabolites play key roles in maintaining cellular homeostasis in the hippocampus of KE-fed mice, including signaling cascades, generating cytosolic NADPH and synthesis of nucleotides.

We've shown that in KE-fed mice the mitochondrial free  $[NAD^+]/[NADH]$  redox state becomes more reduced, favoring NADH (Table 1). A broader examination of these results in light of those of Ghosh et al. (2012) demonstrate that the metabolism of ketones are in essence rescuing a highly oxidized mitochondrial redox state. Through the rescue of the redox potential the cell is more able to prevent and/or repair oxidative damage leading to decreases in both lipid and protein oxidation products observed notably in hippocampal tissue compared to cortex from KE-fed mice (Figure 4). The free  $[NADP^+]/[NADPH]$  redox couple has the single largest electrochemical potential in the cell at  $-0.42V$ . This in turn drives the reduction of glutathione and the ascorbate and tocopherol couples toward their respective reduced states. While the  $[NADP^+]/[NADPH]$  ratio in the hippocampus was not significantly altered by ketone metabolism (Table 1) we observed decreased oxidative damage (Figure 4A–C) suggesting NADPH utilization as well as a demonstrated decrease in amyloid plaques (Kashiwaya et al. 2013). Further, changes in NAD and Q redox states favoring a reduction in NAD and oxidation of Q observed here suggest that ketone metabolism also decreased damaging ROS production by lowering the amount of the semi-oxidized Q-semiquinone species, a major source of mitochondrial superoxide (Chance *et al.* 1979).

Changes in cellular redox states and increases in oxidative damage can stimulate the pathological amyloid precursor protein (APP) metabolism toward  $A\beta$  through induction of BACE1/ $\beta$ -secretase (Kwak et al. 2011). The 3xTgAD mouse produces high levels of APP and its metabolism depends on a family of proteases referred to as  $\alpha$ ,  $\beta$  and  $\gamma$  secretases. The two major breakdown pathways are through either  $\alpha$ -secretase or  $\beta/\gamma$  secretases. The latter pathway is associated with toxic  $A\beta$  aggregation and disease progression, whereas the former is associated with neuronal protection and APP disposal (Mattson 2004). Here, we have shown that mice maintained on the KE diet had increased  $\alpha$ -but lower  $\beta$ -secretase in

the hippocampus (Figure 5), which concurs with previous findings in the same animals of lower A $\beta$  accumulation (Kashiwaya et al., 2013) and with previous work in female 3xTgAD mice maintained on a 2-deoxyglucose diet intended to increase blood ketones (Yao *et al.* 2011).

Membrane cholesterol homeostasis is maintained in the brain by in-situ biosynthesis utilizing NADPH with its removal as 24-S-hydroxycholesterol through an oxidation by CYP36A1. Among several brain diseases and pathologies, human subjects with AD and transgenic animals engineered to accumulate phosphorylated-Tau, brain membrane disequilibrium results in lower plasma concentrations of 24-S-hydroxycholesterol compared to normal controls and to wild type mice, respectively (Bretillon *et al.* 2000, Burlot *et al.* 2015). Here, we demonstrate that mice maintained on the KE diet had nearly a 2-fold higher concentration of 24-S-hydroxycholesterol in the blood. The concentrations of 24-S-hydroxycholesterol from the combined brain regions (hippocampus and cortex) from KE-fed and CON-fed mice were also analyzed. The concentrations of this metabolite appeared to be somewhat elevated in KE-fed animals (CON: 33.0 +/- 3.7 vs KE; 40.9 +/- 5.0 pMol/mg tissue, n=10, p <.06); however, this did not reach the level of significance and therefore leaves open the question whether the brain metabolism of ketones is capable of sustaining membrane homeostasis through its more reduced cellular redox potential (Table 1) as well as supplying the activated carbon substrates (Figure 1D) required for lipid biosynthesis. In a separate analysis for CYP36A1 we found no differences in the level of the enzyme in the hippocampus between groups (data not shown).

