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ARTICLE

The ketogenic diet component decanoic acid increases mitochondrial citrate synthase and complex I activity in neuronal cells

Sean David Hughes,^{*†} Marta Kanabus,^{*} Glenn Anderson,[‡] Iain P. Hargreaves,[§] Tricia Rutherford,[¶] Maura O' Donnell,[¶] J. Helen Cross,^{**} Shamima Rahman,^{*††} Simon Eaton^{‡‡} and Simon J. R. Heales^{*†§}

^{*}Clinical and Molecular Genetics Unit, UCL Institute of Child Health, London, UK

[†]Chemical Pathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

[‡]Electron Microscopy Unit, Great Ormond Street Hospital, Foundation Trust, London, UK

[§]Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, London, UK

[¶]Vitaflo International, Liverpool, UK

^{**}Neurosciences Unit, UCL Institute of Child Health, London, UK

^{††}Metabolic Unit, Great Ormond Street Hospital Foundation Trust, London, UK

^{‡‡}Paediatric Surgery Unit, UCL Institute of Child Health, London, UK

Abstract

The Ketogenic diet (KD) is an effective treatment with regards to treating pharmaco-resistant epilepsy. However, there are difficulties around compliance and tolerability. Consequently, there is a need for refined/simpler formulations that could replicate the efficacy of the KD. One of the proposed hypotheses is that the KD increases cellular mitochondrial content which results in elevation of the seizure threshold. Here, we have focussed on the medium-chain triglyceride form of the diet and the observation that plasma octanoic acid (C8) and decanoic acid (C10) levels are elevated in patients on the medium-chain triglyceride KD. Using a neuronal cell line (SH-SY5Y), we demonstrated that 250- μ M C10, but not C8, caused, over a 6-day period, a

marked increase in the mitochondrial enzyme, citrate synthase along with complex I activity and catalase activity. Increased mitochondrial number was also indicated by electron microscopy. C10 is a reported peroxisome proliferator activator receptor γ agonist, and the use of a peroxisome proliferator activator receptor γ antagonist was shown to prevent the C10-mediated increase in mitochondrial content and catalase. C10 may mimic the mitochondrial proliferation associated with the KD and raises the possibility that formulations based on this fatty acid could replace a more complex diet.

Keywords: decanoic acid, ketogenic diet, mitochondria, PPAR.

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The ketogenic diet (KD) in its various forms has been demonstrated to be effective at reducing seizures in individuals with drug-resistant epilepsy (Neal *et al.* 2009). Despite the effectiveness of the diet, the exact mechanism(s) of action in the context of seizure control are not known. However, a number of mechanisms have been proposed that include alterations in antioxidant status, changes in brain neurotransmitter levels, such as GABA, and the metabolic consequences of the diet on cellular energy metabolism (Rho and Sankar 2008). With regards to the latter, it is of note that mitochondrial dysfunction, at the level of the respiratory chain, has been documented in animal model systems of status epilepticus and in post mortem brain samples obtained

from patients who had epilepsy (Kunz *et al.* 2000; Cock *et al.* 2002). Furthermore, mitochondrial biogenesis has been reported in the brain of rats fed the KD leading to the

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Address correspondence and reprint requests to Simon Heales, Clinical and Molecular Genetics Unit, UCL Institute of Child Health, London, UK. E-mail: s.heales@ucl.ac.uk

Abbreviations used: AcAC, acetoacetate; BADGE, Bisphenol A diglycidyl ether; BHB, beta-hydroxybutyrate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FBS, foetal bovine serum; KD, ketogenic diet; LDH, lactate dehydrogenase; MCT, medium-chain triglyceride; PPAR γ , peroxisome proliferator activator receptor γ .

suggestion that this change has the capacity to elevate the seizure threshold (Bough *et al.* 2006).

A number of versions of the KD exist including the use of a medium-chain triglyceride (MCT)-based diet (Kossoff *et al.* 2009). This diet has been demonstrated to be as effective as the classical KD and can permit a greater proportion of carbohydrate and protein to be consumed when compared with the classical KD. However, compliance and tolerability of the diet is still an issue (Bahassan and Jan 2006). There is therefore a need for refined formulations that can replicate the efficacy of the diet and release patients and families from its current constraints. Critical to the development of such a treatment is better understanding of the biochemical changes that occur as a consequence of the KD.

