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Mitochondrial Pyruvate Carriers are not Required for Adipogenesis but are Regulated by High-Fat Feeding in Brown Adipose Tissue

Jasmine A. Burrell
Louisiana State University

Allison J. Richard
Pennington Biomedical Research Center

William T. King
Pennington Biomedical Research Center

Jacqueline M. Stephens
Louisiana State University

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For chronic weight management in adults with a BMI of ≥ 30 kg/m² (obesity), or ≥ 27 kg/m² (overweight) in the presence of a weight-related comorbidity, as an adjunct to a reduced calorie diet and increased physical activity.

ONCE - WEEKLY

wegovy™

semaglutide injection 2.4 mg

Click here to see the Prescribing Information, including Boxed Warning

Indications and Usage

Wegovy™ (semaglutide) injection 2.4 mg is indicated as an adjunct to a reduced calorie diet and increased physical activity for chronic weight management in adults with an initial body mass index (BMI) of ≥ 30 kg/m² (obesity) or ≥ 27 kg/m² (overweight) in the presence of at least one weight-related comorbid condition (e.g., hypertension, type 2 diabetes mellitus, or dyslipidemia).

Limitations of Use

- Wegovy™ contains semaglutide and should not be coadministered with other semaglutide-containing products or with any GLP-1 receptor agonist.
- The safety and effectiveness of Wegovy™ in combination with other products intended for weight loss, including prescription drugs, over-the-counter drugs, and herbal preparations, have not been established.
- Wegovy™ has not been studied in patients with a history of pancreatitis.

Important Safety Information

WARNING: RISK OF THYROID C-CELL TUMORS

- In rodents, semaglutide causes dose-dependent and treatment-duration-dependent thyroid C-cell tumors at clinically relevant exposures. It is unknown whether Wegovy™ causes thyroid C-cell tumors, including medullary thyroid carcinoma (MTC), in humans as human relevance of semaglutide-induced rodent thyroid C-cell tumors has not been determined.
- Wegovy™ is contraindicated in patients with a personal or family history of MTC or in patients with Multiple Endocrine Neoplasia syndrome type 2 (MEN 2). Counsel patients regarding the potential risk for MTC with the use of Wegovy™ and inform them of symptoms of thyroid tumors (e.g. a mass in the neck, dysphagia, dyspnea, persistent hoarseness). Routine monitoring of serum calcitonin or using thyroid ultrasound is of uncertain value for early detection of MTC in patients treated with Wegovy™.

Contraindications

- Wegovy™ is contraindicated in patients with a personal or family history of MTC or in patients with MEN 2, and in patients with a prior serious hypersensitivity reaction to semaglutide or to any of the excipients in Wegovy™. Serious hypersensitivity reactions, including anaphylaxis and angioedema have been reported with semaglutide.

Warnings and Precautions

- **Risk of Thyroid C-Cell Tumors:** Patients should be further evaluated if serum calcitonin is measured and found to be elevated or thyroid nodules are noted on physical examination or neck imaging.
- **Acute Pancreatitis:** Acute pancreatitis, including fatal and non-fatal hemorrhagic or necrotizing pancreatitis, has been observed in patients treated with GLP-1 receptor agonists, including semaglutide. Acute pancreatitis was observed in patients treated with Wegovy™ in clinical trials. Observe patients carefully for signs and symptoms of acute pancreatitis (including persistent severe abdominal pain, sometimes radiating to the back, and which may or may not be accompanied by vomiting). If acute pancreatitis is suspected, discontinue Wegovy™ promptly, and if acute pancreatitis is confirmed, do not restart.
- **Acute Gallbladder Disease:** In clinical trials, cholelithiasis was reported by 1.6% of Wegovy™ patients and 0.7% of placebo patients. Cholecystitis was reported by 0.6% of Wegovy™ patients and 0.2% of placebo patients. If cholelithiasis is suspected, gallbladder studies and appropriate clinical follow-up are indicated.
- **Hypoglycemia:** Wegovy™ lowers blood glucose and can cause hypoglycemia. In a trial of patients with type 2 diabetes, hypoglycemia was reported in 6.2% of Wegovy™ patients versus 2.5% of placebo patients. Patients with type 2 diabetes taking Wegovy™ with an insulin secretagogue (e.g. sulfonylurea) or insulin may have an increased

risk of hypoglycemia, including severe hypoglycemia. Inform patients of the risk of hypoglycemia and educate them on the signs and symptoms. Monitor blood glucose in patients with type 2 diabetes.

