

Studying regulation of *Mct7* under nutrient deprivation in vitro using a mammalian cell culture model

Caleb R. Volkert

Biochemistry and Molecular Biology 251 Med 1035 University Drive Duluth, MN 55812

University of Minnesota Duluth School of Medicine

May 30th, 2014

Studying regulation of *Mct7* under nutrient deprivation in vitro using a mammalian cell culture model

Introduction:

Monocarboxylate transporters (MCTs) facilitate the diffusion of monocarboxylates such as lactate and pyruvate across the plasma membrane [1,2,4]. These monocarboxylates are paramount in cellular metabolism and metabolic communication between tissues [5]. A recent study by Hugo et al. (2011) demonstrated that the *slc16a6a* gene encodes a transporter of the major ketone body, β -hydroxybutyrate (BHB) in zebrafish hepatocytes, known formally as *Mct7*. These novel findings of MCT7 expression and transport of ketone bodies provide an exciting opportunity to characterize functionally yet another MCT homologue. Although MCT7 expression has been identified in vivo, relevant mammalian models have yet to be explored. Exploring the genetic changes caused by nutrient deprivation will allow us to understand better the role of MCT7. It is the focus of this study to employ gene, and protein expression techniques to expand further our understanding of monocarboxylate transporters.

Heretofore studies have demonstrated that a monocarboxylate transporter (MCT7) is required for hepatocyte secretion of ketone bodies during fasting in zebrafish [2]. The question of whether MCT7 expression and metabolic regulation occurs in relevant mammalian models remains unanswered. Elucidation of *Mct7* will further expand our understanding of changes in metabolic processes during fasting. If indeed MCT7 is observed to change during fasting, the role of BHB as a signaling molecule could potentially be exploited for pharmacological manipulation of ketone body transport. Such exploitation could theoretically treat metabolic diseases such as: type I diabetes, dyslipidemia, and obesity [2].

Methods:

Tissue culture

The H4IIE (hepatocytomas) cell line was chosen for the experiment because of its rapid growth and because zebra fish liver was the focus of the previous study (2). Cells were taken from cryovials in frozen storage and rapidly thawed in a 37°C water bath. Once thawed, cells were slowly diluted with pre-warmed growth medium composed of α MEM, 10% fetal calf serum (FCS) and antibiotics. Once diluted, the cells were plated in a single T75 (75 cm²) tissue culture flask and incubated at 37°C until confluent. The experimental conditions ran in triplicate consisting of normal glucose (5.5mM), low glucose (1.0mM), low glucose with beta-hydroxybutrate (6mM), and normal glucose with BHB (6mM). Later analysis of *Mct7* also included the aforementioned glucose and BHB concentrations with an added 2mM of NH₄Cl in each condition. Treatments were allowed 48 hours of incubation at 37°C. The cells were collected using RLT buffer (Qiagen) and a rubber policeman. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturers instructions. The quality and concentration of the isolated RNA preparations were analyzed using a spectrophotometer (Nanodrop Technologies). RNA (1ug) was taken per sample and used as a template for reverse transcription using Quantiscript Reverse Transcriptase (Qiagen). Total RNA samples were stored at -80°C.

Gene expression: q(RT)-PCR

Quantitative real-time PCR was performed on the Rotor-Gene (Qiagen). All primers were engineered using PrimerQuest from IDT (Table 1). The specificity of all primers was tested using standard PCR and ran on a 1% agarose gel, yielding a single band of expected size. Real-time PCR reactions were performed using the Rotor-Gene SYBR Green RT-PCR kit (Qiagen). A standard reaction vessel contained: 6.25 ul Rotor-Gene SYBR Green, 1ul forward primer, 1ul reverse primer, 1ul template DNA,

and 3.25ul RNase-free water reaching a total volume of 12.5ul. Reaction conditions were: denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s with autoramp time. All reactions were run in triplicate. The expression of gene of interested was normalized to *β-actin* or *Gapdh*. Comparative expression of the gene of interest was calculated as $2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample B}]$. Sample A is the treated sample and sample B is the untreated control, and C_T is the cycle number in the linear range of the amplification curves for all mRNAs in all of the experimental runs [6].