Feeding the KE diet to 3xTgAD mice did not have any significant effect on amino acid concentrations in the cortex (Figure 2A). Previously, we've shown that maintaining rats on a KE-supplemented or high fat diet for 14 days resulted in a decrease in the concentrations of whole brain glutamate and GABA compared to control-fed animals (Kashiwaya et al. 2010). However, in the hippocampus of KE-fed 3xTgAD mice there were significantly lower levels of GABA, the major inhibitory neurotransmitter which corresponded to lower levels of the astrocytic glial fibrillary acidic protein (GFAP). Lower levels of astrocytic-released GABA has been associated with improved long-term potentiation in the hippocampus and better memory outcome in an AD mouse model (Jo et al. 2014). Lower GABA levels in the hippocampi of KE-fed mice here may account, in part, for improved memory and greater exploratory behavior outcome compared to controls as reported previously (Kashiwaya et al., 2013). Whether the KE diet would enhance cognitive performance in non-transgenic mice strains is unknown. However, rats maintained on a KE-diet showed improve cognitive function in tests using an eight arm radial maze compared to animals on other diets (Murray et al. 2016). Also, in the hippocampus there were higher concentrations of both aspartate and n-acetyl aspartate in KE-fed mice. The higher concentrations of the energy-linked n-acetyl aspartate in the hippocampus which follow from the increased availability of the TCA cycle derived acetyl-coA (Figure 1) and carbon units derived from aspartate via oxaloacetate (Figure 2) may also shed light on the potent anxiolytic effect that the KE diet had reducing anxiety in mice which stayed in the open arm portions of the elevated plus maze for notably longer periods of time (figure 3).

Due to potential increases in the uptake of ketones in the hippocampus of aging 3xTgAD mice (Ding et al. 2013b, Yao et al. 2009), attempts have been made to treat AD using ketogenic diets that are non-specific in that they elevate a range of lipids and other nutrients in the fat consumed in humans (Krikorian *et al.* 2012) and in animal models (Brownlow *et al.* 2013, Beckett *et al.* 2013). Due to variability in diet formulation, age of animals, and genetic strain the results have been somewhat mixed. Two studies in mice independent of genotype demonstrated the significance of ketogenic diets in producing increased grip strength but no apparent change in aberrant protein accumulation nor memory effect. However, we noted previously moderate positive changes in memory, substantial reduction in anxiety, and increased exploratory behavior in older 3xTgAD mice maintained on the KE diet (Kashiwaya et al. 2013). Additionally, these changes concur with improved hippocampal cellular energetics, a more favorable redox potential, decreased ROS damage, and lower GABA levels reported here. In order to produce comparable blood ketone concentrations, Brownlow and co-workers virtually eliminated dietary carbohydrate which elicited a decrease in blood glucose in two alternative mouse models of AD. Unlike either of those models the 3xTgAD murine strain has been shown to have increased capacity for ketone metabolism (Yao et al. 2011, Ding et al. 2013a) and responded to ketone utilization with improved amyloid processing (Yao et al. 2011). The ketone ester has had notable success in an APOe4-positive Alzheimer's patient (Newport *et al.* 2015). There is no apparent toxicity of the KE even when elevating blood ketones to 5–6 mM with a single dose or in repeated dosing over 5 days (Clarke *et al.* 2012). Here, we've shown that KE supplementation in 3xTgAD mice increased plasma  $\beta$ -HB by 5-fold and corrected energy deficiencies in the hippocampus which was substantiated by improved biomarkers and reduced oxidative damage. KE supplementation offers a potential therapeutic treatment of the clinical disease as it addresses the fundamental metabolic deficits in both humans and transgenic animals.

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## List of Abbreviations

<b>3PG</b>	3-Phospho-glycerate
<b>4-HNE</b>	4-hydroxynonenal
<b>A<math>\beta</math></b>	Beta-amyloid
<b>AD</b>	Alzheimer's disease
<b>ADAM10</b>	A disintegrin and metalloproteinase domain-containing protein 10
<b>BACE1</b>	Beta secretase 1
<b><math>\beta</math>-HB</b>	Beta-hydroxybutyrate

<b>DHAP</b>	Dihydroxyacetone phosphate
<b>fDG</b>	Fluorodeoxyglucose
<b>GFAP</b>	Glial fibrillary acid protein
<b>G-6-P</b>	Glucose 6-phosphate
<b>KE</b>	Ketone ester or (R)-((R)-3-hydroxybutyl) 3-hydroxybutanoate
<b>PCA</b>	Perchloric acid
<b>PEP</b>	Phosphoenolpyruvate
<b>Q</b>	Coenzyme Q or Ubiquinon
<b>TCA</b>	Tricarboxylic Acid

