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Calcium-insensitive splice variants of mammalian E1 subunit of the 2-oxoglutarate dehydrogenase complex with tissue-specific patterns of expression.

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ABSTRACT

The 2-oxoglutarate dehydrogenase (OGDH) complex is an important control point in vertebrate mitochondrial oxidative metabolism, including in the citrate cycle and catabolism of alternative fuels including glutamine. It is subject to allosteric regulation by NADH and ATP/ADP ratio, and by Ca^{2+} through binding to the E1 subunit. The latter involves a unique Ca^{2+} binding site which includes $D^{114}ADLD$ (site 1). Here, we describe three splice variants of E1 in which either the exon expressing this site is replaced with another exon (Loss of site1, LS1), or an additional exon is expressed leading to the insertion of 15 amino acids just downstream of site1 (Insert), or both changes occur together (LS1/Insert). We show that all three variants are essentially Ca^{2+}-insensitive. Comparison of massive parallel sequence (RNA-Seq) databases demonstrates predominant expression of the Ca^{2+}-sensitive archetype form in heart and skeletal muscle, but substantial expression of the Ca^{2+}-insensitive variants in brain, pancreatic islets and other tissues. Detailed proteomic and activity studies comparing OGDH complexes from rat heart and brain confirmed the substantial difference in expression between these tissues. The evolution of OGDH variants was explored using bioinformatics, and this indicated that Ca^{2+}-sensitivity arose with the emergence of chordates. In all species examined, this was associated with the co-emergence of Ca^{2+}-insensitive variants suggesting a retained requirement for the latter in some settings. Tissue-specific expression of OGDH splice variants may thus provide a mechanism that tunes the control of the enzyme to the specialised metabolic and signalling needs of individual cell types.

SUMMARY STATEMENT

2-Oxoglutarate dehydrogenase plays a central role in the regulation of intramitochondrial energy metabolism. We show that three Ca^{2+}-insensitive splice variants are expressed to varying degrees in different tissues allowing potential important tuning to the metabolic needs of individual cell types.

INTRODUCTION

The OGDH (2-oxoglutarate dehydrogenase) complex is important in the control of the citrate cycle and other intramitochondrial metabolic pathways including glutamine catabolism. The complex from vertebrate sources comprises decarboxylase (E1), dihydrolipoate acetyl transferase (E2) and dihydrolipoate dehydrogenase (E3) subunits [1]. It is exquisitely sensitive to regulation by the ATP/ADP ratio and by NADH, which provide an intrinsic response to changes in cellular energy status, and to Ca^{2+}, which allows responses to external stimuli [2-7].

Recently we successfully expressed recombinant human OGDH E1 and developed an assay for its activity. This allowed us to show that all these important regulators act through binding to the E1 subunit [8], consistent with the stoichiometry of Ca^{2+} and ADP binding to the holo-complex (~3 sites / complex) [9]. In the case of Ca^{2+}, the binding site was shown by a mutagenesis approach to involve the sequence D^{114}ADLD (site 1), a site distinct from other previously described Ca^{2+} binding sites on other proteins [8]. OGDH E1 from vertebrate sources contain another similar site, E^{139}SDLD (site2), close to site 1, but this second potential Ca^{2+}-binding site appears to play no part in the regulation of activity by Ca^{2+} [8].

As part of our exploration of proteins that might contain a Ca^{2+} binding site similar to the OGDH E1 binding sites, we carried out BLAST studies of EST databases. These studies indicated that three splice variants of archetype OGDH E1 exist in man and other mammals (Figure 1a,b). In the variant we refer to as 'Loss of site 1 (LS1)', the exon (exon 4) expressing site 1 in archetype OGDH E1 is replaced by another exon (exon 3) expressing a sequence lacking the DxDxD motif and would be expected to be Ca^{2+}-insensitive. There is another splice variant involving exon 5 we call 'Insert' which gives an insertion of 15 additional amino acids between site 1 and site 2. This insertion can apparently exist with both archetype and LS1 variants giving a total of four sequence alternatives in the site1/site2 region of OGDH E1.   (These variants in human and other species are often referred to as isoforms 1-4 in the various annotated databases but the numbering is very inconsistent and not used in this article).

The aims of the present studies were first to establish the Ca^{2+}-sensitivity and other regulatory properties of these splice variants. We then explore the extent to which the splice variants are expressed across mammalian tissues using RNA-Seq and proteomic approaches and describe studies comparing the Ca^{2+}-sensitivity of the OGDH complex from heart and brain. These tissues were selected as they show very different levels of expression of the various splice variants.

MATERIALS AND METHODS

Materials

$[1-{}^{14}C]$ 2-oxoglutarate sodium salt was obtained from Hartmann Analytic. Other materials were from Sigma-Aldrich or Fisher Scientific.

Expression and purification of human OGDH E1 and splice variant forms; study of their kinetic properties

DNAs encoding human OGDH E1 and its splice variants (lacking the mitochondrial propeptide but containing a tobacco etch virus protease cleavable polyhistidine tag) housed in pJexpress 404 vectors and optimized for expression in *E.coli* were purchased from DNA 2.0. Protein expression and purification was as previously described [8]. The OGDH E1 preparations (0.2-0.4 mg of protein/ml) were divided into small samples and stored at -80°C. Repeated freezing and thawing was avoided as it resulted in some loss of activity. The polyhistidine tag was not cleaved since our previous studies showed that the presence of the tag had no significant effect on the kinetic properties of expressed human OGDH E1 [8].

