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Understanding glucose uptake during methionine deprivation in incubated rainbow trout (*Oncorhynchus mykiss*) hepatocytes using a non-radioactive method

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ABSTRACT

The role of methionine supplementation in fish metabolism remains largely unexplored. This study investigates the effects of methionine deprivation (MD) on glucose uptake in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. To this end, primary hepatocytes were incubated in the presence (+M) or the absence (−M) of methionine for 48 h and glucose uptake was assessed using a novel non-radioactive, fluorescent-linked enzymatic assay. Evidence indicated that glucose uptake increased under methionine deprivation, primarily due to the increased abundance of membrane bound sodium-glucose transporter 2 (SGLT2), which was likely facilitated by the cellular reduction in [ATP] resulting from increased mitochondrial uncoupling, as supported by elevated transcript levels of uncoupling protein 2a (UCP2a). This study is the first to suggest that the mechanisms responsible for the rapid glucose uptake associated with MR are facilitated by the greater abundance of SGLT2 glucose transporter and mitochondrial uncoupling.

1. Introduction

Research into dietary methionine restriction (MR) has demonstrated numerous beneficial effects in mammalian studies, including the decreased generation of mitochondrial reactive oxygen species (ROS) thereby reducing the potential for oxidative damage, increased fat oxidation which reduced fat mass accretion, improved glucose and insulin homeostasis, and extending lifespan (Orentriech et al., 1993; Malloy et al., 2006; Sanz et al., 2006; Caro et al., 2008; Ables et al., 2012). However, despite the numerous studies addressing the beneficial effects of MR in mammals, the mechanisms explaining how MR alters energy expenditure and metabolism remains elusive. The impact of MR was also recently examined in rainbow trout (*Oncorhynchus mykiss*), an economically important fish species. Of particular interest, Craig and Moon (2012) observed a rapid uptake of glucose 6 h post-feeding in trout fed both nominal (12%) and high (22%) carbohydrate-MR diets, thus abolishing the glucose intolerant phenotype associated with this carnivorous species. Glucose intolerance in trout is characterized by elevated plasma insulin levels and hyperglycemia for a prolonged period of time after a meal and has remarkable similarities to the human metabolic syndrome, type II diabetes (Del sol Novoa et al., 2004). However, Craig and Moon (2012) were not able to establish a mechanism for the rapid uptake of glucose associated with MR.

A prominent feature of MR in mammalian studies is the increased energy expenditure related to uncoupled respiration and metabolic inefficiency (Hasek et al., 2010). Simply put, there is increased glucose uptake that is not efficiently converted to ATP, resulting in substrate cycling, which leads to the de novo synthesis of lipids and their rapid oxidation (Hasek et al., 2010). Likewise, Craig and Moon (2012) demonstrated increased oxygen consumption in MR-rainbow trout, irrespective of carbohydrate load, coupled with rapid glucose uptake and increased markers of β-oxidation. Combined, these results suggest that MR alters both glucose uptake mechanisms and mitochondrial uncoupling.

In fish, as in mammals, glucose is transported across cellular membranes by two families of glucose transporters; the Na^+-dependent glucose transporters (SGLTs or SLC5A family) and the facilitative glucose transporters (GLUT or SLC2A family). Although both the muscle and the liver can take-up glucose, this study focuses on the liver, as Craig and Moon (2012) showed that the greatest fluctuations in MR glucose uptake were liver specific. In rainbow trout, it is the GLUT2 isoform (Krasnov et al., 2001) that is specifically found in the liver, and as of yet, there is no conclusive evidence for SGLTs in the liver (Sugiura et al., 2003). In contrast, mammalian literature supports the presence of the SGLT2 isoform in the liver (Turk et al. 1994). Given that MR impacts glucose uptake, a change in either the abundance or type of glucose transporters is likely. Alternatively, the activity of glucokinase (GK; EC 2.7.1.2), the primary hepatic enzyme that phosphorylates glucose in the first step of glycolysis, may also be significantly altered by MR, since GK activity can be induced by dietary carbohydrates (Panserat et al., 2000a, 2000b).

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In addition to changes in glucose uptake dynamics, MR affects mitochondrial uncoupling. Uncoupling proteins (UCP) dissipate the proton gradient across the inner mitochondrial membrane, reducing the efficiency of ATP production (Ledesma et al., 2002). The classical UCP1, discovered in brown adipose tissue in mammals, plays a primary role in non-shivering thermogenesis (Nicholls, 2001), and has multiple homologues found in other vertebrates and plants (Ricquier and Bouillaud, 2000; Borecky et al., 2001). For example, the expression of UCP2 genes in rainbow trout muscle was affected by fasting, suggesting their involvement in energy balance (Coulibaly et al., 2006). Given that MR alters both energy expenditure and mitochondrial uncoupling, it is likely to increase the expression of UCP2a, the predominant isoform in the rainbow trout liver, and similar to UCP2A expression changes in the muscle as described by Coulibaly et al. (2006).