Membrane preparation and western blot analysis

Cells were collected from tissue culture flasks via a rubber policeman and SDS Boiling Buffer and homogenized using a sterile 22-G needle and syringe by passing the cell suspension through the needle 5 times. Samples were centrifuged at 1,000 g for 10 min, and the supernatant was saved. Subsequently, total protein concentration was ascertained using BCA protein assay (Pierce Thermo Scientific). Protein samples were solubilized in Laemmli sample buffer, and SDS-PAGE was performed on gradient 5-15% polyacrylamide gels. For immunoblotting, the proteins were transferred electrophoretically to nitrocellulose membranes (BIOrad). SeaBlocking Buffer (SBB) (Pierce ThermoScientific) was applied for one hour at room temperature. Chicken anti-MCT1 antibody diluted 1:5,000 in TBST 1% SBB applied overnight at 4°C with agitation. The membranes were then washed three times and incubated for one hour at room temperature with the secondary antibody HRP conjugated rabbit anti-Chicken IgY. The membranes were then washed three times and exposed to Super Signal West Pico (Pierce ThermoScientific) for 5 mins and imaged using FluorChem (Alpha Innotech). The blots were also evaluated using mouse monoclonal anti-*β-actin* antibody (Millipore) diluted 1:10,000 and HRP conjugated goat anti-mouse secondary (ThermoScientific) following the same protocol.

Table 1: shows the primer design for all primers used

Gene	Primers
<i>Mct7</i>	F: 5'-AGTTTCCTCCCGACTGTGACCATT-3' R: 5'-GCGAAAGCAAACATCGCAAAGCAC-3'
<i>Mct7</i>	F: 5'-AAACACGGACCTCAATCGACTCCA-3' R: 5'-AAGATGGAGAAGTCCAGCAACGGA-3'
<i>β-actin</i>	F: 5'-ATCGTGCGTGACATTAAGAGAAG-3' R: 5'-GGACAGTGAGGCCAGGATAGAG-3'
<i>Gapdh</i>	F: 5'-GGTGTGAACCATGAGAAGTATGA-3' R: 5'-GAGTCCTTCCACGATACCAAAG-3'
<i>Mct1</i>	F: 5'-GCTGCTTCTGTTGTTGCGAATGGA-3' R: 5'-AAAGGCAAATCCAAAGACTCCCGC-3'

Results:

Tissues harvested from a rat were used for PCR analysis; *Mct7* and *Mct1* were both found in abundance in heart, liver, and brain samples (Fig. 1).

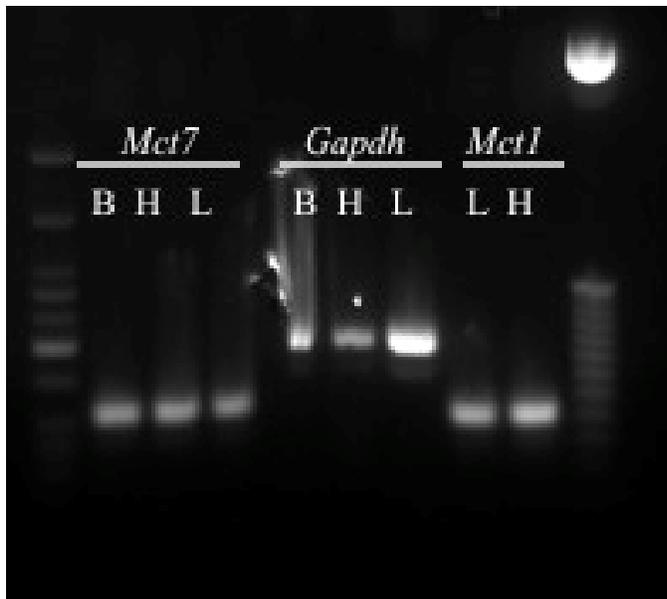


Figure 1: shows agarose gel electrophoresis of MCT7 (left), GAPDH (center), and MCT1 (right) from brain, heart and liver samples from rat tissue.

Using engineered primers for *Mct1*, *Mct7*, *Gapdh*, and β -actin (Table 1), we were able to measure the change in gene expression of *Mct1* and *Mct7* after 48h under assigned conditions. Low glucose when combined with either BHB or NH₄Cl, showed an approximate 2-fold increase in *Mct1* and *Mct7* expression. Interestingly, when BHB and NH₄Cl were combined with low glucose, both *Mct1* and *Mct7* increased 7.5 and 8.2-fold respectively. A similar fold increase was also present in the normal glucose condition with BHB and NH₄Cl (Fig. 2).