Patients on the KD diet have raised plasma levels of ketone bodies (β -hydroxybutyrate and acetoacetate), but these correlate poorly with seizure control, suggesting the likely involvement of other factors with regards to conveying therapeutic benefit (Likhodii *et al.* 2000; Thavendiranathan *et al.* 2000). In addition to ketones, the medium-chain fatty acids octanoic (C8) and decanoic (C10) acid are also raised in the plasma of patients who are on the MCT-based diet (Haidukewych *et al.* 1982). However, the contribution of these fatty acids to efficacy of the KD has not been extensively studied. In view of the suggestion that increased cellular mitochondrial content may be beneficially associated with KDs, we have in this study investigated the effects of C8 and C10 on neuronal mitochondrial content with an aim to provide further insight into the mechanism whereby the MCT KD may exert its beneficial effects.

Methods

Materials

Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient mixture (DMEM/F-12) (1 : 1), L-glutamine, heat-inactivated foetal bovine serum (FBS) and 2.5 g/L trypsin EDTA were purchased from Invitrogen Ltd (Paisley, UK). Unless otherwise stated all other chemicals were from VWR international (Lutterworth, UK) or Sigma (Poole, UK). SH-SY5Y cells were purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK).

Cell culture

SH-SY5Y cells were stored frozen and only cells with passage numbers between 20 and 24 were utilized. When required, cells were thawed at 20–25°C and quickly seeded at a density of 13.3×10^3 cell/cm² in 75 cm² flasks. Cells were then grown in media containing 1 : 1 DMEM/F-12 + 100 mL/L FBS + 10 mL/L L-glutamine at +37°C and 5% CO₂. Growth medium was replaced the day after seeding, owing to the presence of dimethyl sulphoxide (DMSO) in the freezing media, and every other day thereafter. Cells were passaged by washing once with 6 mL/flask Hank's balanced salt solution and then lifting with 4 mL/flask trypsin EDTA. Media of 8 mL/flask were added to each flask and the cells were transferred

to conical tubes for centrifugation at 500 g for 3 min. Supernatant was removed and cells were re-suspended in a known volume of medium. Cell suspension of 10 μ L was mixed 1 : 1 with 4 g/L trypan blue before cells were counted on an Invitrogen Countless automated cell counter. Cells were then added to 75 cm² flasks at a density of $5\text{--}10 \times 10^3$ cells/cm². The total volume in the flask was made up to 10 mL with medium before incubating at 37°C and 5% CO₂. Cells were passaged at 80–90% confluence.

Cell treatment conditions

Stock solutions of C8, C10 and sebacic acid were made up in DMSO, sterile-filtered and stored at –20°C. These were then added to 10 mL growth medium in the culture flasks. Stock solution of 50 μ L was added to the medium to give final concentrations between 50 and 300 μ M. DMSO of 50 μ L alone was used as a control incubation. For some incubations with C10, 25- μ M Bisphenol A diglycidyl ether (BADGE, Tocris Bioscience, Bristol, UK) was also added. The effects of a range of C8 + C10 mixtures (final combined concentration 250 μ M) were also evaluated. Experimental incubations were for 1–6 days. The potential reversibility of any effect was evaluated by removing the fatty acid from the medium and incubating for a further 4 days. Medium was changed every 48 h, along with the treatment compound/vehicle. For comparison, beta-hydroxybutyrate or acetoacetate (5-mM final concentration) was added to the cell culture medium and incubations were performed as above. Cells were harvested with 2.5 g/L trypsin EDTA. Trypsin EDTA was diluted and inhibited with FBS, removed by centrifugation and cell pellets washed once with Hanks balanced salt solution. For enzymatic assays and total protein determination, cell pellets were re-suspended in isolation buffer consisting of 10 mM Tris (pH 7.4), 1 mM EDTA and 320 mM sucrose in HPLC grade water. Samples were stored at –80°C until analysis.

Cell storage

Cells were passaged, as described above, except after centrifugation the cells were re-suspended in a freezing medium containing 700 mL/L 1 : 1 DMEM/F-12 + 200 mL/L FBS + 100 mL/L DMSO. The cells were then counted, as described above, and stored in 1-mL aliquots containing 1×10^6 cells/mL; the aliquots were then frozen at –20°C for 2 h before being transferred to the –80°C freezer. For long-term storage, the cells were transferred into liquid nitrogen the next day.