In this study, the rainbow trout hepatocyte preparation was chosen as an in vitro model to assess the mechanistic impact of MD, with specific interest in examining changes in glucose uptake. Additionally, we selected a non-radioactive method to measure glucose uptake, using an enzymatic fluorometric assay modified for trout hepatocytes as previously described (Yamamoto et al., 2006). Unlike the more costly radioactive [^{3}H]2-deoxyglucose method for glucose uptake, this assay allows one to mimic the postprandial glucose concentrations. Furthermore, this assay is more desirable for laboratories who do not wish to use radioactive materials, or institutions that are not licensed for radioactive materials. We also employed specific glucose transporter blockers to determine the specificity and abundance of transporters related to MD. Specifically, cytochalasin-B, a potent inhibitor of the GLUT transporters (Estensen and Plagemann, 1972; Taverna and Langdon, 1973), and phlorizin, a competitive inhibitor of glucose uptake by Na^+-dependent glucose transporter (Alvarado and Crane, 1964), were used in equal concentrations to identify the presence and specific changes in transporter abundance. Additionally, the mitochondrial uncoupler, 2,4-dinitrophenol was employed to assess the degree of mitochondrial uncoupling in hepatocytes associated with MR. Finally, quantitative PCR was used to analyze the changes in the gene expression profiles of GLUT2, SGLT2, UCP2a, and glucokinase (GK) in response to MR.

2. Methods and materials

2.1. Fish

Female rainbow trout (*O. mykiss*) were purchased locally (Linwood Acres Trout Farm; Campbellcroft, ON, Canada) and housed in 3 m^3 (270 L tanks). Tanks were supplied continuously with water from the City of Ottawa that was oxygenated and dechloraminated at the University of Ottawa Aquatic Care Facility. During this time, fish were fed commercial trout fed ad libitum (3 PT classic floating grower feed; Martin Mills, Elmira, ON, Canada). All fish were held under a photoperiod that mimicked the City of Ottawa natural light cycle (Fall). Fish were selected based on size (>300 g), taken from their tank and euthanized with an overdose of anesthetic (benzocaine) prior to hepatocyte recovery. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and undertaken in accordance with institutional animal care guidelines that adhere to those of the Canadian Council on Animal Care.

2.2. Hepatocyte isolation

Hepatocytes were isolated according to Mommsen et al. (1994). Briefly, Hanks' medium was prepared (in mM: NaCl, 136.9; KCl, 5.4; MgSO4, 0.8; NaHCO3, 5; Na2HPO4, 0.33; KH_2PO_4, 0.44; Hepes, 5; Na-Hepes, 5) and used to prepare the rinsing, collagenase, and resuspension solutions all adjusted to pH 7.63 at room temperature. A mid-ventral incision was made and a cannula was inserted into the hepatic portal vein. Using a perfusion pump (2 mL/min) the liver was perfused with rinsing solution containing 1 mM EGTA (the heart was cut to prevent pressure build-up in the liver). Once the liver was cleared of blood it was perfused with a collagenase solution containing 0.15 mg/mL collagenase (Sigma type IV). Once the liver has expanded and softened (approximately 25 min), it was removed and carefully cut to remove adhering vessels/tissues. It was then diced with a razor blade in a glass Petri dish and sequentially filtered using 250 and 75 mm nylon mesh. The collected hepatocytes were poured into 50 mL Falcon tubes and centrifuged for 2 min at 1000 rpm (Sorvall RC centrifuge with SS-34 rotor) at 4 °C. Cells were resuspended in media containing 1% bovine serum albumin and centrifuged as above. This was repeated twice more and cells were finally re-suspended in Eagle minimum essential medium containing Earl salts (2 g/L $NaHCO_3$) with or without L-methionine (Cat#1641454, MP Biomedical), antibiotic and antimycotic solutions (Invitrogen), and 1% BSA, and adjusted to pH 7.63. After a 30 min rest period on ice the cells were re-suspended and counted using a Bright-Line hemocytometer (Spencer); viabilities were determined using the Trypan Blue exclusion method (Mommsen et al., 1994). Only cell suspensions with >90% live cells were used in the experiments. The cells (0.4 mL at 25 mg/mL) were plated in 48-well plates (Corning 3338) and allowed to adhere for 48 h at 13 °C on a shaker prior to glucose uptake experiments.

2.3. LDH leakage assay

The viability of hepatocytes was determined by the lactate dehydrogenase (LDH; EC 1.1.1.27) leakage assay modified from Feng et al. (2003). Briefly, after treatment the cell media was collected and LDH activity was estimated with NADH (0.35 mM) and pyruvate (4.5 mM) in imidazole buffer (50 mM, pH 7.5). The reaction was monitored spectrophotometrically at 340 nm over 15 min. The activity was normalized to the control samples.