Gene expression: using Gapdh as housekeeping gene

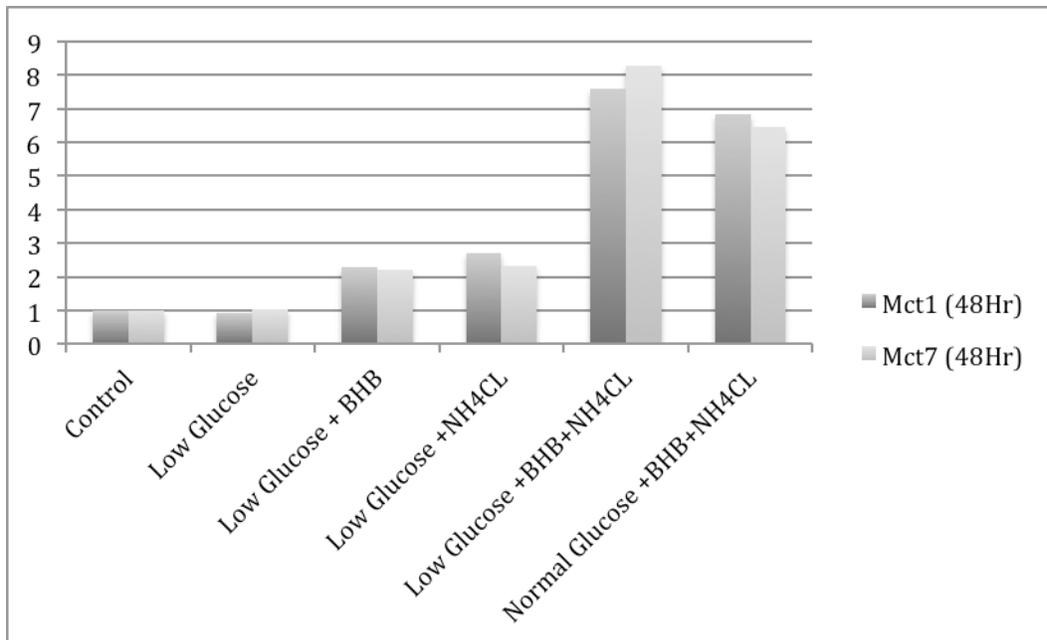


Figure 2: Confluent H4IIE cells were incubated for 48 hours under the shown conditions in triplicate. Control is 5.5 mM glucose, low glucose is 1 mM, BHB concentration was 6 mM and NH₄CL was 2 mM.

An attempt to replicate the dramatic increase in *Mct1* and *Mct7* was made to establish whether the patterns described were significant and reproducible. A similar pattern was observed in the combined treatment of low glucose with BHB and NH₄Cl. Additionally, the groups consisting of low glucose with either NH₄Cl or BHB also showed an approximate 1.5-fold increase of *Mct1* but not *Mct7* (Fig. 3).

Upon review of the data it was decided that *Gapdh* was not a suitable housekeeping gene due to the variability of its threshold cycles between the experimental conditions.

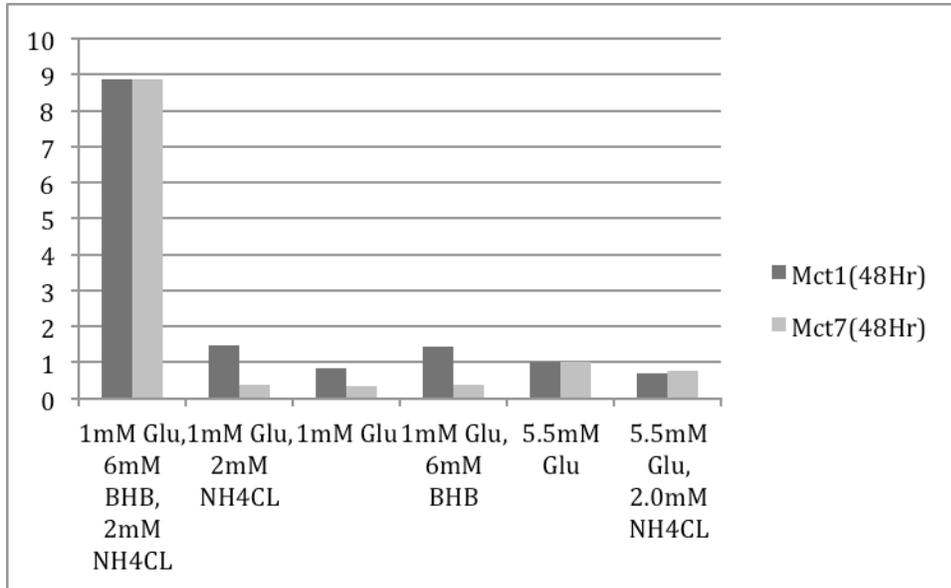


Figure 3: H4IIE were incubated for 48 hours under the shown conditions in triplicate after grown to confluence in normal growth media.