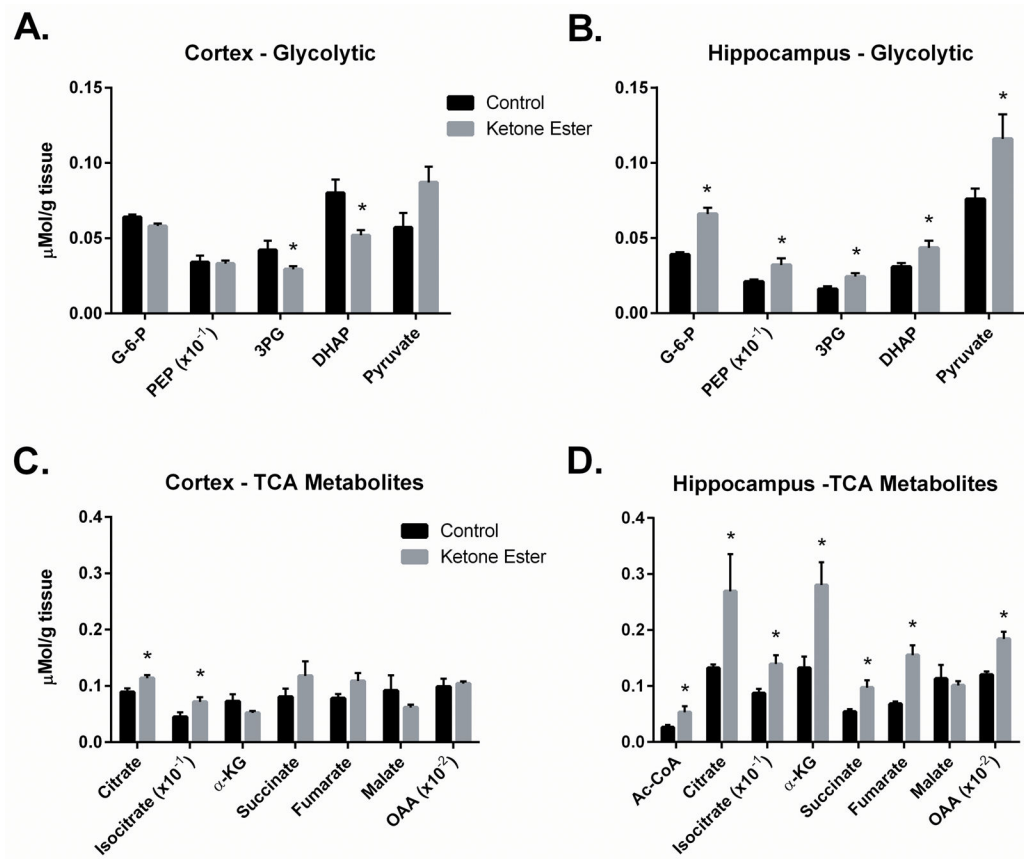
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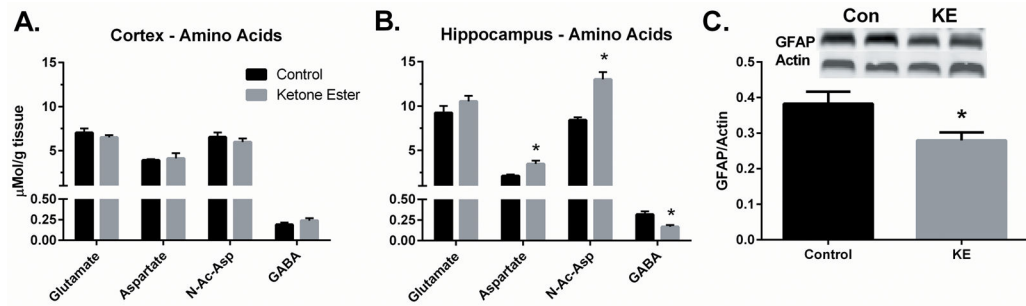