2.4. Hepatocyte glucose uptake and inhibition experiments

After 48 h of incubation, hepatocytes (n = 4 with 8 wells measured per repeat) were washed twice with Hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.63). The washed hepatocytes were then incubated for 30 min in their respective culture media with or without (control) 20 mM 2-deoxyglucose (2-DG). Additional hepatocyte cultures were performed under the same treatments as above, with the addition of a known mitochondrial uncoupler (50 μM 2,4-dinitrophenol dissolved in 0.1% DMSO), a general GLUT blocker (25 μM cytochalasin-B dissolved 0.1% DMSO), or a SGLT blocker (25 μM phlorizin dissolved 0.1% DMSO). This approach was used as there are no suitable antibodies for rainbow trout GLUT2 and SGLT to determine protein abundance. For insulin stimulation, cells were incubated with insulin (4 nM bovine insulin) for 20 min in respective culture media prior to the addition of 20 mM 2-DG. After incubation, the cells were washed twice with Hepes buffered saline to remove residual 2-DG, collected in 0.1 N NaOH, and frozen at −80 °C for analysis of 2-DG uptake and cellular ATP concentrations. Cells used for enzymatic analysis of glucokinase and quantitative PCR were frozen at −80 °C in Hepes buffered saline.

2.5. 2-Deoxyglucose uptake and ATP assay

Uptake of 2-DG was determined using an enzyme-fluorometric assay modified for trout hepatocytes based on Yamamoto et al. (2006). The following is the assay performed to determine the 2-DG uptake using a standard curve of 2-DG ranging from 0 to 100 μM. Cells frozen in 100 μL 0.1 N NaOH were thawed and subsequently incubated at 85 °C for 40 min to degrade any residual cellular NADP/NADPH, which could interfere with the stoichiometry of the

assay. 100 μL 0.1 N HCl and 100 μL Tris assay buffer (60 mM Trizma base, 40 mM Tris–HCl, 50 mM KCl, pH 8.1) were added to neutralize the sample. The neutralized sample or the standard (50 μL; prepared in an identical manner) was then added to 200 μL assay buffer consisting of 0.8 mM $MgSO_4$, 0.02% BSA, 0.1 mM NADP, 0.5 mM ATP, 0.2 U/mL diaphorase, 5 mM resazurin sodium salt, 2 U/mL hexokinase, and 15 U/mL G6PDH (from *Leuconostoc* sp.). The assay was incubated in a 96-well white assay plate for 90 min at 37 °C. At the end of the incubation, fluorescence at 590 nm with excitation at 530 nm was measured by a Gemini XPS fluorescence microplate reader (Molecular Devices, Menlo Park, CA, USA) in order to detect the resorufin derived from the reduced resazurin. The 2-DG uptake was determined using the standard curve and expressed as a percent increase over control cells that were incubated in the absence of 2-DG. The neutralized sample was additionally assayed spectrophotometrically for cellular concentrations of ATP according to the methods described by Bergmeyer (1983).

2.6. Glucokinase assay

Glucokinase (GK; EC 2.7.1.2) enzyme activity was assessed in the hepatocytes (n = 4 with 8 wells measured per repeat). Activities were determined by coupling ribulose-5-phosphate formation from glucose 6-phosphate to the reduction of $NADP^+$ at 340 nm as described previously (Panserat et al., 2000a, 2000b). Activities were determined using a 96-well microplate at 37 °C and absorbance was measured and recorded on a SpectraMAX Plus 384 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA, USA). GK activity was corrected for glucose dehydrogenase activity (G1DH; EC 1.1.1.47) as described (Panserat et al., 2000). Briefly, GK activity was determined as the total HK activity – low Km activity – 1/3 G1DH activity. Enzyme activity was normalized to protein content, which was assessed using the BCA assay (Sigma), and expressed as mU/mg protein.

2.7. qPCR

Total RNA from the hepatocytes (n = 4 with 6 wells pooled per repeat) was extracted using Trizol (Invitrogen) following the manufacturer's protocol. Total RNA concentrations and purity were measured using the Nanodrop ND-1000. First-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). The mRNA abundance was quantified in duplicate using a Rotor-Gene Q real-time PCR machine (Qiagen). Each reaction contained 6.25 μL Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 1 μL of each forward and reverse gene specific primer for a final concentration of 1 μM, 3.25 μL RNase/DNase-free purified H_2O (Roche), and 1 μL cDNA product as described above. Cycling conditions were: 5 min initial denaturation at 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 10 s. Additionally, a melt curve analysis was used at the end of each run to validate the amplification of only one product. To account for differences in cDNA production and loading differences, standard curves were constructed for each target gene using serial dilutions of a reference pool of representative cDNA from all experiments. To account for the differences in cDNA production and loading differences, all samples were normalized to the expression of the housekeeping gene elongation factor 1 alpha (ef1α), which did not change over the experimental treatments. Transcript level data were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Both RNase/DNase-free H_2O and non-reverse transcribed RNA were assayed on each plate to ensure no contamination was present in the reagents or in the primers used. Primers were designed using Primer 3 software. Target genes of interest were: glucose transporter 2 (GLUT2), glucokinase (GK), sodium-coupled glucose transporter 2 (SGLT2), and mitochondrial uncoupling protein 2a (UCP2a). All primer sequences and accession numbers can be found in Table 1.