Gene expression using β -actin as housekeeping gene

Using β -actin as the new housekeeping gene, we again performed a 48h incubation under the annotated conditions in Fig. 4. The low glucose condition with BHB and NH4Cl showed a 1.6-fold increase (*Mct1*) and 2.3-fold increase (*Mct7*). Additionally the normal glucose (5.5mM) with NH4Cl also showed a moderate increase for both *Mct1* and *Mct7*. The findings using the new housekeeping gene were parallel to the findings using the previous housekeeping gene, the distinction being that the increases were more moderate and predictable. Still, more trials using the established parameters were needed to ascertain any significant patterns of gene change.

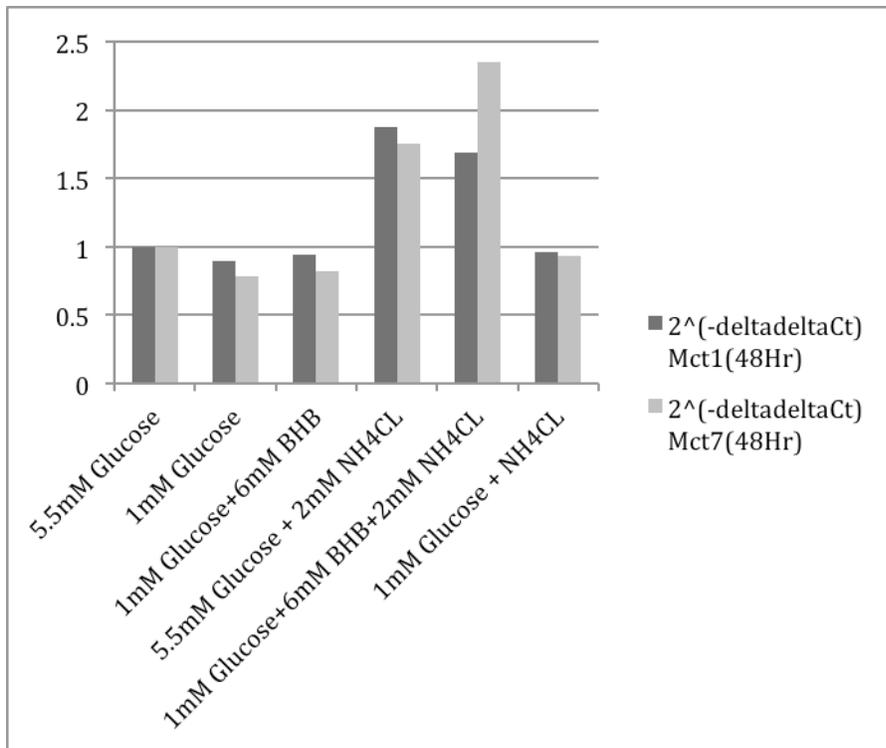


Figure 4: H4IIE cells were incubated for 48 hours under the shown conditions after grown to confluence in regular growth media. Beta-actin was used as a housekeeping gene.

After conducting two more trials of 48h nutrient deprivation studies, low glucose supplemented with BHB and NH4Cl continued to show an increase in expression of *Mct1* and *Mct7* (Fig. 5 & 6).

Interestingly, BHB or NH4Cl alone with low glucose had a 1.5-fold increase of *Mct1* but not *Mct7* (Fig. 5). However when the two were both present in low glucose conditions, over 3-fold increase in *Mct1* was detected (Fig. 5). A latter experiment did however detect a moderate 1.5-fold increase of *Mct7* (Fig. 6).