Control newborn human foreskin fibroblasts were obtained from GlobalStem, Rockville, MD, USA (Cat Number: GSC-3002) and were grown in DMEM containing 25 mM glucose and 3.97 mM L-glutamine. DMEM was supplemented with 10% FBS, uridine 50 mg/L and 10 μ g/mL penicillin streptomycin. Upon reaching confluence, cells were split into 4×182 cm² flasks and treated for 6 days with 250- μ M C10 or DMSO (as described above) in a total of 20 mL of supplemented DMEM. Medium was changed once, on the third day. On day 6, cells were trypsinized, washed once with phosphate-buffered saline, the cell pellet was re-suspended in isolation buffer and stored in –80°C until analysis.

Citrate synthase, respiratory chain enzymes, Acyl CoA dehydrogenase and catalase-specific activities

Immediately prior to analysis, samples were thawed at 37°C. Enzyme activities were assessed using an Uvikon XL spectrophotometer

(Secomam, Ales, France). Citrate synthase (EC 4.1.3.7) activity was determined according to the method of Shepherd and Garland (1969). Complex I (EC 1.6.5.3) and complex II+III (EC 1.8.3.1) activities were assessed as described previously by Schapira *et al.* (1990). Assessment of complex IV (EC 1.9.3.1) activity was as described by Wharton and Tzagoloff (1967). Acyl CoA dehydrogenase (EC 1.3.8.7) activity was determined as described by Ijlst and Wanders (1993) using decanoyl CoA as a substrate. Catalase (EC 1.11.1.6) activity was measured using the method of Beers and Sizer (1952). Total protein was determined using the Peterson modified Lowry method (Peterson 1977). Bovine serum albumin was used as a standard.

Lactate dehydrogenase release

The percentage of lactate dehydrogenase (LDH) released into the cell culture medium from the SH-SY5Y cells was determined on the last day of incubation, as previously described (Bolaños *et al.* 1995).

Reduced glutathione

Reduced glutathione (GSH) was determined using HPLC and electrochemical detection in cell pellets, as previously described (Bolaños *et al.* 1995).

Electron microscopy

Cells were detached from culture flasks by trypsin digestion, and centrifuged to form a pellet for EM processing. All samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer followed by secondary fixation in 1.0% osmium tetroxide. Tissues were dehydrated in graded ethanol, transferred to propylene oxide and then infiltrated and embedded in Agar 100 epoxy resin. Polymerization was at 60°C for 48 h. Using a Diatome diamond knife on a Leica (Milton Keynes, UK) Ultracut UCT ultramicrotome, 90-nm-ultrathin sections were cut. Sections were picked up on copper grids and stained with alcoholic uranyl acetate and Reynold's lead citrate. The samples were examined in a JEOL (Welwyn Garden City, UK) 1400 transmission electron microscope and images recorded using AMT XR80 digital camera and software.

Statistical analysis

All results are expressed as mean \pm SEM. n = the number of independent cell culture preparations and was between 3 and 7 separate experiments. Statistical comparisons between two groups were carried out using Student's *t*-test. Multiple comparisons were made using a one-way ANOVA and Tukey's test for *post hoc* analysis. In all cases $p < 0.05$ was considered to be significant.

Results

Citrate synthase activity following C8 and C10 exposure

The activity of citrate synthase is frequently used as a biochemical marker for the mitochondrial enrichment of a tissue/cellular homogenate (Selak *et al.* 2000; Bernier *et al.* 2002; Itkonen *et al.* 2013). Time- and dose-response experiments revealed that exposure of the SH-SY5Y cells, for 6 days, to 250- μ M C10 elicited a maximal significant increase in citrate synthase activity. In contrast, incubations with C8 under comparable conditions revealed no significant effect

with regards to citrate synthase activity (Table 1). The use of DMSO as a vehicle was shown to have no effect upon citrate synthase activity, i.e. when compared with cells incubated with just cell culture medium. To ascertain whether this effect of C10 upon citrate synthase activity could be demonstrated in another cell type, cultured human skin fibroblasts were also exposed to 250- μ M C10 for 6 days; again, a significant increase in activity was recorded (Table 1).