2.8. Statistical analysis

All data are presented as mean ± SEM. For statistical determination, a one-way ANOVA and Tukey's post-hoc test was performed to determine statistical significance between treatments within a methionine condition. A Student's *t*-test was used to compare within treatments for the differences between methionine nominal (+M) and methionine deficient (−M) hepatocytes. The level of significance for all tests was set at $p < 0.05$, and all statistical analyses were performed on SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Methionine restriction and 2-DG uptake

The fluorometric assay for glucose uptake allowed the use of glucose concentrations, which matched postprandial plasma levels from previous studies (Craig and Moon, 2012). The LDH leakage assay indicated similar cell viability across treatments, which was not significantly different from the control group (data not shown). Rainbow trout hepatocytes incubated in methionine-deficient (−M) media displayed a significant 4-fold increase in the uptake of 2-DG compared to +M-incubated cells (Fig. 1A). Similar trends were apparent with insulin stimulation, with both +M and −M incubated hepatocytes increasing their rate of 2-DG uptake (Fig. 1A). In an effort to relate mitochondrial uncoupled stimulation of glucose uptake, the cells were exposed to 50 μM 2,4-dinitrophenol, which significantly increased 2-DG uptake in both treatments, with no significant difference between +M and −M (Fig. 1A). Under these conditions, there was a significant 30–45% decrease in cellular ATP concentrations in insulin and non-insulin treated −M cells (Fig. 1B). Upon exposure to 2,4-dinitrophenol, there was a 75% reduction in cellular ATP concentrations in both +M and −M treated cells, with no significant difference between the two groups (Fig. 1B).

Table 1
Forward and reverse primers for glucokinase (GK), glucose transporter 2 (GLUT2), sodium/glucose transporter (SGLT), mitochondrial uncoupling protein 2a (UCP2a), and the housekeeping gene elongation factor 1 alpha (ef1a) used for qPCR analysis in rainbow trout hepatocytes, including amplicon length and GenBank accession numbers.

Gene	Description		Primer	Length	GenBank accession no.
GK	Glucokinase	F:	CAG GAG GCC AGT GTC AAA AT	124	NM001124249
		R:	CAA CCT TCA CCA ACA TCA CA		
GLUT2	Glucose transporter 2	F:	GGC ATG ACC TTC CCT TAC ATA G	125	NM001124289
		R:	AAT GTC TTC CCT TTG GTC TCC		
SGLT2	Sodium/glucose transporter 2	F:	CTA TGT GGC TGT GAT AGC CCT C	119	NM001124432
		R:	TG AAG GCG CCA GCG ATG ATG		
UCP2	Uncoupling protein 2	F:	TGT GAA GTT TAT CGG TGC TGG	89	NM001124654
		R:	GGA TCT GTA GCC GGA CTT TG		
ef1α	Elongation factor 1 alpha	F:	TCC TCT TGG TCG TTT CGC TG	159	NM001124339
		R:	ACC CGA GGG ACA TCC TGT G		