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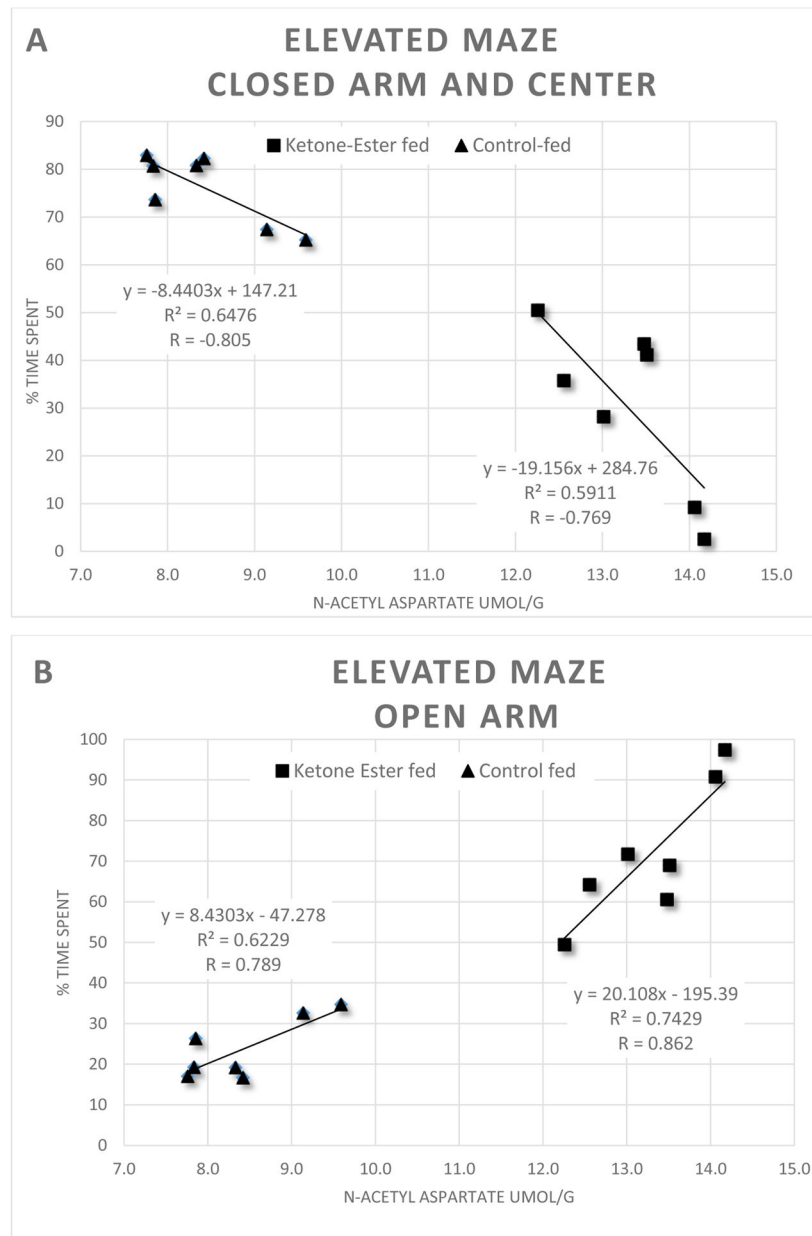
**Figure 1.**

Effect of KE diet on the concentrations of glycolytic and TCA cycle intermediates. These metabolites were measured in the cortex (A, C) and hippocampus (B, D) of 3XTgAD mice fed ketone ester (KE) or control (CON) diets using isotope dilution mass-spectrometry. Oxaloacetate (OAA) which was calculated as shown in the Methods section. Values plotted are average  $\pm$  SEM,  $n=5-6$ , \* indicates significant at  $p < 0.05$  in Students t-test.