Activities of mitochondrial respiratory chain enzymes and acyl CoA dehydrogenase activities following C10 incubations

In view of the possible increase in mitochondrial enrichment that follows exposure to C10, activities of the mitochondrial respiratory chain enzymes were determined to ascertain whether there was a concomitant increase in their activities. Acyl CoA dehydrogenase activity was also determined, following C10 exposure, to ascertain whether there were any effects upon activity, as this enzyme is also located in the mitochondria and could potentially be up-regulated as a result of increased metabolic flux through β -oxidation. After 6 days of treatment with 250- μ M C10, only complex I activity was significantly increased (Table 2).

Effect of 250- μ M C10 upon GSH status and catalase activity

Mitochondria are a source of reactive oxygen species such as superoxide (Jacobson *et al.* 2005). GSH is a major cellular

Table 1 Citrate synthase activity following exposure of either SH-SY5Y cells to C8 or C10 or fibroblasts to C10

Cell type and treatment	Activity – nmol/min/mg protein
SH-SY5Y – Control	113 \pm 4
SH-SY5Y + C8	115 \pm 5
SH-SY5Y + C10	146 \pm 7**
Fibroblast – Control	52 \pm 6
Fibroblast + C10	79 \pm 9*

** $p < 0.005$, * $p < 0.05$, when compared with the appropriate control incubation.

Cells were exposed to 250 μ M of the fatty acid and for 6 days.

Table 2 Complex I, II+III, IV and acyl CoA dehydrogenase activity in control and C10-exposed (250 μ M for 6 days) SH-SY5Y cells

Enzyme	Control	C10
Complex I	8.8 \pm 0.25	12.0 \pm 0.77*
Complex II+III	8.6 \pm 0.95	8.1 \pm 0.75
Complex IV	0.36 \pm 0.026	0.33 \pm 0.019
Acyl CoA Dehydrogenase	48.3 \pm 4.54	61.2 \pm 6.56

* $p < 0.005$ when compared to control incubation.

Enzyme activity recorded as nmol/min/mg protein, except for complex IV which is expressed as the first-order rate constant/mg protein.

antioxidant, levels of which can reflect cellular oxidative stress (Gegg *et al.* 2003). Catalase, also forms part of the cellular antioxidant defences by removing hydrogen peroxide, formed from superoxide and is located within the mitochondrion and peroxisome (Bai and Cederbaum 2001; Walton and Pizzitelli 2012). In view of the pivotal role these systems play with regards to combating oxidative stress, GSH status and catalase activity were determined after 6 days exposure to C10.

No significant difference in cellular GSH content could be demonstrated between control and C10-treated (250 μ M for 6 days) cells; control, 16.6 ± 1.25 ; and C10 treated, 15.3 ± 0.63 nmol/mg protein. However, following the 6-day incubation with 250- μ M C10, catalase activity was found to be significantly elevated (Fig 1a).

Effect of 250- μ M C10 on cellular LDH release

Loss of cell viability is associated with compromised plasma membrane integrity and increased appearance of LDH into the extracellular medium (Bolaños *et al.* 1995). For cells incubated in the presence of C10, the percentage of total cellular LDH present in the cell culture medium was $23.7 \pm 0.3\%$. For control incubations this was $23.4 \pm 0.5\%$.

Citrate synthase and catalase activities following co-incubation of C10 with the peroxisome proliferator activator receptor γ antagonist, BADGE

C10 is reported to display agonist activity towards peroxisome proliferator activator receptor γ (PPAR γ) (Malapaka *et al.* 2012). Furthermore, glitazone-type drugs, which are PPAR γ ligands, have been shown to cause mitochondrial proliferation in SH-SY5Y cells (Miglio *et al.* 2009). Therefore, we assessed the effect of co-incubations of C10 with the PPAR γ antagonist, BADGE (Wright *et al.* 2000). In the presence of the antagonist, after a 6-day incubation with 250- μ M C10, catalase and citrate synthase were no longer significantly elevated (Fig. 1a and b).