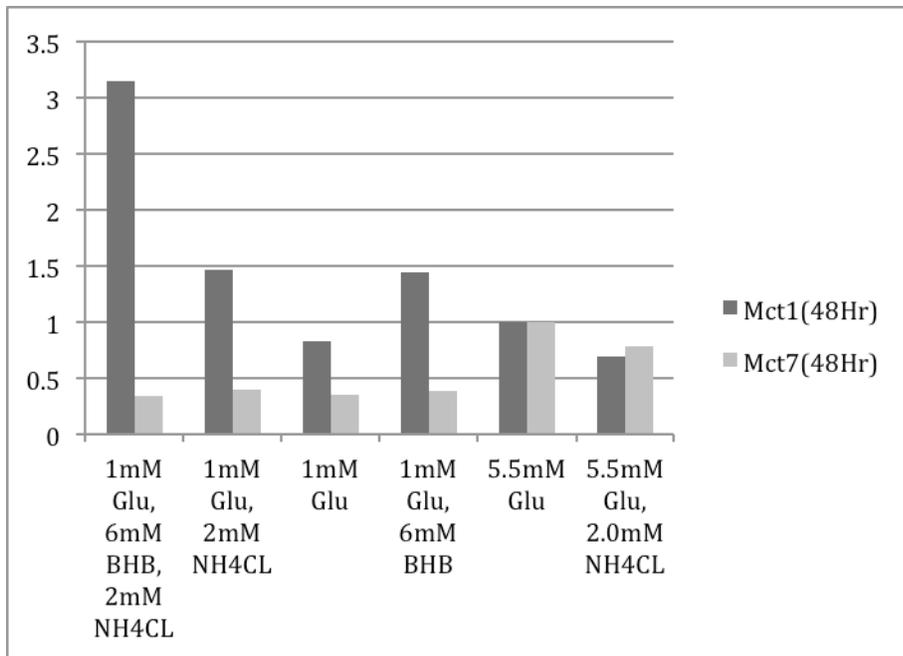


Figure 5: H4IIE cells were incubated for 48 hours under the shown conditions after grown to confluence in regular growth media. Beta-actin was used as a housekeeping gene.

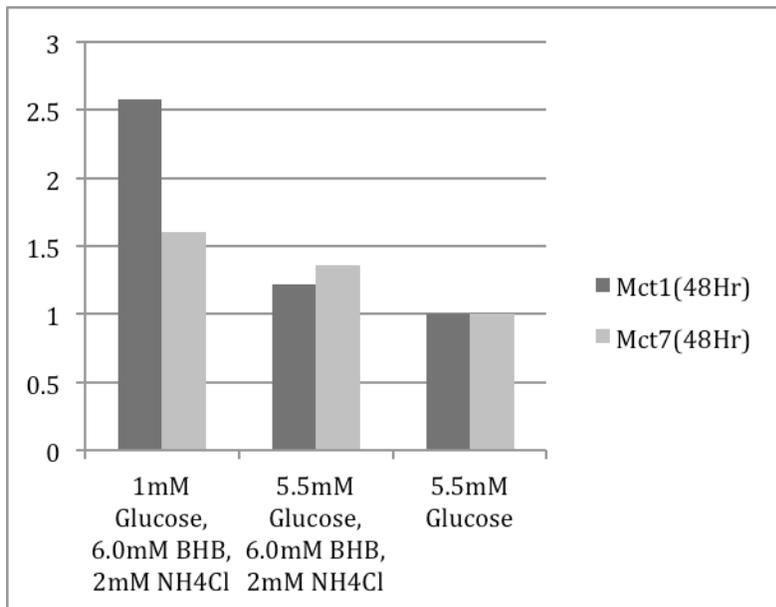


Figure 6: H4IIE cells were incubated for 48 hours under the shown conditions after grown to confluence in regular growth media. Beta-actin was used as a housekeeping gene.

Averages for three experiments using the *β-actin* housekeeping gene were taken and the differences were annotated using error bars as shown in Fig. 7. Due to time constraints all of the conditions were not able to be replicated enough times to produce data that might reveal significant changes in gene expression for *Mct1* and *Mct7*. The gene expression profiles of both *Mct1* and *Mct7* in low glucose with BHB and NH₄Cl did however depict the recurring trend of an increase in expression compared to the controls (Fig.7). To this point we have taken multiple samples of *Mct1* and *Mct7* mRNA, reverse transcribed them into cDNA, and performed real-time PCR to detect the level of gene expression (amount of transcripts present in a sample). Because it is known that mRNA abundance does not necessarily correlate to an increase in translation, our next objective was to take samples of protein via immunoblot detection.