Activity of expressed OGDH E1 variants was assayed by following the decarboxylation of $[1-{}^{14}C]$ 2-oxoglutarate in the presence of glyoxylate at 30°C, usually for 30 min [8]. Final assay conditions used throughout were:- 50 mM Mops (pH 7.2) containing 75 mM KCl, 0.2 mM EGTA, 1 mM HEDTA (*N*-hydroxyethylethylenediaminetri-acetic acid), 1 mM DTT, 1µg/ml oligomycin, 1µg/ml rotenone, 1 mM TPP, plus $MgCl_2$ and $CaCl_2$ to give 1 mM Mg^{2+} and the appropriate concentration of Ca^{2+} together with the stated concentrations of adenine nucleotides, NADH and $[1-{}^{14}C]$ 2-oxoglutarate plus 5mM sodium glyoxalate. The amounts of added $MgCl_2$ and $CaCl_2$ were determined using the computer program METLIG [10]. Maximum activity (V_{max}) was measured at 0.33 mM Ca^{2+} and 2mM 2-oxoglutarate in the absence of added adenine nucleotides and NADH.

Extraction of OGDH complex from rat heart and brain mitochondria and study of its kinetic properties

Mitochondria were prepared from whole hearts and brains of 250-350 g male Wistar rats following homogenization in sucrose isolation buffer (250 mM sucrose, 20 mM Tris–HCl, 2 mM EGTA, pH 7.4) using a Polytron PT10 (heart) or a Dounce Potter homogenizer (brain) and purified by Percoll® density-gradient centrifugation. The mitochondria were then sedimented by centrifugation at 10,000*g* for 5 min in an Eppendorf minicentrifuge and the pellet immediately frozen in liquid N_2. The mitochondria were then extracted by freezing

and thawing three times in 100 mM potassium phosphate buffer pH 7.2 containing 1mM DTT plus 30 ul.ml^{-1} foetal calf serum and 5 ug.ml^{-1} each of pepstatin A, aprotinin and leupeptin to prevent proteolysis.

The extracts were then centrifuged at 10,000$\textbf{\textit{g}}$ for 5 min before assay of OGDH complex activity in the supernatant by following the production of NADH at 340 nm and 30^{0}C.   Final conditions of the assay were the same as those used for the assay of OGDH E1 activity described above but with the omission of sodium glyoxalate and the use of non-radioactive 2-oxoglutarate together with the addition 1 mM NAD$^+$ and 0.2 mM CoA.   See Armstrong et al. [8] for more details.

Curve fitting and calculation of kinetic data

Data were fitted to the equation given in figure legends using GraFit5.

Determination of amounts of OGDH E1

Concentrations of subunit E1 protein was determined by quantifying the intensity of the 110 kDa band separated by SDS/PAGE (10% gel) using ImageJ [8].

RNA-Seq analysis

Publicly-available RNA-Seq data for various tissues from adult C57BL/6 mice and embryonic stem cells (Bruce-4) (GEO Accession No GSE36026) were combined with RNA-Seq data for pancreatic islets and skeletal muscle (Accession Nos E-MTAB-2791, E-MTAB-3725 and GSM1020654). Reads were assigned to the annotated mouse transcriptome (Ensembl assembly GRCm38) using the Sailfish algorithm [11]. cDNA sequences for the open reading frames of the four isoforms identified in this study were assembled and reads mapped to these, also using Sailfish. Expression levels were calculated as Reads Per Kilobase of transcript per million Mapped reads (RPKM) [12]. To study individual exon expression levels, the DEXSeq tool [13] was used on RNA-Seq data from brain cortex and heart samples (GEO Accession No GSE36026 and GSE36025). A very large number of reads mapped to the last exon of *Ogdh* possibly due to mis-mapping or an overlapping transcript. To avoid this skewing of the normalisation of exon usage, the last exon was ignored in this analysis.

Phylogenetic analysis

Ogdh and related genes were identified in sequenced genomes of representative species using published gene annotations (ENSEMBL) and Blast searching. The full sequences of each of the major transcript variants were aligned using MUSCLE [14] and a phylogenetic tree constructed with the maximum likelihood model using MEGA (version 6.0) software [15].

Proteomic analysis

Proteomic analyses were performed within the University of Bristol Proteomics Facility.

Samples of expressed human OGDH splice variants were separated by SDS/PAGE (10% gel) and the 110 kDa band excised.

Samples of OGDH complex partially purified from rat brain and heart mitochondria: mitochondrial extracts were prepared as described above except that foetal calf serum was not added to the mitochondrial extraction buffer. These samples were centrifuged at 33,000 g for 20 min and the supernatants then centrifuged at 250,000 g for 60 min in a Beckman Optima TLX Ultracentrifuge using TLA55 and TLA100 heads respectively. Proteins in the pellets were then separated by SDS/PAGE (10% gel) and the band corresponding to 100-120 kDa excised.

The excised bands were subjected to in-gel tryptic digestion and the resulting peptides fractionated using an Ultimate 3000 nanoHPLC system in line with either an LTQ-Orbitrap Velos or Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). The raw files were processed with the Proteome Discoverer software v1.4 (Thermo Scientific) using the SEQUEST algorithm. Searches were filtered to satisfy a false discovery rate of less than 1%.