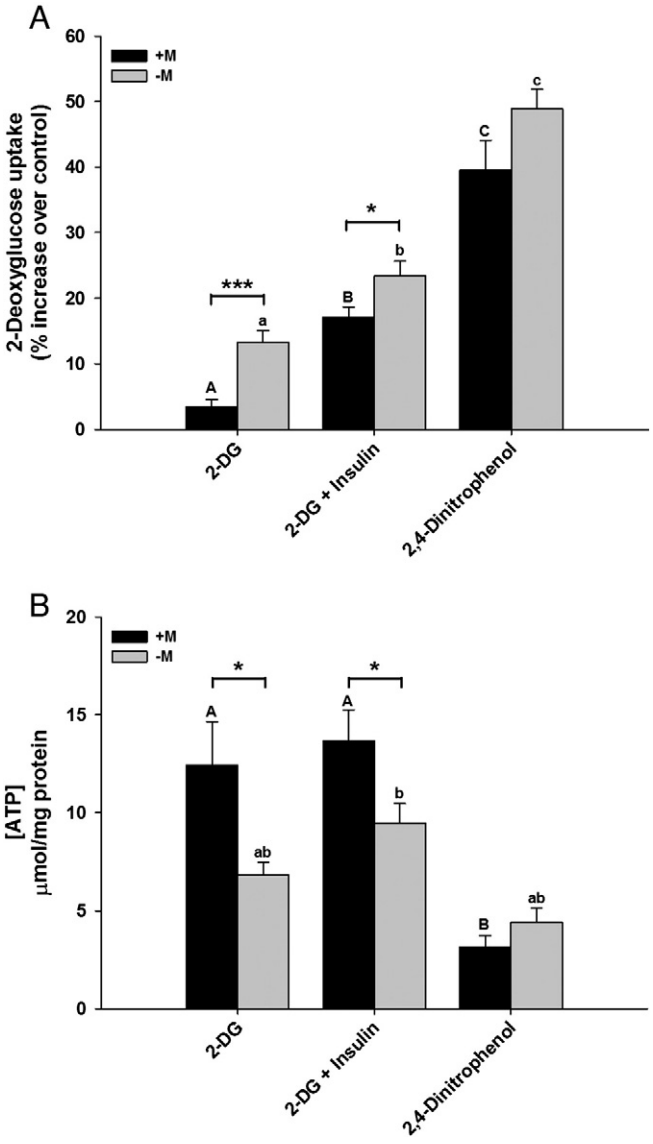

Fig. 1. Methionine nominal (+M) and deficient (−M) rainbow trout hepatocyte uptake (A) of 20 mM 2-deoxyglucose (% increase vs control) and (B) cellular concentration of ATP (mmol/mg protein) alone and under the influence of insulin (4 nM) or the mitochondrial uncoupler 2,4-dinitrophenol (50 µM) after a 30 min incubation. N = 4, with 8 wells measured per repeat. Bars that do not share the same symbol are significantly different from each other as determined by a one-way ANOVA ($p < 0.05$) followed by a Tukey's post hoc test. An * indicates a significant difference within a treatment between +M and −M, as determined by a Student's *t*-test (* = $p < 0.05$; *** = $p < 0.001$).

3.2. Glucose transport blockers

In order to tease apart differences in transporter abundance or type in response to methionine restriction, blockers of glucose transporter 2 (cytochalasin-B) and sodium-glucose transporter (phlorizin) were employed. Using comparable concentrations, we determined that (1) SGLT2 was in greater abundance in hepatocytes as phlorizin significantly decreased 2-DG uptake with greater efficacy than cytochalasin-B in both +M and −M cells (Fig. 2), and (2) that methionine restricted hepatocytes had a greater reliance on SGLT2 for glucose uptake since phlorizin reduced 2-DG ~50% more in −M cells than in +M cells (Fig. 2).

3.3. Quantitative PCR and enzyme analysis

Given that the observed difference in glucose uptake may in part be related to transporter type and abundance, we examined the gene

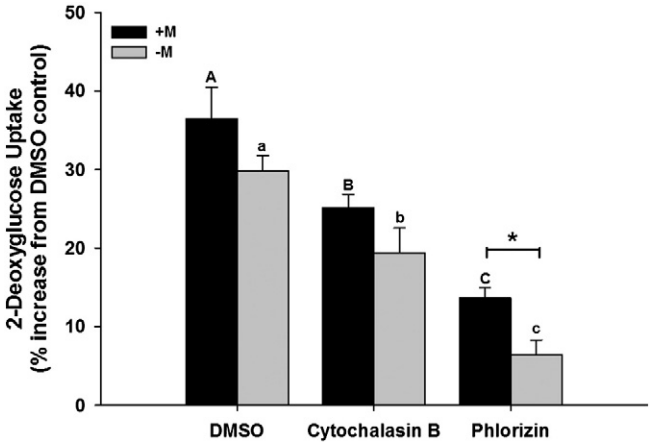

Fig. 2. Methionine nominal (+M) and deficient (−M) rainbow trout hepatocyte uptake of 20 mM 2-deoxyglucose (% increase vs DMSO control) alone or under the influence of a glucose transporter blocker, cytochalasin-B (25 µM), or the sodium glucose transporter inhibitor, phlorizin (25 µM) after a 30 min incubation. N = 4, with 8 wells measured per repeat. Bars that do not share the same symbol are significantly different from each other as determined by a one-way ANOVA followed by a Tukey's post hoc test ($p < 0.05$). An * indicates a significant difference within a treatment between +M and −M, as determined by a Student's *t*-test (* = $p < 0.05$).

expression profile of SGLT2 and GLUT2 in the absence and presence of insulin. Transcript levels of GLUT2 were not affected by MR or insulin (Fig. 3A). Conversely, the gene expression of SGLT2 in the absence of insulin was 68% higher in −M hepatocytes (Fig. 3B). Addition of insulin significantly increased the gene expression profile of SGLT2 in both +M and −M hepatocytes, which was 40% higher in −M hepatocytes (Fig. 3B).

To verify whether MR affected mitochondrial uncoupling, we examined the transcript levels of UCP2a. MR increased the expression of UCP2a by 57% in the absence of insulin; however, this increase was abolished after insulin stimulation (Fig. 3C).

To further examine how MR affects 2-DG, we assessed the activity and gene expression of glucokinase. Interestingly, glucokinase gene expression was significantly lower in −M hepatocytes independent of insulin (Fig. 4A). However, GK activity was not affected by MR, whereas insulin increased its activity in both +M and −M cells to the same extent (Fig. 4B).

4. Discussion

To our knowledge this is the first paper to characterize the possible mechanisms responsible for understanding our previous in vivo studies where MR abolished the postprandial hyperglycemic phenotype in rainbow trout. After a 48 h incubation in methionine deficient (−M) culture media, the hepatocytes increased glucose uptake, independent of insulin stimulation, by a substantial 400% (Fig. 1A). Through the use of specific glucose transporter blockers and qPCR analysis, we provide evidence that the increase in glucose uptake is facilitated by the SGLT2 whose abundance was apparently increased by MD (Figs. 2, 3B). Moreover, we further suggest that MD induced mitochondrial uncoupling as evidenced through an increased expression of UCP2a and decreased concentration of cellular ATP (Figs. 1B, 3C). These results support that the MD uncoupling action triggers increased glucose uptake in response to decreased cellular ATP, although this hypothesis needs further testing. Paradoxically, a decrease in GK gene expression in response to MD was observed, although GK enzyme activity was unchanged, suggesting that alternate mechanisms of regulation may play a greater role in glycolysis associated with MD (Fig. 4).