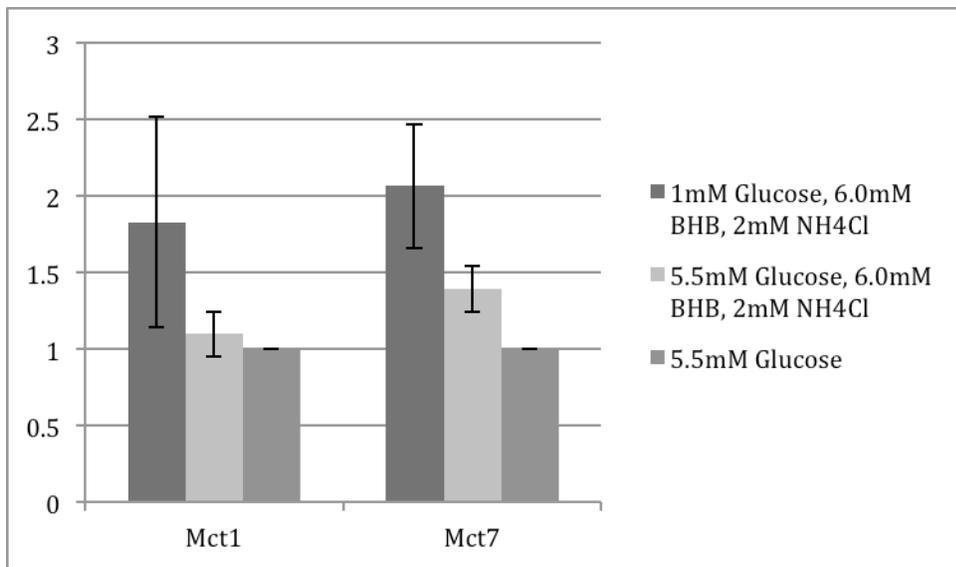


Figure 7: Depicts n=3 for each condition shown. Error bars show the difference between threshold cycles after using the comparative $2^{-\Delta\Delta C_T}$ method. Conditions were normalized using the *β-actin* housekeeping gene.

Immunoblot detection of MCT1 revealed changes in protein abundance (Fig. 8). Compared with the normal glucose condition, all of the experimental groups showed an increase in protein concentration of MCT1 (Fig.9). However, the additive transcriptional pattern observed when NH₄Cl and BHB are both present in low glucose was not obtained in the protein analysis. In contrast, Fig. 9 depicts the largest increase in protein concentration in low glucose with BHB only. Fig. 8 and 9's protein samples were taken from the trial shown in Fig. 4.

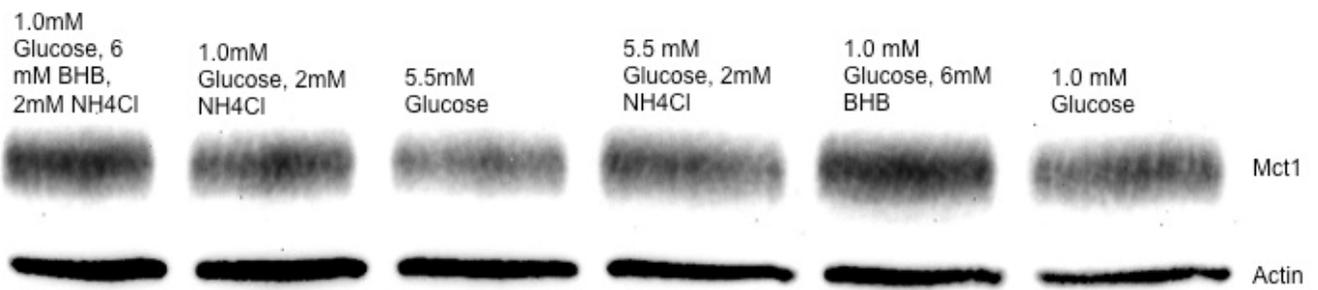


Figure 8: MCT1 immunoblot detected protein abundance under the indicated experimental conditions.