RESULTS

Comparison of the kinetic properties of the expressed human OGDH E1 splice variants with those of archetype OGDH E1

All three splice variants were active when expressed as recombinant proteins and estimates were made of their specific activities compared to archetype E1. Relative amounts of protein in archetype and variant E1 were measured from scans of the stained 110 kDa bands separated by SDS/PAGE. Specific activities were then calculated using measurements of V_{max} determined at 2mM 2-oxoglutarate and 0.33 mM Ca^{2+} in the absence of added adenine nucleotides and NADH. Values as % of archetype activity were LS1 85, Insert 78 and LS1/Insert 115 indicating that there were only small differences, if any, in the specific activities of the variants compared to archetype. Since SDS/PAGE indicated that the E1 bands accounted for much of the protein in the preparations, specific activities were also calculated based on the total protein in the preparations measured by the Pierce BCA assay using BSA as the standard. The values obtained were (μmol/min per mg of protein): WT, 0.10; LS1, 0.11; Insert, 0.053; LS1/Insert, 0.21. Again the specific activities for the variants were similar to archetype but more variable than those based on gel scans, reflecting some differences in the purity of the preparations.

The effects of Ca^{2+}, adenine nucleotides and NADH on the spice variants and archetype OGDH E1 were explored using surveys in which assays were carried out at 50 μM 2-oxoglutarate under 12 different combinations of these regulators (Figure 2 and Supplementary Table S1). Results with archetype OGDH E1 were very similar to those obtained in our previous study under the same conditions [8] and showed activation by Ca^{2+} and ADP and inhibition by ATP and NADH. In particular, activation by 0.33mM Ca^{2+} was evident under all conditions. In contrast, all three of the three splice variants exhibited little, if any, Ca^{2+} activation under any of the conditions studied while effects of adenine nucleotides and NADH were still clearly evident.

Confirmation of the extent of the loss of Ca^{2+} activation in the splice variants was obtained in a series of further studies in which the activity of archetype and splice variants

were compared at $<10^{-5}$, 0.33 and 1 mM Ca^{2+} in the presence of 0.2 mM NADH and 50 µM 2-oxoglutarate (Figure 3). Archetype E1 exhibited a 13.4 fold increase in activity with 0.33 mM Ca^{2+} compared to $<10^{-5}$ mM whereas the corresponding values for the variants were much smaller at 1.1, 2.2 and 1.4 fold for LS1, Insert and LS1/Insert respectively (similar effects were evident in Supplementary Table S1). At 1 mM Ca^{2+}, appreciable increases of 2.5 and 4.8 fold respectively in activity of the variants LS1 and Insert were evident but only a 1.9 fold increase was apparent for the LS1/Insert; these were all considerably less than the 15.6 fold increase observed with archetype E1.

Studies into the effects of varying the NADH concentration at 50 µM 2-oxoglutarate in the absence of Ca^{2+} and adenine nucleotides revealed that the calculated K_i for NADH was increased by modest amounts in the splice variants, with LS1 showing the largest increase (Figure 4a). Parallel studies on the effects of varying the ATP/ADP ratio at the same 2-oxoglutarate concentration in the absence of Ca^{2+} and NADH revealed the ratio giving 50% inhibition was also slightly increased with the largest increases seen with Insert and LS1/Insert (Figure 4b). Previous studies with archetype E1 in the absence of Ca^{2+} showed that NADH (0.2 mM) increased the K_m for 2-oxoglutarate while markedly decreasing the V_{max} [8]. All three splice variants also showed similar changes (Figure 5), though the K_m for 2-oxoglutarate in the absence of NADH was deceased.

Expression of archetype and splice variants of OGDH E1 in a range of mouse tissues explored using RNA-Seq.

The expression of the splice variants was investigated at the RNA level in mouse tissues using publicly-available RNA-Seq data (see Methods). Initially, expression of the four different isoforms was estimated using the Sailfish tool (Figure 6a). These data are also presented as the proportional expression of the four isoforms (Figure 6b). Heart and skeletal muscle showed higher overall archetype expression than the other mouse tissues, and this was associated with very low expression of the three Ca^{2+} insensitive splice variants. In several other tissues there was a markedly different pattern of expression with the sum of expression of the three splice variants being comparable to that of archetype. These included islet, cerebellum, cortex, thymus and spleen. However, within this group appreciable differences in the relative expression of the three splice variants were evident. In islet and thymus tissue the LS1 variant was predominant, whereas in cerebellum, cortex and thymus similar expression of the three splice variants was observed. In a third group of tissues, the sum of expression of the three splice variants was in the range of 10-30%. These were ESC (embryonic stem cells), kidney, liver and lung. Again, there were differences in the relative expression of the variants.

Given the clearly contrasting pattern of expression at the RNA level, the data from heart and brain cortex were examined in more detail. Expression at the exon level was calculated using DEXSeq, which detects significantly differentially spliced exons while controlling for the overall expression level of the gene. The archetype and LS1 domains are encoded by a pair of exons (exons 4 and 3 respectively), one of which is included in each mRNA. The Insert domain is encoded by the subsequent exon (exon 5), and these three exons are highlighted in Figure 6c. Much greater usage of both the LS1 and Insert exons is

apparent in the brain cortex compared to the heart. Since DEXSeq measures the usage of each exon individually but not splicing between them, this tool does not indicate whether the Insert exon is associated with Archetype or LS1 exon usage. The Sailfish analysis (Figure 6a,b), which does take account of splicing, indicates roughly equal quantities of Insert and LS1/Insert transcripts in cortex, although the ratio does vary across other tissues.

Expression of archetype and splice variants of OGDH E1 in rat brain and heart explored using a proteomic approach.

Figure 7 summarizes the peptides expected to be generated by the action of trypsin on the various splice variants of human OGDH E1. Each of the forms has one unique peptide (peptides numbered 3 in archetype, 6 in LS1, 7 in Insert and 10 in LS1/Insert). In addition, peptides 1 and 2 are restricted to archetype and Insert, peptides 4 and 5 to LS1 and LS1/Insert and peptides 8 and 9 to Insert and LS1/Insert. Peptides a and b are shared by all four forms. The figure also indicates two amino acid differences that occur in this region in rat and mouse compared to human.