Citrate synthase activity after 6 days incubation with C10 followed by 4 days incubation in the absence of C10

To test whether the observed increase in citrate synthase activity could persist after removal of C10, activity was determined in the SH-SY5Y cells after a 4-day washout period immediately following the 6-day incubation with C10. After this period, in the absence of C10, the activity of citrate synthase was no longer significantly elevated and returned to levels comparable with control cells (activity following 6 days incubation with C10, plus 4 days in absence: 184 ± 5 ; 10-day control incubation: 191 ± 6 nmol/min/mg protein).

Exposure of SH-SY5Y cells to varying ratios of C8 and 10 – Effects upon citrate synthase activity

C8 and C10 are found together in nutritional preparations for incorporation into the MCT-based ketogenic diet

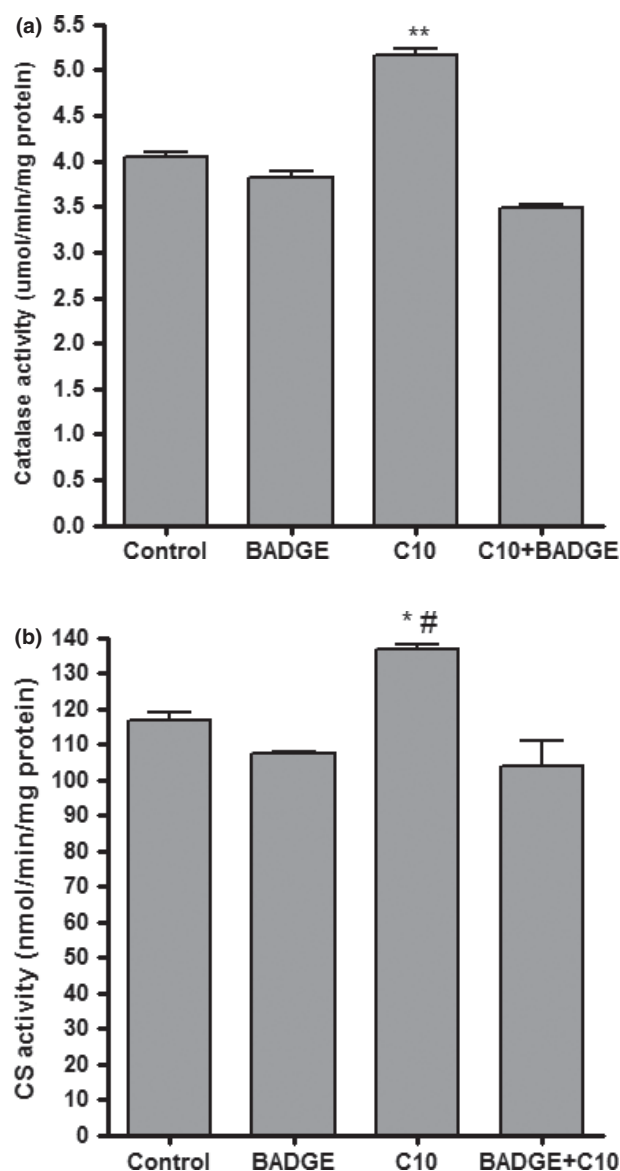


Fig. 1 Effect of co-incubation of the peroxisome proliferator activator receptor γ (PPAR γ) antagonist, bisphenol A diglycidyl ether (BADGE) (25 μ M), with C10 (250 μ M) for 6 days on SH-SY5Y catalase (a) and citrate synthase (CS) activities (b). Catalase activity was significantly increased (** $p < 0.001$) in the presence of C10, when compared with control incubations and C10 incubations performed in the presence of BADGE. Citrate synthase activity was significantly greater in the presence of C10, when compared with control (* $p < 0.05$) incubations and C10 incubations performed in the presence of BADGE (# $p < 0.01$). In all cases, incubations with BADGE alone had no significant effect upon citrate synthase or catalase activities.