Carnivorous fish species have long been identified as glucose intolerant, with the notable phenotype of prolonged plasma hyperglycemia after a carbohydrate rich meal, concomitant with increased

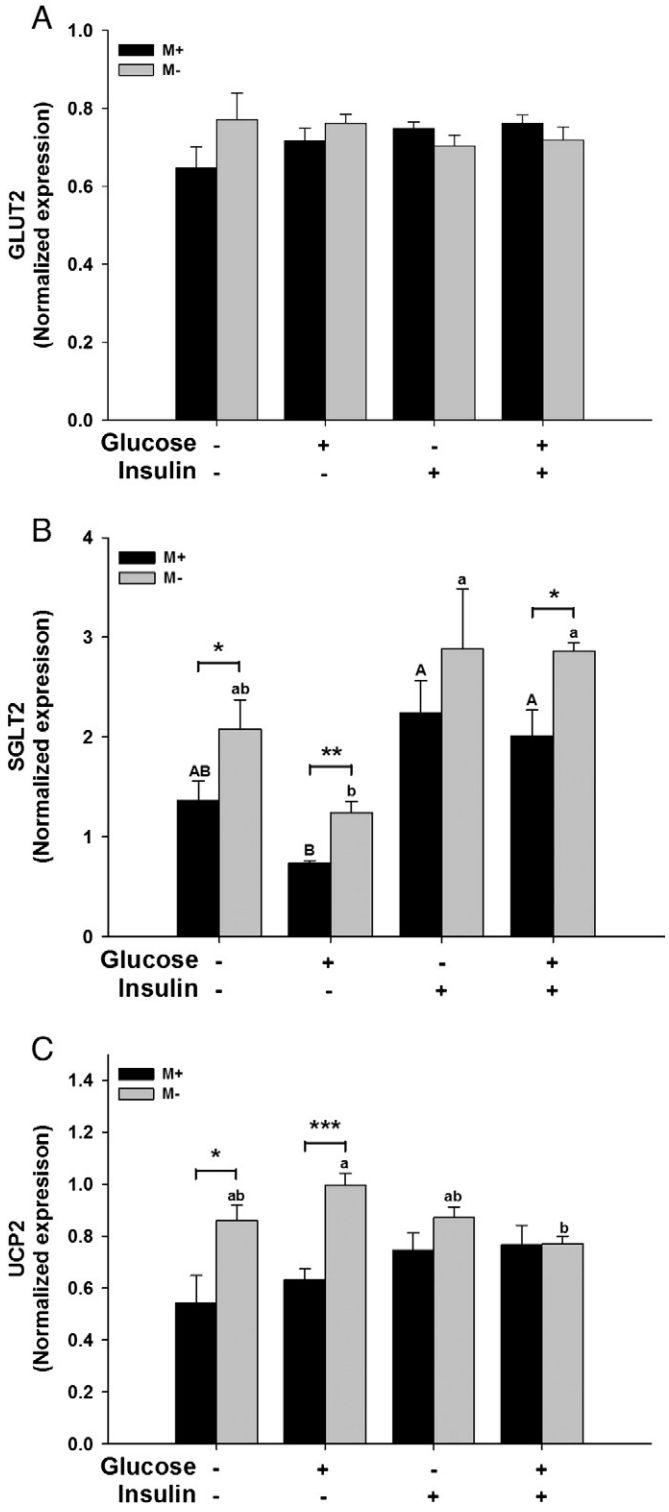

Fig. 3. Relative transcript expression levels of (A) GLUT2, (B) SGLT2, and (C) UCP2 from 48 h primary incubated methionine nominal (+M) and deficient (−M) rainbow trout hepatocytes exposed to either 20 mM glucose for 30 min or pre-incubated with 4 nM insulin for 20 min, followed by a 30 min exposure to 20 mM glucose. Transcript expression is normalized to the housekeeping gene ef1α and values expressed as a fold change compared to +M control. N = 4, with 6 wells pooled per repeat. Bars that do not share the same symbol are significantly different from each other as determined by a one-way ANOVA ($p < 0.05$) followed by a Tukey's post hoc test. An * indicates a significant difference within a treatment between +M and −M, as determined by a Student's *t*-test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