Using either LTQ-Orbitrap Velos or the more sensitive Orbitrap Fusion Tribrid mass spectrometer, peptides 1,4,5 and 8 were never detected because their mass/charge ratios were less than the minimal detected ratio of 300 for both machines. Peptides 6 and 10 were also not observed in samples of human splice variant LS1 and LS1/Insert, possibly because of their high mass/charge ratio. However, their calculated ratios based on an expected net charge of 2^+ are 1830 and 1496 respectively, and these values are within the maximum detected ratio of 2000 for both machines. Whatever the reason the lack of detection of these peptides from human sources that are unique to LS1 and LS1/Insert respectively may limit to some extent the application of proteomics in the determination of the expression of the four forms of OGDH E1 in tissue samples.

The number of peptide spectral matches (PSM) can be used as a measure of the amount of a particular peptide [16-18]. Figure 8a shows the values of PSM for the various relevant peptides with mass/charge ratios in the range 300-2000 which were detected in studies using the Orbitrap Fusion Tribrid mass spectrometer on samples of expressed human archetype and the three splice variants. These have been expressed as percent of the total PSM observed for all the peptides in the relevant protein to correct for any variations in the amount of protein studied. Results are also compared with the mean PSM observed in peptides a and b which are present in all four forms. As expected peptides 2 and 3 were observed in archetype OGDH E1 but not peptides 6,7,9 and 10. None of these peptides were observed in LS1, whereas peptides 2,7 and 9 were found in Insert and peptide 9 in LS1/Insert. This is the pattern of peptides predicted given the lack of detection of peptides 6 and 10.

When the same proteomic approach was applied to OGDH complex in rat heart and brain mitochondrial extracts very different spectra of peptides were detected (Figure 8b). In the case of rat heart, peptides 2 and 3 were found with barely detectable amounts of peptides 7 and 9 whereas in rat brain peptides 2 and 3 were found together with significant

amounts of peptides 6,7,9 and 10. These results are compatible with the conclusions of the RNA-seq studies; namely, that in the heart the OGDH complex contains predominantly archetype E1 whereas in the brain there is also comparable expression of the calcium-insensitive splice variants. The considerably greater amounts of peptide 9 compared to peptide 7 indicates that splice variant LS1/Insert may be expressed in greater amounts than Insert in rat brain. It should be noted that peptide 6 was detected in rat brain indicating some expression also of LS1. (The reason this peptide is detected in rat brain but not in expressed human LS1 is likely to be because an aspartate in the human sequence is changed to histidine thus lowering the mass/charge ratio.)

The preparation of rat brain and heart mitochondrial extracts used in these studies had involved sedimentation of OGDH complex at 250,000g for 60 min. Thus it can be concluded that the Ca^{2+} -insensitive splice variants are part of a high molecular weight complex, most likely OGDH complex. It is noteworthy that two other proteins that have a high degree of sequence identity with OGDH E1 were also found in these preparations. These are OGDHL (OGDH-like) and DHTDK1 (dehydrogenase E1 and transketolase domain containing 1) which may also replace E1 in OGDH complexes [19-21] but the identity of their substrates has not been established.   The amounts of both OGDHL and DHTDK1 in rat brain expressed as PSM as % archetype OGDH E1 PSM were 51±1.8 and 20±1.5% respectively which was considerably greater than the corresponding values for rat heart of 14±1.8 and 3±0.2% respectively (results are mean±S.E.M. for observations on four different preparations in each case). These observations are in general agreement of those of Bunik and colleagues [19].

OGDH complexes from rat heart and brain mitochondria have different Ca^{2+}-sensitivity.

The RNA-seq and proteomic studies above both indicated that the OGDH complex in mammalian heart mitochondria contained predominately the Ca^{2+}-sensitive archetype E1 whereas that in mammalian brain mitochondria contained comparable amounts of archetype E1 and a mix of the Ca^{2+}-insensitive splice variants. It follows that OGDH complex from mitochondria from the two tissues should exhibit differences in sensitivity to Ca^{2+}. In particular, the activity of the brain complex measured in the presence of Ca^{2+} and at a low concentration of 2-oxoglutarate should be less than that of the heart complex when expressed as a % of the V_{max} (measured at 2 mM 2-oxoglutarate and 326 µM Ca^{2+}).

Comparisons of the activity of OGDH complex in extracts of rat and brain mitochondria showed that this was the case. The data in Figure 9a are from studies on single extracts of rat heart and brain mitochondria in which OGDH complex was assayed at 50 µM 2-oxoglutarate and a range of concentrations of Ca^{2+}. Increasing [Ca^{2+}] from <0.01 to 326 µM increased activity by 63% of V_{max} in the rat heart preparation but only 38% in that from rat brain. If the assumption is made that the heart complex only contains the Ca^{2+} -sensitive archetype E1, then the proportion of Ca^{2+} -insensitive forms in the brain can be estimated as (1-(38/63)) = 0.40. This is in close agreement with the RNA-seq and proteomic data (Figures 6 and 8). Figure 9b summarises data on four separate preparations of rat

brain and heart mitochondria. The activities at 50 µM 2-oxoglutarate and at 0.25, 0.59 and 326 µM Ca^{2+} are all significantly lower in the brain whereas the activity at <0.01 µM is not significantly different. The estimate of the proportion of Ca^{2+} - insensitive forms from these data is (1-(41/63)) = 0.35 confirming the conclusion from Figure 9a. Our studies also showed that there may be a small increase in the $K_{0.5}$ for Ca^{2+} for the OGDH complex from the brain compared with the heart. The values for the brain OGDH complex calculated from the data of Figure 9a and Figure 9b were 0.74 µM and 0.57 µM respectively. The corresponding values for OGDH complex from the heart were both 0.36 µM.