- **Acute Kidney Injury:** There have been postmarketing reports of acute kidney injury and worsening of chronic renal failure, which in some cases required hemodialysis, in patients treated with semaglutide. Patients with renal impairment may be at a greater risk of acute kidney injury, but some events have been reported in patients without known underlying renal disease. A majority of the events occurred in patients who experienced nausea, vomiting, or diarrhea, leading to volume depletion. Monitor renal function when initiating or escalating doses of Wegovy™ in patients reporting severe adverse gastrointestinal reactions and in patients with renal impairment reporting any adverse reactions that could lead to volume depletion.
- **Hypersensitivity:** Serious hypersensitivity reactions (e.g., anaphylaxis, angioedema) have been reported with semaglutide. If hypersensitivity reactions occur, discontinue use of Wegovy™, treat promptly per standard of care, and monitor until signs and symptoms resolve. Use caution in a patient with a history of anaphylaxis or angioedema with another GLP-1 receptor agonist.
- **Diabetic Retinopathy Complications in Patients with Type 2 Diabetes:** In a trial of patients with type 2 diabetes, diabetic retinopathy was reported by 4.0% of Wegovy™ patients and 2.7% of placebo patients. Rapid improvement in glucose control has been associated with a temporary worsening of diabetic retinopathy. Patients with a history of diabetic retinopathy should be monitored for progression of diabetic retinopathy.
- **Heart Rate Increase:** Mean increases in resting heart rate of 1 to 4 beats per minute (bpm) were observed in Wegovy™ patients compared to placebo in clinical trials. More Wegovy™ patients compared with placebo had maximum changes from baseline of 10 to 19 bpm (41% versus 34%) and 20 bpm or more (26% versus 16%). Monitor heart rate at regular intervals and instruct patients to report palpitations or feelings of a racing heartbeat while at rest. If patients experience a sustained increase in resting heart rate, discontinue Wegovy™.
- **Suicidal Behavior and Ideation:** Suicidal behavior and ideation have been reported in clinical trials with other weight management products. Monitor patients for depression, suicidal thoughts or behavior, and/or any unusual changes in mood or behavior. Discontinue Wegovy™ in patients who experience suicidal thoughts or behaviors and avoid in patients with a history of suicidal attempts or active suicidal ideation.

Adverse Reactions

- The most common adverse reactions reported in $\geq 5\%$ of patients treated with Wegovy™ are nausea, diarrhea, vomiting, constipation, abdominal pain, headache, fatigue, dyspepsia, dizziness, abdominal distention, eructation, hypoglycemia in patients with type 2 diabetes, flatulence, gastroenteritis, and gastroesophageal reflux disease.

Drug Interactions

- The addition of Wegovy™ in patients treated with insulin has not been evaluated. When initiating Wegovy™, consider reducing the dose of concomitantly administered insulin secretagogues (such as sulfonylureas) or insulin to reduce the risk of hypoglycemia.
- Wegovy™ causes a delay of gastric emptying and has the potential to impact the absorption of concomitantly administered oral medications. Monitor the effects of oral medications concomitantly administered with Wegovy™.

Use in Specific Populations

- **Pregnancy:** May cause fetal harm. When pregnancy is recognized, discontinue Wegovy™. Discontinue Wegovy™ in patients at least 2 months before a planned pregnancy.

Click here to see the Prescribing Information, including Boxed Warning.

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Mitochondrial Pyruvate Carriers are not Required for Adipogenesis but are Regulated by High-Fat Feeding in Brown Adipose Tissue

Jasmine A. Burrell^{1,2}, Allison J. Richard², William T. King³, and Jacqueline M. Stephens^{1,2}

Objective: The objectives of this study were to assess the role of mitochondrial pyruvate carriers (MPCs) in adipocyte development *in vitro* and determine whether MPCs are regulated *in vivo* by high-fat feeding in male and female C57BL/6J mice.

Methods: This study utilized small interfering RNA-mediated knockdown to assess the requirement of MPC1 for adipogenesis in the 3T3-L1 model system. Treatment with UK-5099, a potent pharmacological MPC inhibitor, was also used to assess the loss of MPC activity. Western blot analysis was performed on adipose tissue samples from mice on a low-fat diet or a high-fat diet (HFD) for 12 weeks.

Results: The loss of MPC expression via small interfering RNA-mediated knockdown or pharmacological inhibition did not affect adipogenesis of 3T3-L1 cells. *In vivo* studies indicated that expression of MPCs was significantly decreased in brown adipose tissue of male mice, but not female, on an HFD.

Conclusions: Although MPCs are essential for pyruvate transport, MPCs are not required for adipogenesis *in vitro*, suggesting that other substrates can be used for energy production when the MPC complex is not functional. Also, a significant decrease in MPC1 and 2 expression occurred in brown fat, but not white fat, of male mice fed an HFD.

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Introduction

The mitochondrial pyruvate carriers (MPCs) are transmembrane proteins, found on the inner mitochondrial membrane, that transport pyruvate from the cytosol into the mitochondrial matrix where pyruvate is oxidized to acetyl coenzyme A (acetyl CoA) and carbon dioxide via the pyruvate dehydrogenase complex. Acetyl CoA is utilized in the citric acid cycle to form reduced nicotinamide adenine dinucleotide (NADH) for oxidative phosphorylation to create a proton gradient for the production of ATP via the ATP synthase complex. There are two MPC proteins in mammals (MPC1 and MPC2), but there are three that have been identified in *Saccharomyces cerevisiae* (MPC1, MPC2, and MPC3) (1). In mammals, MPC1 and MPC2 form a heterodimer to actively transport pyruvate into the mitochondria (2). Because MPC1 and MPC2 act in a codependent manner, the loss or deletion of one of these proteins results in the loss of the other MPC because of destabilization (2–4). The study

of MPC1 mutants has confirmed that dimerization of MPC1 and MPC2 in the inner mitochondrial membrane is essential for pyruvate uptake into the mitochondria (3).