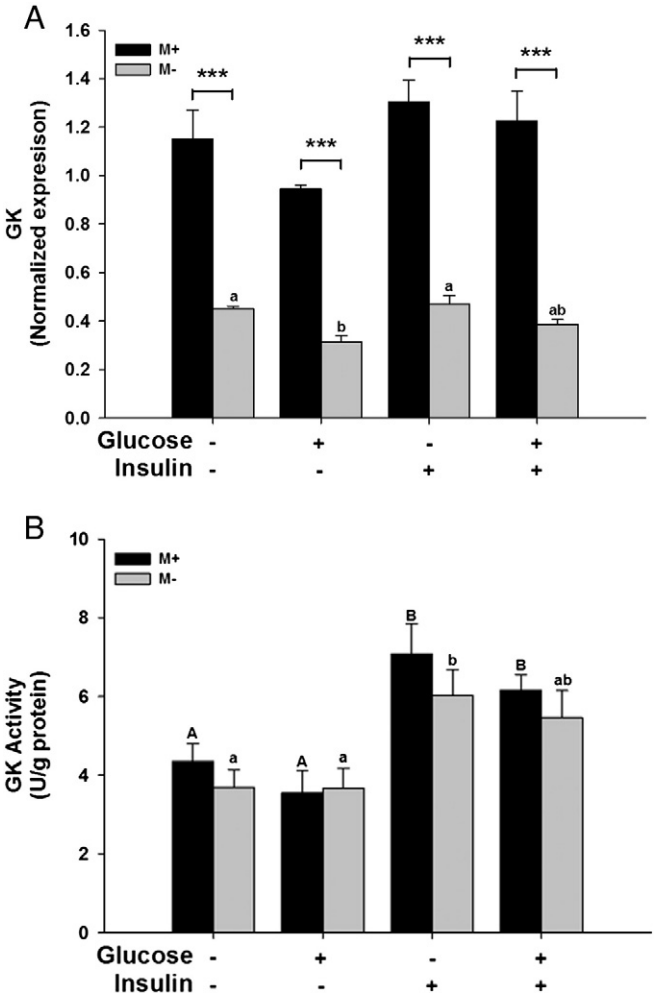

Fig. 4. Relative transcript expression levels of (A) GK and (B) enzyme activities of GK (U/g protein) from 48 h primary incubated methionine nominal (+M) and deficient (−M) rainbow trout hepatocytes exposed to either 20 mM glucose for 30 min or pre-incubated with 4 nM insulin for 20 min, followed by a 30 min exposure to 20 mM glucose. Transcript expression is normalized to the housekeeping gene ef1α and values expressed as a fold change compared to +M control. N = 4, with 6 wells pooled per repeat for transcript measurements and N = 4, with 8 wells measured per repeat. Bars that do not share the same symbol are significantly different from each other as determined by a one-way ANOVA ($p < 0.05$) followed by a Tukey's post hoc test. An * indicates a significant difference within a treatment between +M and −M, as determined by a Student's *t*-test (*** = $p < 0.001$).

hepatic glycolytic capacity without a reduction in gluconeogenic machinery (Furuichi and Yoon, 1982; Wilson and Poe, 1987; Brauge et al., 1995; Panserat et al., 2000a, 2000b, 2001; Hemre et al., 2002). Craig and Moon (2012) demonstrated that rainbow trout fed a methionine-deficient diet high in carbohydrate (22% carbohydrate load) for 8 weeks could eliminate this hyperglycemic phenotype 6 h post-feeding, although the authors could only speculate that the liver played an essential role in the rapid uptake of glucose, which contributed to the reduced plasma glycemic index post-feeding. By extending this study to an in vitro hepatocyte preparation, we were able to better identify the pathways involved in the rapid glucose uptake associated with MD.

Our primary goal was to establish a viable MD primary cell culture that could be used to examine glucose uptake using a non-radioactive method. Glucose uptake in cell culture has primarily used non-metabolizable radioactive hexoses, such as [^{3}H]2-deoxyglucose, as this is converted to an impermeable and stable derivative [^{3}H] 2-deoxyglucose-6-phosphate (2DG6P) through phosphorylation driven by glucokinase or hexokinase, depending on the cell type examined

(Sokoloff et al., 1977; Sasson et al., 1993). Although a reliable method, quantities of this radioactive analog are kept low due to high costs, making it difficult to use comparable concentrations of postprandial plasma glucose. Furthermore, this approach may be more desirable for institutions that are not permitted with radioactive licenses. By using a non-radioactive 2DG fluorometric assay, we were able to administer a concentration of glucose (20 mM) to hepatocytes that mimics the 6 h peak in hyperglycemia noted in trout fed a carbohydrate-rich meal (Craig and Moon, 2012) and obtain reliable glucose uptake rates under the influence of MD. After a 30 min exposure to 20 mM 2DG under MD, there was a marked increase in glucose absorption, which was exacerbated by pre-incubation with 4 nM insulin (Fig. 1A). However, despite the abundance of fuel available for oxidative phosphorylation, there was a significant depression in the concentration of ATP in the −M treated hepatocytes (Fig. 1B), which suggested the possibility that MD induced mitochondrial uncoupling. Mammalian studies showed that MR induced uncoupled respiration and metabolic inefficiency (Hasek et al., 2010, Perrone et al., 2010; Plaisance et al., 2010). This inefficient fuel utilization comes with the added benefit of reduced ROS production, as methionine is known to interact with complexes I and III, resulting in increased ROS production (Gomez et al., 2011). As a means of comparison, we used the known mitochondrial uncoupler 2,4-dinitrophenol, which resulted in a similar, albeit greater response, to MD in hepatocytes, with both increased glucose uptake and reduced cellular ATP concentrations (Fig. 1B). From this series of experiments, we can show that MD in trout hepatocytes increased glucose uptake yet reduced cellular ATP production, however we needed to establish the means through which this was accomplished. In short, we examined the transcript response of UCP2 to MD. Given that UCP2a is found in the liver and does respond effectively to energy balance and nutritional status in rainbow trout muscle (Coulibaly et al., 2006), we examined the transcript response of UCP2a and found increased expression in −M hepatocytes (Fig. 3C). Although the abundance of UCP2a protein was not assessed, we can still suggest that MD in trout hepatocytes may induce mitochondrial uncoupling, as the increased UCP2a transcript levels were associated with decreased ATP concentrations. Further validation of the UCP2a protein response and degree of mitochondrial uncoupling through mitochondrial respiration experiments is necessary to further establish this connection.