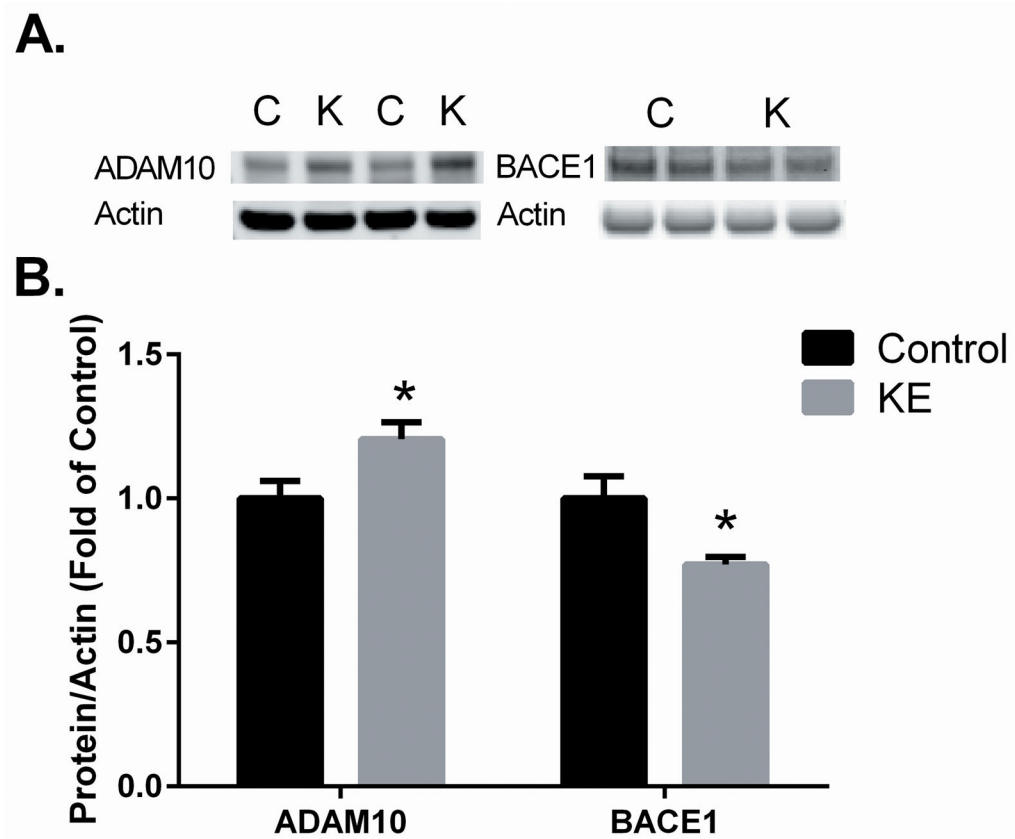


**Figure 2.**

Effect of KE diet on the concentrations of amino acids; glutamate, aspartate, n-acetyl aspartate, and  $\gamma$ -amino butyric acid (GABA). These amino acids were quantified in cortex (A) and hippocampus (B) from 3XTgAD mice fed KE or CON diets using isotope dilution mass-spectrometry. GFAP, a marker for activated astrocytes, was determined in the hippocampus (C) using western blotting, quantifications were made as a fluorescence intensity ratio between the GFAP and actin bands. Values plotted are average  $\pm$  SEM, n=5–6, \* indicates significant at  $p < 0.05$  in Students t-test.

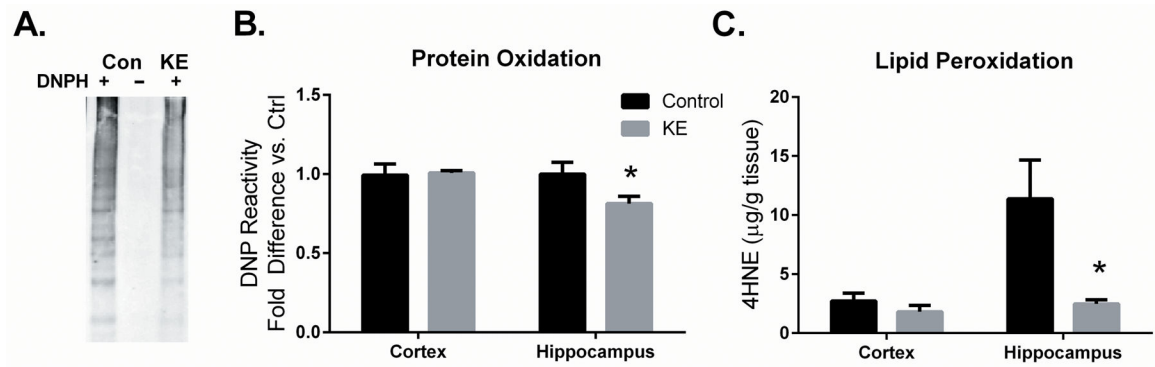


**Figure 3.** Linear correlation diagrams that depict a relationship between hippocampal n-acetyl aspartate (NAA) from 3xTgAD mice that were maintained on either a ketone ester diet (n=7) or control diet (n=7) versus the percent time spent that each animal remained in either the closed arm and center (A) or the open arm (B) areas of an elevated (60 cm from floor) plus sign-shaped platform maze from phase I trials (Kashiwaya et al., 2013). There were strong correlations between the concentrations of hippocampal NAA and the percent time that animals spent in either areas of the maze. Control diet-fed mice had lower concentrations of hippocampal NAA and remained for a greater percentage of time in the closed arms whereas mice fed the ketone-ester diet preferred the open area portions of the maze.