(Haidukewych *et al.* 1982; Kossoff *et al.* 2009). In view of this, we examined various ratios of C8 to C10 and determined the effects upon citrate synthase activity in the SH-SY5Y cells exposed to such mixtures (final combined concentration 250 μ M) for 6 days. Only incubations con-

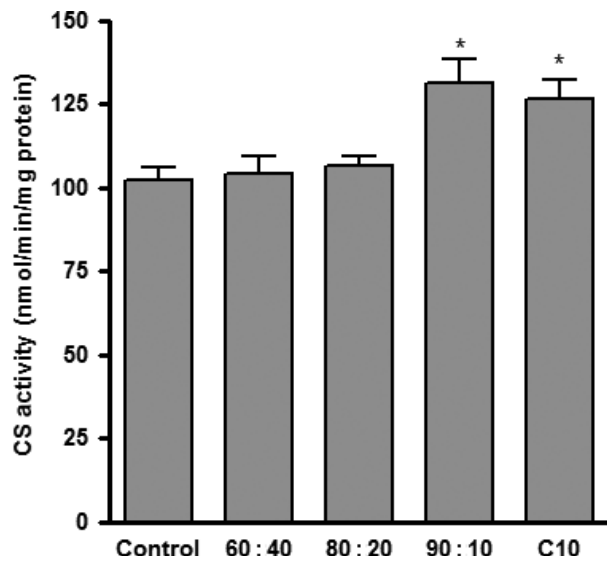


Fig. 2 Effect of 6-day incubation with varying ratios of C8 and C10 (final combined concentration 250 μ M) on SH-SY5Y cell citrate synthase (CS) activity. Ratios ranged from 60% C10 through to 100%. * $p < 0.05$ when compared with control incubation. No significant difference between the incubations performed with 90% C10 (10% C8) and 100% C10 could be demonstrated.

sisting of 10% C8 and 90% C10, together with the 100% C10 preparation, resulted in a significant increase in citrate synthase activity (Fig 2).

Citrate synthase activity following exposure to sebamic acid

In addition to metabolism via β -oxidation, C10 can also be metabolized via ω -oxidation to its dicarboxylic metabolite sebamic acid, which has been reported to display biological activity including improvement of fasting plasma glucose levels in diabetic mice. (Membrez *et al.* 2010). Consequently, SH-SY5Y cells were exposed, in an identical manner to the incubations involving C10, to sebamic acid. Under these conditions, sebamic acid was found to have no significant effect upon citrate synthase activity (sebamic acid: 115 \pm 5; control: 109 \pm 6 nmol/min/mg protein).

Citrate synthase activity following exposure to BHB or acetoacetate

Beta-hydroxybutyrate (BHB) and acetoacetate (AcAc) are both elevated to millimolar levels in the plasma of patients on the KD (Huttenlocher 1976). We therefore also evaluated the effect of these ketone bodies upon SH-SY5Y cell citrate synthase activity. After a 6-day exposure to either 5 mM BHB or AcAc, neither BHB nor AcAc was found to have a demonstrable effect upon citrate synthase activity (BHB: 110 \pm 6; AcAc: 103 \pm 5; control: 104 \pm 2 nmol/min/mg protein).

Electron microscopy – effects of C10 upon mitochondrial content/morphology

To build upon our biochemical data suggesting that there may be an alteration in cellular mitochondrial content following C10 exposure, we utilized transmission electron microscopy to determine whether subcellular differences in mitochondrial number or morphology occurred following exposure of the SH-SY5Y cells to 250- μ M C10 for 6 days. When comparing images from C10-treated cells and controls, an increase in the number of particularly electron dense relatively small mitochondria could be observed in the C10-treated cells (Fig 3).

Discussion

The MCT KD is widely and successfully used for the treatment of pharmaco-resistant epilepsy. Despite this, the biochemical mechanisms responsible for its effectiveness are poorly understood. Seizure control does not appear to correlate strongly with ketosis. However, plasma levels of the medium-chain fatty acids, C8 and C10, are also elevated (C8: 104–859 μ M; C10: 87–552 μ M) in patients on this form of the KD (Haidukewych *et al.* 1982), and we sought here to determine the relative efficacy of these molecules in increasing mitochondrial function.