The greater proportion of Ca^{2+}-insensitive splice variants in rat brain OGDH complex would be expected to effect the sensitivity to 2-oxoglutarate in the presence of Ca^{2+} compared with that of rat heart. This was observed (Figure 10). All the data fitted simple Michaelis kinetics reasonably well (solid lines). The K_m ± S.E.M. in the presence of 326 µM Ca^{2+} was increased from 0.067 ± 0.005 in the heart to 0.183 ± 0.019 mM in the brain preparation. In contrast, there was no appreciable difference at <0.01 µM Ca^{2+} where the values were 1.29±0.19 mM for brain and 1.17±0.12 mM for heart. The data for rat brain in the presence of Ca^{2+} were also fitted to a 2 K_m model (dashed line) assuming a mix of Ca^{2+} - insensitive (K_m = 1.17 mM) and Ca^{2+} -sensitive (K_m = 0.067 mM) forms. The calculated proportion of the Ca^{2+} -insensitive form from this approach was 45%, in agreement with comparable estimates using the Ca^{2+} -sensitivity data of Figure 9. However, the fit was not as good as the single K_m model and this is discussed further below.

DISCUSSION

Regulation of the splice variants of OGDH E1

Our previous study showed that site 1 is an essential part of the Ca^{2+}-binding site on archetype OGDH E1 [8]. In particular, replacement of D^{114}ADLD with A^{114}AALA resulted in essentially complete loss of Ca^{2+}-activation of archetype OGDH E1. We therefore predicted that the splice variant LS1, which lacks site 1, would be Ca^{2+}-insensitive. This prediction has been confirmed in the present study. Perhaps a little surprisingly, the splice variant Insert which has the insertion of 15 amino acids just downstream of site 1 was also found to exhibit much attenuated Ca^{2+} activation. It seems likely that this insertion causes a major disruption of Ca^{2+}-binding in some way. The binding of Ca^{2+} to proteins invariably involves six or seven co-ordinations [22] and thus Ca^{2+}-binding to OGDH E1 is very likely to involve co-ordinations beyond site 1 which may be disrupted by the insertion of 15 amino acids. However, it should be noted that it is, at least theoretically, possible that Ca^{2+}-binding is still intact but its influence on the activity of the enzyme is greatly decreased. At high concentrations of Ca^{2+} (0.33 and 1.0 mM), we found some evidence of activation with all three splice variants with the greatest activation with splice variant Insert and the least with variant LS1/Insert. However, the activation was markedly less with all three splice variants than that observed with archetype E1. In any case, the small differences between the variants are probably of little physiological importance since over the range of intramitochondrial concentrations of Ca^{2+} that usually occur physiologically (0.1 – 50 µM;

[23-26] or up to 500 µM in the case of caffeine-stimulated chromaffin cells [27]) all three splice variants will be essentially Ca^{2+}-insensitive, with the possible exception of the insert variant (Figure 3) which represents the most minor form in most tissues (Figure 6b). Whether these differences assume more importance in pathophysiological circumstances (ischaemia, hypoxia etc), where mitochondrial Ca^{2+} levels may exceed this range [28], is unclear.

All three splice variants still exhibit inhibition both by NADH and by increasing ATP/ADP ratios that is similar in extent to archetype E1 but the K_i and $K_{0.5}$ values are somewhat higher. In summary, the major loss of Ca^{2+}-sensitivity in the variants is accompanied by modest decreases in sensitivity to inhibition by both NADH and elevated ATP/ADP ratios.

Differential expression of splice variants in various mammalian tissues

Our RNA-seq studies showed substantial differences in relative expression of the Ca^{2+}-activated archetype OGDH E1 and the Ca^{2+}-insensitive splice variants (at the mRNA level) across a wide range of mouse tissues. In heart and skeletal muscle, archetype E1 accounted for some 95% of the total expression whereas its expression in brain, spleen and pancreatic islets was only about 50% with the expression of the three variants taken together making up the other 50%. In embryonic stem cells, liver, kidney and lung the expression of the three variants in total ranged from 15 – 33% of that of archetype OGDH E1. In all cases the splice variant LS1 appeared to be the predominant variant expressed.

The proteomic approach we used to explore expression at the protein level in rat heart and brain confirmed the marked difference in expression of archetype and its splice variants seen in the RNA-seq studies. In particular, there was little evidence of any expression of the Ca^{2+}-insensitive splice variants in heart OGDH complex whereas in the complex from brain the total amount of the insensitive variants appeared to be comparable with that of archetype OGDH E1. However, as explained under Results, it is more difficult to get an accurate measure of the LS1 variant than the other variants because a large unique peptide may be poorly detected.

We have also shown that there are clear differences in the Ca^{2+}-sensitivity of the OGDH complex from rat brain and heart. Our observations are compatible with the presence of only the Ca^{2+}- activated archetype OGDH E1 with little or no Ca^{2+}-insensitive splice variants in the heart while in the brain the Ca^{2+}- insensitive splice variants are present in equivalent activity to that of archetype. In heart and brain tissue, the agreement between the RNA-seq, proteomic and activity studies is both impressive and reassuring. It should be noted that we found, in agreement with Bunik et al. [19], that the brain OGDH complex contains considerably more OGDHL and DHTDK1 than the heart complex and so the possibility arises that this difference may contribute to the observed differences in Ca^{2+}-sensitivity. However, we are unaware of any evidence that 2-oxoglutarate is actually a substrate for either OGDHL or DHTDK1. We have expressed human OGDHL using the same approach that we successfully used for the expression of archetype OGDH E1 but the

preparation was completely inactive with 2-oxoglutarate as substrate (Denton & Armstrong, unpublished work).