**Figure 4.**

Effect of KE diet on protein carbonylation and 4-hydroxynonenal (4-HNE) concentrations in cortex and the hippocampus from 3XTgAD mice fed KE or CON diets. DNP derivatization of protein carbonyl groups were detected using western blotting. (A) The representative blot of the diet effect on DNP staining in the hippocampus includes an underderivatized lane for background quantification. (B) shows the quantification of these blots relative to control levels. (C) 4-HNE was quantified in the cortex and hippocampus using isotope dilution mass-spectrometry. Values plotted are average  $\pm$  SEM,  $n=5-6$ , \* indicates significant at  $p < 0.05$  in Students t-test.



**Figure 5.**

Effect of KE diet on the amyloid metabolizing enzymes ADAM10 ( $\alpha$ -secretase) and BACE1 ( $\beta$ -secretase protein levels. Levels of ADAM10 (A) and BACE1 (B) were measured using Western Blot in hippocampus from 3XTgAD mice fed KE or CON diets, using fluorescence intensity ratios between the target band and actin. Representative blots are shown. Values plotted are average  $\pm$  SEM, n=4–6, \* indicates significant  $P < 0.05$  in Students t-test.

**Table 1**

Effect of KE diet on the cytosolic and mitochondrial redox potentials and the concentrations of metabolites used to determine them. The metabolites lactate, pyruvate,  $\beta$ -hydroxybutyrate, acetoacetate, isocitrate and  $\alpha$ -ketoglutarate were used for these determinations in the cortex and hippocampus of mice fed either a control diet (CON) or a diet containing and ester of  $\beta$ -hydroxybutyrate (KE). Quantities in  $\mu\text{mol/g}$  tissue are listed as average  $\pm$  SEM, n=5–6, \* indicates significant at  $p < 0.05$  in Students t-test.

	Cortex		Hippocampus	
	CON	KE	CON	KE
<b>Lactate (<math>\mu\text{Mol/g}</math>)</b>	1.31 $\pm$ 0.144	1.35 $\pm$ 0.07	1.80 $\pm$ 0.21	1.58 $\pm$ 0.07
<b>Pyruvate (<math>\mu\text{Mol/g}</math>)</b>	0.057 $\pm$ 0.097	0.087 $\pm$ 0.011	0.076 $\pm$ 0.007	0.116 $\pm$ 0.016*
<b>cyto NAD/NADH</b>	230 $\pm$ 21	388 $\pm$ 58*	250 $\pm$ 23	411 $\pm$ 48*
<b><math>\beta</math>-hydroxybutyrate (<math>\mu\text{Mol/g}</math>)</b>	0.112 $\pm$ 0.008	0.103 $\pm$ 0.001	0.116 $\pm$ 0.027	0.182 $\pm$ 0.011*
<b>Acetoacetate (<math>\mu\text{Mol/g}</math>)</b>	0.034 $\pm$ 0.003	0.038 $\pm$ 0.005	0.084 $\pm$ 0.008	0.048 $\pm$ 0.008*
<b>mito NAD/NADH</b>	3.97 $\pm$ 0.48	4.74 $\pm$ 0.60	9.27 $\pm$ 0.84	3.44 $\pm$ 0.64*
<b>iso-citrate (<math>\mu\text{Mol/g}</math>)</b>	0.0045 $\pm$ 0.0009	0.0072 $\pm$ 0.0011*	0.0087 $\pm$ 0.0039	0.0139 $\pm$ 0.0020*
<b><math>\alpha</math>-ketoglutarate (<math>\mu\text{Mol/g}</math>)</b>	0.073 $\pm$ 0.013	0.052 $\pm$ 0.004	0.132 $\pm$ 0.022	0.280 $\pm$ 0.040*
<b>NADP+/NADPH</b>	0.034 $\pm$ 0.008	0.014 $\pm$ 0.003*	0.032 $\pm$ 0.008	0.039 $\pm$ 0.008
<b>G<sub>ATP</sub> Hydrolysis (kJ/mol)</b>	-62.3 $\pm$ 0.5	-61.6 $\pm$ 0.2	-59.3 $\pm$ 0.4	-64.3 $\pm$ 0.5*