Among the potential mechanisms whereby KDs convey their efficacy is via improvement of brain energy metabolism

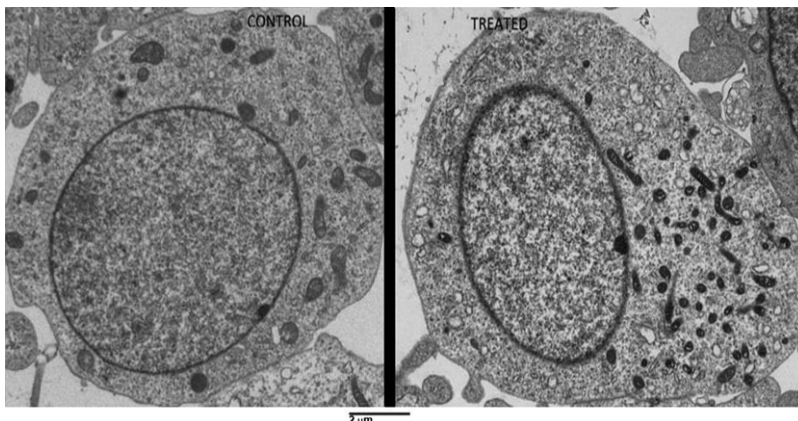


Fig. 3 Electron micrograph prepared from control and C10-treated (250 μ M for 6 days) SH-SY5Y cells. Considerably more electron dense, relatively smaller, mitochondria appear to be present in the treated SH-SY5Y cell (right image).

and mitochondrial function. Brain mitochondrial proliferation has been demonstrated following exposure to the KD (Bough *et al.* 2006). This observation may be particularly relevant given the loss of respiratory chain enzyme activity associated with seizure activity (Kunz *et al.* 2000; Cock *et al.* 2002). In view of these observations, and the efficacy of the MCT-based KD, we studied the effects of C8 and C10 on neuronal cell mitochondrial content. Using a concentration (250 μM) of these fatty acids comparable to that reported in the plasma of patients on a MCT-based KD (Haidukewych *et al.* 1982), we observed, for C10 but not C8, a significant increase in citrate synthase activity after 6 days of exposure. Citrate synthase is exclusively located in the mitochondrion and can be utilized as a marker enzyme for this organelle. Alterations in citrate synthase activity can therefore be used to reflect the mitochondrial enrichment of a cellular/tissue homogenate (Selak *et al.* 2000; Bernier *et al.* 2002; Itkonen *et al.* 2013). Consequently, the data reported here suggest an increase in the mitochondrial content of the SH-SY5Y cell homogenates. The SH-SY5Y neuronal cell line is widely used in neurological research and in the study of mitochondrial function (Wang *et al.* 2012; Hong *et al.* 2013). However, in view of the fact that this is a cell line, a degree of caution is required when interpreting our results. Our observation that C10 can also elicit increased citrate synthase activity in cultured skin fibroblasts suggests that one of the key findings of this study is not restricted to SH-SY5Y cells.

Mitochondrial proliferation and increase in citrate synthase activity may also occur as a consequence of decreased respiratory chain activity (Bernier *et al.* 2002). However, this is unlikely to be the situation here as there was no evidence of impaired respiratory chain enzyme activity following treatment with C10. Moreover, complex I activity was significantly increased. It could also be argued that the effect of C10 upon citrate synthase activity/mitochondrial content occurs as a consequence of the increased availability of an energy substrate. Our finding that C8, BHB and AcAC were unable to elicit a response with regards to citrate synthase suggests that our observation is specific to C10 and not related to provision of energy substrates. The lack of response with the ketone bodies also implies that BHB and AcAC are unlikely to be responsible for the mitochondrial proliferation associated with the KD.

Consistent with our citrate synthase data, the electron microscopy findings indicate an increase in mitochondrial number following C10 treatment. However, the mitochondria appear smaller and more electron dense when compared with the untreated cells. Although the number of mitochondria in the C10-treated cells had increased, their smaller morphology may mean that, overall, the proportion of mitochondria occupying the cytosol has not increased. It remains to be demonstrated whether the appearance of the mitochondria in

the C10-treated cells reflect alterations in metabolic activity and/or “new” mitochondria formed as a consequence of an increased biogenesis.

Loss of respiratory chain enzyme activity, in particular complex I, is associated with seizure activity (Chuang *et al.* 2012). In contrast to the other components of the respiratory chain and decanoyl CoA dehydrogenase activity, complex I activity was significantly elevated by treatment with C10. Although the mechanism for this increase in complex I activity has not been addressed here, it is of interest to note that activity of this complex can, in neuronal cells, exert significant control over ATP synthesis (Davey *et al.* 1998). Whether this specific increase in complex I activity leads to an increase in cellular oxygen consumption and ATP production will be the subject of further studies.