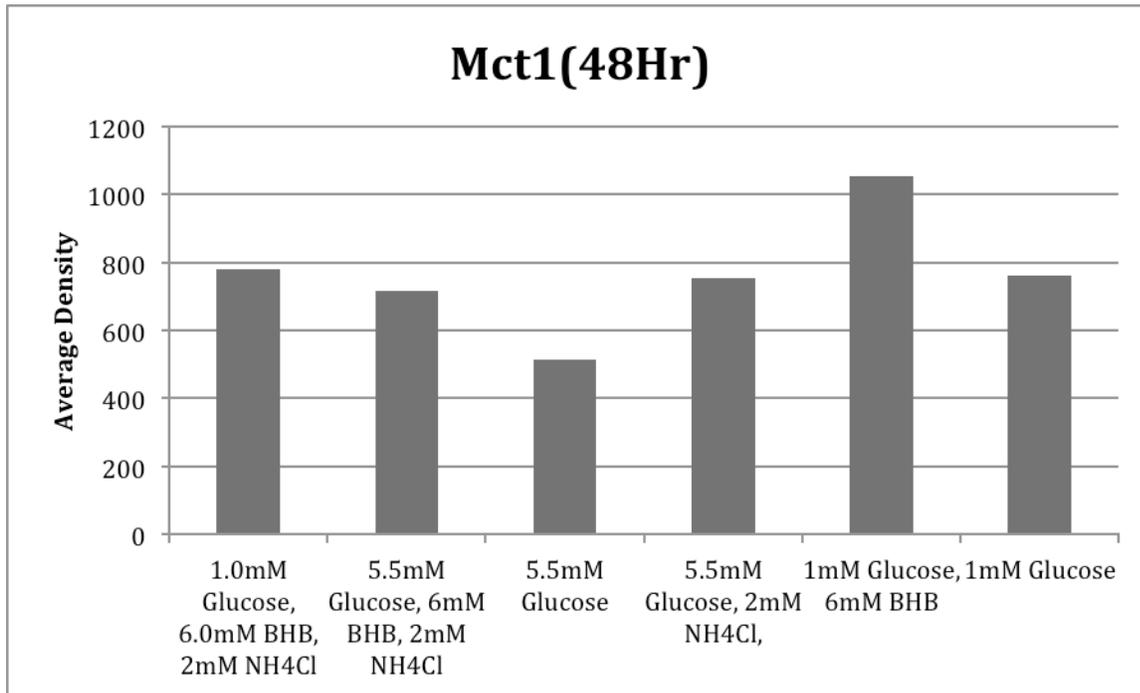


Figure 9: Densitometry analysis was used to analyze the relative abundance of MCT1.

Protein samples taken from the same trials shown in Fig 6 were immunoblotted for MCT1 (Fig 10). Subsequent densitometry analysis (Fig 11) revealed changes in MCT1 abundance consistent with the *Mct1* changes previously shown in Fig 6. The densitometry analysis also showed the same pattern of protein abundance observed in Fig 9: low glucose with BHB and NH4Cl had the highest MCT1 abundance, normal glucose with BHB and NH4Cl had the second highest, and the control had the lowest abundance of MCT1.

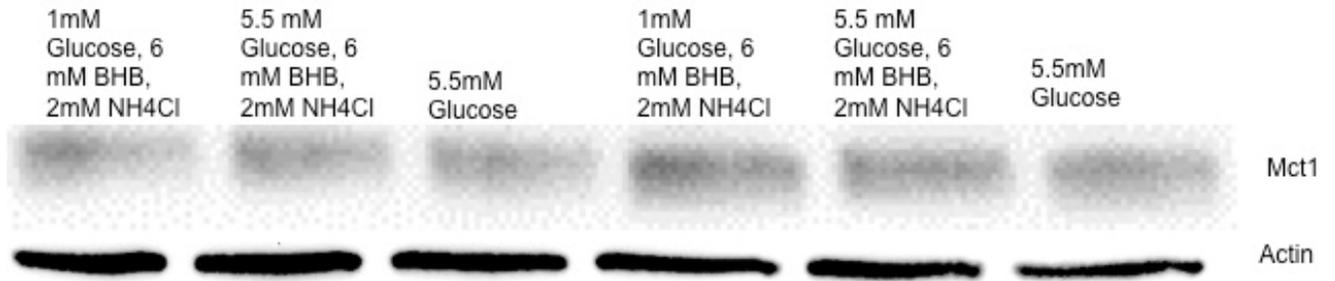


Figure 10: MCT1 immunoblot detected protein abundance under the shown conditions. The three conditions were blotted twice to take averages for densitometry.

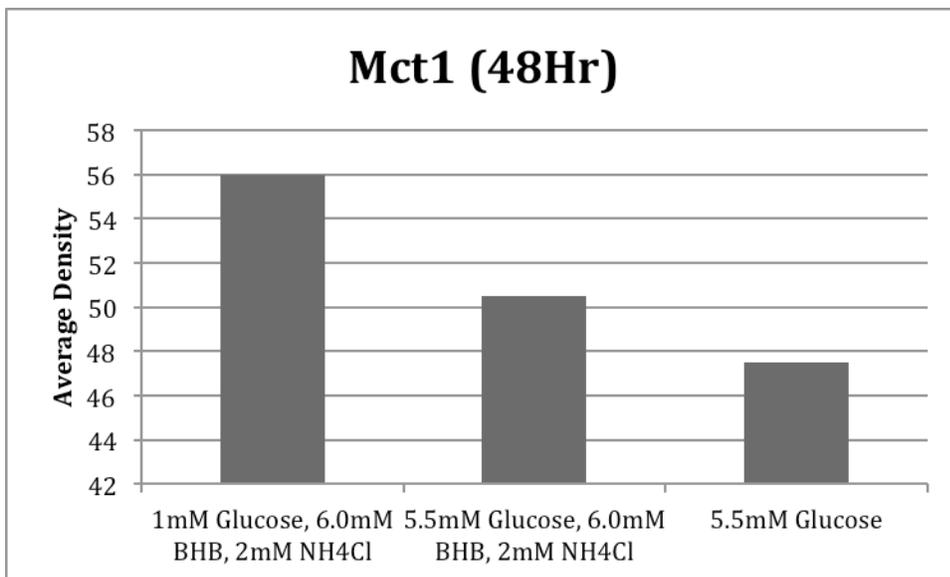


Figure 11: Densitometry analysis was used to analyze the relative abundance of MCT1.