An interesting area for future study in the brain and other tissues will be the extent to which archetype OGDH E1 and the splice variants are present in distinct OGDH complexes, perhaps in different cells, or are present in the same complex as homo- or mixed E1 dimers. Our studies have not addressed this aspect in any direct way but the relatively poor fit of the 2-oxoglutarate sensitivity of the OGDH complex from rat brain to a simple two K_m model might reflect the presence of the homo- or mixed E1 dimers on the same OGDH complexes. Useful future direct approaches may include immunocytochemical techniques coupled to super-resolution optical imaging [29] or electron microscopy.

Evolution of the calcium insensitive splice variants

Based on direct studies of Ca^{2+}- sensitivity, our previous studies have shown that the OGDH complexes from human, rat, mouse, pig, frog, pigeon and zebra-fish are activated by Ca^{2+} whereas those from drosophila, locust, *C. elegans*, yeast and *E. coli* are not [4,8,30,31]. These observations suggest that Ca^{2+}-sensitivity may be restricted to vertebrates. This view is strengthened and refined by the presence of DADLD within site 1 of OGDH E1 from most chordate (including vertebrate) sources but not from non-chordate sources. An interesting exception is the elephant shark (*Callorhinchus milii*) which has AADLD and hence would be expected to exhibit greatly attenuated activation as found when DADLD within human OGDH E1 is mutated to this sequence [8].

Using published annotations of sequenced genomes combined with Blast searches (Materials and Methods), we have further explored the evolution of exons capable of giving rise to the splice variants LS1 and Insert. An evolutionary tree of protein sequences alongside the motifs around Site 1, and the corresponding sequence in LS1 for these illustrative species, are shown in Figure 11a and in more detail in Supplementary Table S2. The key changes are shown in an evolutionary tree of species in Figure 11b. The first of these (A) is a duplication of an exon (equivalent to exon 4) to give rise to exon 3 (LS1). The alternative splicing of these two exons is observed in both sea urchins and chordates and so presumably arose before the divergence of echinoderms and chordates. Next (B) the evolution of the DADLD motif occurred early in chordate evolution. Since the DADLD motif appears to be essential for Ca^{2+} sensitivity, this suggests that the development of Ca^{2+}-sensitive OGDH was preceded by the development of an alternatively-spliced variant which retained the ancestral Ca^{2+} insensitivity. The gain of the Insert exon (C) occurred in a common ancestor of cartilaginous and bony fish, giving rise to two further Ca^{2+} insensitive splice variants in this lineage. It might therefore be speculated that the ability to express a Ca^{2+}-insensitive variant is essential, presumably in a tissue-specific or developmentally-regulated manner. Nonetheless, the reason for the existence of *three* nominally Ca^{2+} insensitive variants, which differ in their response to very high (usually pathological) concentrations of the ion, remains unclear (see above). Of note, NAD^+-isocitrate dehydrogenase also shows a broad range of Ca^{2+} sensitivities depending on ATP/ADP and NADH/NAD^+ ratios [6] suggesting that a retained ability of the insert variant of OGDH (Figure

3) to respond to very high (> 100 µM) Ca^{2+} levels might be of relevance under some circumstances.

The duplication of the *Ogdh* gene giving rise to *Ogdhl* occurred before the divergence of cartilaginous fish, and presumably before the gain of the Insert exon (C) since this is not found in any *Oghdl* genes. In the ray-finned fish, a further gene duplication (D) gave rise to two OGDH E1 genes (*Ogdha* and *Ogdhb*). Subsequently, the S1 exon was lost in *Ogdha* (E) in a subgroup, presumably rendering it Ca^{2+} insensitive. This may indicate that the balance in the expression of Ca^{2+}-sensitive *versus* insensitive OGDH isoforms is regulated to a greater extent in these fish at the transcriptional level rather than through alternative splicing.

Role of calcium insensitive splice variants in mammalian tissues

The current studies have shown at the RNA and protein level, as well as by activity studies, that the Ca^{2+}-sensitive archetype OGDH E1 is the predominant form (over 95%) of E1 in the heart and in skeletal muscle whereas in the brain about half is a calcium insensitive variant. The amounts of calcium insensitive variants in some other tissues such as liver and kidney based on RNA levels would appear to be about 25% of the archetypal form. These tissue specific variations and likely roles of the archetype and variant forms are summarised in Figure 12.

We have previously argued that in vertebrates the Ca^{2+}- sensitivity of the OGDH complex coupled with that of two other intramitochondrial enzymes (pyruvate dehydrogenase phosphatase and NAD-isocitrate dehydrogenase, NAD-ICDH) is an important mechanism for matching ATP supply and demand [4,32-34]. The results of the present study fully support this view for the heart where the principal role of the OGDH complex is to regulate the citrate cycle. Supporting this are recent studies [35,36] involving the inactivation of the mitochondrial Ca^{2+}-uniporter (MCU) [37,38] selectively in the heart. The latter studies demonstrated a role for mitochondrial Ca^{2+} uptake in the acceleration of heart rate during fight or flight [35], with inactivation of the transporter through the over-expression of a dominant-negative variant also leading to a suite of compensatory changes including an elevation in cytoplasmic Ca^{2+} levels [36]. Correspondingly, mice globally deleted for the MCU gene display an impaired ability to perform strenuous work and changes in the phosphorylation state of the pyruvate dehydrogenase complex in skeletal muscle [39].