Glucose is transported across the membrane in vertebrates through glucose transporters that fall into two classes: the Na^+-dependent glucose transporter (SGLT) and the facilitative glucose transporter (GLUT or SLC2A family). Previous studies identified that the GLUT2 isoform in rainbow trout is specifically linked to the liver, although no conclusive evidence for any hepatic-associated SGLTs is available (Krasnov et al., 2001; Sugiura et al., 2003). In an effort to tease apart which transporter may be (1) predominant in trout hepatocytes and (2) significantly affected by MR, we employed known pharmaceutical blockers for each of the respective glucose transporters, coupled with an analysis of the transcript levels under +M and −M conditions. Using comparable concentrations of cytochalasin-B (known GLUT blocker) and phlorizin (known blocker of SGLTs), we observed a stronger response to 25 μM phlorizin (Fig. 2), suggesting not only the presence of SGLTs in the trout hepatocytes, but also their greater abundance in −M hepatocytes.

To further validate the presence and response of SGLTs, we examined the transcript abundance after incubating hepatocytes in +M and −M media. Not only was there a significant increase in the abundance of the SGLT2 transcript in −M incubated hepatocytes, but also its expression was further increased with 4 nM insulin pre-incubation (Fig. 3B). Combined, these results indicate that MR in trout hepatocytes induces increased gene expression of SGLT2, although we cannot say that this is a direct effect of MR.

Considering the reduced ATP concentration within −M hepatocytes (Fig. 1B), there is likely an increased cellular AMP concentration, which could increase the phosphorylation of AMP-activated kinase (AMPK). Craig and Moon (2012) demonstrated reduced liver ATP concentrations coupled with increased AMP concentrations and AMPK transcript abundance. Furthermore, AMPK does stimulate glucose uptake in both liver and muscle tissues through increased transcript response of glucose transporters (Polakof et al., 2011; Magnoni et al., 2012). Conceivably, the increase in SGLT2 demonstrated here could in part be due to increased AMPK phosphorylation in response to increased cellular AMP concentrations, although further studies are warranted to validate this hypothesis.

Another plausible explanation for the increased glucose uptake is its rapid removal upon transport into the cell by the enzyme glucokinase (GK). High carbohydrate loads in trout stimulate both the GK transcript response and its enzyme activity (Panserat et al., 2000a, 2000b). Interestingly, we saw no difference in GK enzyme activities associated with MR, however there was a significant repression in the transcript abundance of GK in −M hepatocytes (Fig. 4). Craig and Moon (2012) demonstrated a significant reduction in both liver GK enzyme activity and transcript response associated with an 8-wk MR in rainbow trout, which is in contrary to our present results. However, the MD in hepatocyte lasted only for 48 h, potentially implicating that post-transcriptional modification stabilizes the GK protein, which may, in part, explain the disconnect between transcript levels and enzyme activity. Moreover, recent evidence in mammals suggest that post-transcriptional modification is essential for the stability and function of this enzyme in hepatic and pancreatic cells (Aukrust et al., 2013), although this has yet been tested in trout models. Together these results suggest that the increased glucose uptake is primarily due to the increased abundance of SGLT2 in −M hepatocytes, although protein abundance of both classes of glucose transporters should be assessed to support this hypothesis.

In summary, this study provides further insight into the mechanisms responsible for the increased glucose uptake associated with MR in rainbow trout hepatocytes. We demonstrated that MR increased mitochondrial uncoupling linked to decreased cellular ATP production. Furthermore, the rapid uptake of glucose from the culture media is likely due to the increased abundance of membrane bound SGLT2. This information may be useful as farmed trout, and other salmonid species, are a valuable asset to the aquaculture industry. There is significant pressure to reduce the use of protein-rich, fishmeal-based diets in favor of more sustainable plant based meals, which requires the use of amino acid supplementation (Naylor et al., 2000; New and Wijkstroem, 2002). Understanding the role that methionine plays in aquatic nutrition is essential to develop diets that can accelerate growth and facilitate moving the product to market in an efficient manner. We have demonstrated here that methionine deprivation may accelerate glucose uptake, yet this rapid absorption may not be reflected in optimal growth.

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