Discussion:

Hepatic adaptation to metabolic acidosis involves the regulation of various enzymes and transport proteins. Although the mechanisms and proteins involved in regulating metabolic acidosis are known, the adaptive response by the liver to modulate *Mct* expression for BHB transport is still poorly understood. In the present study we used q(RT)-PCR and Western blotting to characterize transcriptional changes of *Mct1* and *Mct7* in vitro.

Once it was established that *Mct7* was indeed present in rat tissue, the goal of the study was to begin to develop technical parameters by which the gene of interest could be detected using q(RT)-PCR. Specifically, the goal was to find out if *Mct7* responds in a predictable manner, to metabolic changes. Initial observations of *Mct7* under normal and low glucose conditions with and without BHB showed no significant changes in gene expression (data not shown). Subsequent attempts to study the gene expression of *Mct7* entailed using variable glucose concentrations with BHB in addition to ammonium chloride. Figures 2 and 3 suggest a dramatic increase in *Mct1* and *Mct7* under low glucose, with BHB and NH₄Cl. However, large variability of Gapdh threshold cycles between experimental conditions seemed to account for the provocative increases shown in figures 2 and 3. The β -actin housekeeping gene was later selected for use as a normalizer in this study because of its success in similar studies of *Mct* expression [3,5].

Using variable glucose concentrations with BHB in addition to ammonium chloride yielded a more predictable pattern of *Mct1* and *Mct7* regulation during the 48h trials of nutrient deprivation (Fig 4-6). The consistent increases of *Mct1* and *Mct7* transcripts during enhanced nutrient deprivation support previous findings by Hugo et al (2012). If these transporters function to mobilize ketone bodies during fasting, one would predict that their expression would increase during nutrient deprivation conditions. Indeed, our data parallel the predicted pattern. Furthermore, immunoblot detection revealed an increase in protein consistent with mRNA detection (Fig 10 & 11).

Although MCT7 expression has been identified in vivo, relevant mammalian models have yet to be explored. Exploring the genetic changes caused by nutrient deprivation will allow us to understand better the role of MCT7. It was the focus of this study to employ gene, and protein expression techniques to expand further our understanding of monocarboxylate transporters. Our analysis of MCT7 revealed that it is present in mammalian tissues, and it is also highly regulated by metabolic conditions.

Recurring trends throughout our data suggest both MCT1 and MCT7 play a critical role maintaining sufficient metabolite transport. Further investigation into the expression and function of MCT1 and MCT7 is required to understand the cell's dependence on ketone transporters during nutrient deprivation. Future studies should focus on replicating this study. Due to time constraints we were not able to establish statistically significant results. In summary, our data provide a glimpse into the functionality of MCT7, a largely unknown metabolite transporter that we have shown is well at home in mammalian tissues.

References

1. Enerson, B. E., and L. R. Drewes. "Molecular Features, Regulation, and Function of Monocarboxylate Transporters: Implications for Drug Delivery." *Journal of Pharmaceutical Sciences* 92.8 (2003): 1531-44. Print.
2. Halestrap, A. P., and N. T. Price. "The Proton-Linked Monocarboxylate Transporter (Mct) Family: Structure, Function and Regulation." *Biochemical Journal* 343 (1999): 281-99. Print.
3. Hugo, Sarah E., et al. "A Monocarboxylate Transporter Required for Hepatocyte Secretion of Ketone Bodies During Fasting." *Genes & Development* 26.3 (2012): 282-93. Print.
4. Leino, R. L., et al. "Diet-Induced Ketosis Increases Monocarboxylate Transporter (Mct1) Levels in Rat Brain." *Neurochemistry International* 38.6 (2001): 519-27. Print.
5. Newman, John C., and Eric Verdin. "Ketone Bodies as Signaling Metabolites." *Trends in Endocrinology and Metabolism* 25.1 (2014): 42-52. Print.

6. Nowik, Marta, et al. "Induction of Metabolic Acidosis with Ammonium Chloride (NH₄Cl) in Mice and Rats - Species Differences and Technical Considerations." *Cellular Physiology and Biochemistry* 26.6 (2010): 1059-72. Print.
7. ---. "Genome-Wide Gene Expression Profiling Reveals Renal Genes Regulated During Metabolic Acidosis." *Physiological Genomics* 32.3 (2008): 322-34. Print.