In non-muscle tissues, the role of the OGDH complex in metabolism has more dimensions including an important role in glutamine and glutamate metabolism. It seems most likely that the Ca^{2+}- insensitive splice variants facilitate this multifaceted role by modifying (attenuating) large Ca^{2+}-dependent changes in OGDH complex activity during cellular stimulation. In the brain, glutamate serves as an essential neurotransmitter [40] whose intracellular levels may need to be maintained during action potential firing and Ca^{2+}-dependent exocytosis. Ca^{2+} activation of OGDH, which would be expected to increase glutamate catabolism after conversion to 2-oxoglutarate by glutamate dehydrogenase, may therefore need to be minimised under these conditions. We note, however, that our

measurements in brain are likely to be affected by the presence of glial cells [41] and future studies may be required to determine the distribution of OGDH variants in these *versus* neurons.

Glutamate also has important roles in the pancreatic islet β cell both as an extracellular [42] and intracellular [43-45] signal, and is implicated in the control of insulin secretion by glucose and by incretin hormones. Whilst levels of the splice variants in islets may not precisely reflect those in β cells (which comprise 60-70% of rodent islets) [46], it seems possible that the lower levels of archetype OGDH E1 in these cells may, in common with neurons, reflect a requirement to avoid large Ca^{2+}-dependent changes in glutamate catabolism during stimulation with secretagogues.

We have also considered the possibility that changes in the ratio of splice variants may occur in cancer cells given the dependence upon glutamine metabolism apparent in tumours [47,48] . Whilst we did not perform a comprehensive screen across multiple cancer types our analysis (not shown) of RNASeq data from transformed *versus* untransformed mouse embryonic fibroblasts [49] did not show any significant differences in the expression of splice variants of OGDH E1.

In summary, the existence of Ca^{2+} sensitive and insensitive variants of OGDH appears to be a relatively early evolutionary adaptation, which allows the tissue-specific regulation of the enzyme in mammals and presumably in other chordates.

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DECLARATIONS OF INTEREST

None

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AUTHOR CONTRIBUTION

Richard Denton conceived and directed the work with Guy Rutter and carried out the enzyme activity studies plus, with Kate Heesom, the proteomic studies. Timothy Pullen carried out the RNA-seq studies and phylogenetic analyses and Craig Armstrong the

expression of archetype OGDH E1 and its splice variants. Richard Denton prepared the paper with Guy Rutter and Timothy Pullen.

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FIGURE LEGENDS

Figure 1   Alignment of human archetype and splice variants of OGDH E1 and arrangement of exons.

(a) Sequence of archetypal is from Leu^{94} – Leu^{148} (using sequence numbering after cleavage of mitochondrial propeptide). Sequence corresponding to the archetypal site 1 exon (exon 4) shown in green with site 1 in bold type. Sequences corresponding to loss of site 1 exon (exon 3) is shown in blue and Insert exon (exon 5) in red. Site 2 sequence is underlined and is present in all the variants. (b)   Arrangement of exons in vertebrate OGDH genes.

Figure 2   Survey of effects of adenine nucleotides, NADH and Ca^{2+} on the activity of human OGDH E1 splice variants.

(a) Archetype. (b) LS1. (c) Insert. (d) LS1/Insert. All activities were measured at 50 μM 2-oxoglutarate in the presence and absence of adenine nucleotides, NADH and Ca^{2+} as indicated and are expressed as % V_{max}. Values are mean ± S.E.M. for four determinations. Further details of the observations are given in Supplementary Table S1.

Figure 3   Effects of Ca^{2+} on the activity of human OGDH E1 splice variants.

All activities were measured at 50 μM 2-oxoglutarate in the presence of 0.2 mM NADH (but in the absence of adenine nucleotides) at <0.01, 326 and 996 μM Ca^{2+} and are expressed as % V_{max}. Values are mean ± S.E.M for 4-6 observations. *P<0.05, **P<0.01 (Student's t test) compared to <0.01 μM Ca^{2+}. Note, under the same conditions used in the present study the $K_{0.5}$ for Ca^{2+} activation of archetype OGDH E1 was 7.81±0.87 μM [8].

Figure 4   Inhibition of human OGDH E1 splice variants by NADH and ATP/ADP.

 (a) Effects of increasing concentrations of NADH in the presence of 50 μM 2-oxoglutarate, <0.01 μM Ca^{2+} and the absence of adenine nucleotides. Points are the mean of 3-5 observations. Dated fitted to :- $v = v_0 – ((v_0-v_{ni}).[NADH]/([NADH]+K_i))$ where V_0 is the rate in the absence of NADH, v_{ni} is the non-inhibitable rate and K_i is the concentration of NADH giving half-maximal effects. **(b)** Effects of increasing ratios of ATP to ADP (with the ATP plus ADP concentration constant at 1.5 mM) in the presence of 50μM 2-oxoglutarate, <0.01 μM Ca^{2+} and the absence of NADH. Points are the mean of 2-6 observations. Data fitted to same equation as in (a) but with ATP/ADP ratio replacing [NADH]. **(c)** Values of K_i for NADH and the ratio of ATP/ADP giving 50% inhibition calculated from the data plotted in parts (a) and (b) and given as parameter mean ± S.E.M. (based on 7-11 mean observations).

Figure 5   Effects of 2-oxoglutarate concentration in the presence and absence of NADH (0.2 mM) and [Ca^{2+}] <0.01 μM on the activity of human OGDH E1 splice variants.