In addition to key functions that include ATP production, calcium homeostasis and regulation of apoptosis, mitochondria are a source of reactive oxygen species. Consequently, mitochondrial proliferation could lead to an increase in oxidative stress. However, improved antioxidant status has been reported in rats fed a KD (Ziegler *et al.* 2003). In this study, we determined the cellular content of GSH and found it to be unaffected by the C10 treatment. Neuronal GSH content has previously been shown to be decreased by exposure to oxidizing species (Gegg *et al.* 2003). In view of this, the preservation of GSH status observed here indicates that significant cellular oxidative stress may not occur in the C10-treated cells. A contributing factor to minimizing oxidative stress may be the increased catalase activity seen in the C10-treated cells. Catalase catalyses the decomposition of hydrogen peroxide into oxygen plus water thereby minimizing cellular oxidative stress (Meilhac *et al.* 2000). Increased generation of reactive oxygen/nitrogen species is also associated with loss of cell viability and an increase in LDH release (Bolaños *et al.* 1995). Our finding that LDH release was comparable for control and C10-treated cells implies that the cells were not markedly stressed by exposure to this fatty acid.

To explore further the potential mechanism responsible for the above findings, we considered the involvement of PPAR γ . Recently, C10, but not C8, has been shown to act as a ligand and activator of PPAR γ (Malapaka *et al.* 2012). Furthermore, stimulation of PPAR γ , using pioglitazone or rosiglitazone, has been shown to promote mitochondrial biogenesis in SH-SY5Y cells (Miglio *et al.* 2009). Our finding that the PPAR γ antagonist BADGE prevented the C10-mediated increase in citrate synthase implicates PPAR γ activation in our study and in addition explains the lack of effect of C8. Alterations in gene expression may also explain the finding that exposure to C10 for a number of days is required before significant effects upon mitochondrial function can be observed. In addition, our observation that removal of C10 from the cell culture medium led to a return of citrate synthase to control levels implies that persistent exposure to the fatty acid and/or PPAR γ activation is

required to maintain an increased mitochondrial content. The relative specificity of PPAR γ towards C10, when compared with C8, also provides an explanation of our finding that only a very high ratio of C10 to C8 is capable of eliciting an increase in citrate synthase. PPAR γ activation has also been shown to cause increased catalase activity, thereby providing a further explanation of our findings relating to this antioxidant enzyme (Diano *et al.* 2011).

Concerning PPAR γ and mitochondrial function, activation, using rosiglitazone as an agonist, has been shown to increase complex I activity in a seizure model (Chuang *et al.* 2012). This observation is also consistent with our finding relating to the effects of C10 upon complex I activity discussed above. While C10 has been shown to occupy a novel binding site on PPAR γ , it is also capable of binding, with less affinity, to PPAR α and PPAR β/δ (Malapaka *et al.* 2012). Thus, it is possible that downstream metabolic events beyond PPAR γ may occur as a consequence of C10 exposure.

Plasma levels of C8 and C10 are elevated in patients adhering to the MCT form of the KD. However, there are a number of metabolic fates with regards to these fatty acids, e.g. β -oxidation and ω -oxidation. This latter process generates the dicarboxylic acid form of C10, sebacic acid, which has been shown to exhibit biological activity including improving glucose tolerance (Membrez *et al.* 2010). In contrast to such studies, sebacic acid in our experimental system showed no effect which again suggests the specificity of C10 with regards to causing mitochondrial proliferation.

In conclusion, we have considered the role the medium-chain fatty acids C8 and C10 may play with regards to explaining the efficacy of the MCT KD. In this study, we have focussed upon mitochondrial involvement. Our finding that C10 has the potential to increase cellular mitochondrial number raises the possibility that simplified/more easily managed C10-based forms of the KD diet could be developed. Support for exploring the use of C10 further also comes from recent work that has demonstrated that C10, but again not C8, may acutely control seizure activity (Chang *et al.* 2013). Although this acute response would suggest a different mechanism to that being considered in our study, it does point to the fact that there may be many biological targets for C10, activation of which may be beneficial with regards to seizure control.

Acknowledgements and conflict of interest disclosure

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All experiments were approved by Great Ormond Street Hospital Children's Charity and were conducted in compliance with the ARRIVE guidelines. A patent relating to this work has been filed by UCL Business PLC and Vitafllo.

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