(a) Comparison of archetype with LS1 splice variant. Each point is the mean of three observations. Data fitted to:- $v = V_{max} . [OG]/([OG] + K_m)$.   **(b)** Values of K_m calculated from the data plotted in (a) and given as parameter mean ± S.E.M. (based on 6 mean

observations). Also included are values for variants Insert and LS1/Insert calculated from data obtained under the same conditions as in (a) and again given as mean±S.E.M based on 6 mean observations.

Figure 6 Expression patterns of archetype and splice variants determined from RNA-Seq data.

(a) Expression levels of mRNA encoding archetype and the three splice variants of OGDH E1 were measured across a range of adult mouse tissues and embryonic stem cells (ESC) from publicly available RNA-Seq data using the Sailfish tool. Data presented as mean ± S.E.M. (n=2-3). **(b)** These data are also presented to show the proportion of the variants expressed in each tissue. **(c)** Exon-level expression analysis of archetype and splice variants in mouse cortex and heart using DEXSeq. The exons encoding LS1, archetype and Insert are highlighted and expanded in the bottom part. Due to differences in the LS1 boundaries present in the reference annotation, this exon was split into two counting bins. However, the predicted exon usage for both bins is almost identical in agreement with a single LS1 exon. (n=3) * Significantly different exon usage with false discovery rate <10^{-6}.

Figure 7 Trypsin derived peptides from archetype and splice variants of human OGDH E1

Tryptic peptides from Leu^{84} to Arg^{164} for archetype OGDH E1 together with corresponding peptides for the three splice variants. Peptides have been assigned a letter or number and these are shown under the peptides. Peptides a and b are present in all four forms of OGDH E1. Underlined peptides are unique to a single form. Peptides from the four forms in rat and mouse are identical to those from human except *asp is replaced by his and †ser is replaced by pro.

Figure 8 Proteomic analysis of tryptic peptides from (a) expressed archetype and splice variants of human OGDH E1 and (b) OGDH E1 separated from rat brain and heart mitochondria.

Results are shown as peptide spectral matches (PSM) for the indicated peptide or the mean of peptides a and b expressed as a % of the total E1 protein PSM and are mean±S.E.M. of three or four observations. Values of total E1 protein PSM were archetype 3571±552 (4), LS1 3719±552 (3), Insert 3782±1961 (3), LS1/Insert 3407±156 (3), rat brain OGDH E1 620±126 (4) and rat heart OGDH E1 1086±126 (4).   All observations were obtained using an Orbitrap Fusion Tribrid mass spectrometer. The number of PSM is a measure of the amount of a peptide.

Figure 9   Comparison of the effects of Ca^{2+} on the activity of OGDH complex in extracts of rat heart and brain mitochondria.

Activity of the OGDH complex was assayed in the presence of 50µM 2-oxoglutarate (in the absence of added adenine nucleotides and NADH) and expressed as % V_{max} (taken as the activity in the presence of 2mM oxoglutarate and 0.33mM Ca^{2+}). **(a)** Observations on single

preparations of mitochondrial extracts using 11-12 different Ca^{2+} concentrations; data fitted to:- $v = V_{max} \cdot [Ca^{2+}]/([Ca^{2+}] + K_{0.5})$. **(b)** Mean results with four preparations each of rat heart and brain mitochondria measured at four concentrations of Ca^{2+}.

Figure 10   Effects of 2-oxoglutarate concentration on the activity of OGDH complex from rat brain and heart mitochondria.

Activity of the OGDH complex was assayed in the presence of <0.01 or 326 µM Ca^{2+} (without added adenine nucleotides or NADH) and expressed as % V_{max}. Data points are the means of 2-4 observations on two separate preparations of mitochondria from each tissue. Data fitted to :- $v = V_{max} \cdot [OG]/([OG] + K_m)$ shown as solid lines. Dashed line is data from brain at 326 µM Ca^{2+} fitted assuming a mix of Ca^{2+}-sensitive and Ca^{2+}-insensitive forms with K_m for 2-oxoglutarate of 0.067 and 1.17 mM respectively; the calculated proportion of the Ca^{2+}-insensitive form is 45±7%.

Figure 11. Phylogenetic development of OGDH E1 and OGDHL

a Evolutionary tree of OGDH protein sequences. Within each species, variants have been collapsed into a single node for clarity. An alignment of the S1, LS1 and insert motifs is provided. *Ogdhl* genes have similarly been collapsed. Mammals are highlighted in pink and ray-finned fish in blue. **b** Evolutionary tree of species highlighting the major changes to the OGDH and OGDHL genes. The underlying tree was constructed using data from ENSEMBL as described under Materials and Methods.

Figure. 12. Scheme indicating the likely roles of tissue-specific expression of Ca^{2+}-sensitive archetype OGDH E1 (blue) and Ca^{2+}-insensitive splice variants (LS1, insert, LS1-insert; green) in representative mammalian tissues.

Approximate relative abundances and predicted fluxes of each are represented by the size of the circles and thicknesses of the arrows, respectively. Citrate cycle flux towards increased ATP synthesis (shown for heart, skeletal muscle) is implied for the other indicated tissues. See the Discussion for further explanation.

Table S1 Survey of effects of adenine nucleotides, NADH and Ca^{2+} on the activity of human OGDH E1 splice variants.

These data were used to prepare Figure 2 of the main text.

Table S2 Sequences expressed by LS1, site 1 (archetype) and Insert exons in selected vertebrates.   Data was obtained by use of BLASTp and tBlastn together with sequences of

human archetype E1 and its splice variants in conjunction with species genomic DNA sequence information. The sequence of site 1 is shown in bold.