MPC1 expression may also play a role in cancer metabolism as inhibition of MPC complex formation, via UK-5099, hinders pyruvate's entry into the mitochondria and is associated with increased glycolysis (5). Increased glycolysis is a distinct metabolic feature of cancer cells, along with mitochondrial reprogramming and decreased lipid metabolism (6). The MPC inhibitor 2-Cyano-3-(1-phenyl-1H-inodl-3-yl)-2-propenic acid (UK-5099) binds to MPCs, which deactivates the MPC complex and inhibits pyruvate oxidation (7). Thiazolidinediones inhibit MPCs in several cell types, and in skeletal muscle, inhibition of MPCs is associated with increased glucose uptake and increased insulin sensitivity. Using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system, a heterozygous

Study Importance

What is already known?

- ▶ Mitochondrial pyruvate carriers (MPCs) are essential for pyruvate to be transported into the mitochondria and utilized as substrate by the pyruvate dehydrogenase complex for energy production.

What does this study add?

- ▶ This study is novel because it demonstrates that MPCs are not required for the adipogenesis of 3T3-L1 cells, suggesting that other substrates may compensate for energy production during *in vitro* adipogenesis.
- ▶ This study is also novel because it shows that MPCs are regulated by diet-induced obesity in brown adipose tissue.

¹ Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA. Correspondence: Jacqueline Stephens (jsteph1@lsu.edu)

² Adipocyte Biology Laboratory, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA ³ Integrated Physiology and Molecular Medicine Laboratory, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA.

MPC1 knockdown model was generated, and these mice had reduced lipid accumulation, increased lipolysis, enhanced fatty acid oxidation, and decreased energy expenditure (8). During adipogenesis, mitochondrial density is increased by 20- to 30-fold along with increases in mitochondrial gene expression and oxidative capacity to meet increasing energy requirements (9-13). Insufficient or reduced mitochondrial density in tissues with a high energy demand (such as the brain, heart, and muscles) and endocrine organs (such as adipose tissue) is associated with disease states (14). Although MPCs have been studied in several cell and tissue types, the role of these proteins in adipocyte development and their regulation in conditions of obesity are not known.

In our novel studies, we examined the requirement of MPC1 during adipocyte development using two independent approaches. Both pharmacological inhibition of MPCs as well as small interfering RNA (siRNA)-mediated knockdown of MPC1 demonstrated that the expression and/or activity of MPC1 was not required for adipogenesis in 3T3-L1 cells. To our knowledge, this is the first siRNA-mediated MPC1 knockdown reported in 3T3-L1 cells. However, siRNA-mediated knockdown of MPC1 and MPC2 in 832/13 β -cells resulted in impaired insulin secretion in response to glucose and reductions in glucose-stimulated oxygen consumption (15). We also examined the modulation of MPC expression in both brown (BAT) and white adipose tissue (WAT) depots of male and female mice in a rodent model of diet-induced obesity (DIO). Expression levels of MPC1 and MPC2 were substantially decreased in the BAT of male mice following high-fat feeding, but not in female mice under the same conditions. In conclusion, our results indicate that loss of MPC1 does not have any effect on adipogenesis of 3T3-L1 preadipocytes, MPC1 and MPC2 are highly expressed in BAT, and MPC1 and MPC2 are modulated by high-fat feeding in BAT of male mice.

Methods

Animals and diets

Four-week-old male and female C57BL/6J mice were purchased from Jackson Laboratories (Stock #000664; Bar Harbor, Maine). Animals were housed in a temperature-controlled ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity-controlled (45%-55%) room under a 12-hour light/dark cycle. At 6 weeks of age, mice were placed on respective diets. Mice were allowed ad libitum access to food and water. For 12 weeks, mice had access to either a low-fat diet (LFD) containing 20% of kilocalories from protein, 70% of kilocalories from carbohydrate, and 10% of kilocalories from fat (D12450J; Research Diets, Inc., New Brunswick, New Jersey) or a high-fat diet (HFD) containing 20% of kilocalories from protein, 20% of kilocalories from carbohydrate, and 60% of kilocalories from fat (D12492; Research Diets). Body weights were obtained biweekly. Mice were fasted for 4 hours prior to euthanasia. All animal studies were performed with approval from the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

Animal body composition and glucose tolerance measurements

Nonfasting body composition was measured by nuclear magnetic resonance (NMR) (Bruker Minispec, Bruker BioSpin Corp., Billerica, Massachusetts) at 6 weeks of age (before beginning LFD or HFD intervention) and at 19 weeks of age (after 12 weeks of LFD or HFD feeding). Adiposity was calculated as total fat mass $\div$ total body weight $\times 100$. Following a 4-hour fast, an intraperitoneal glucose tolerance test (GTT) was performed on each animal at 17 weeks of age (10

weeks on diet). A baseline blood glucose measurement was obtained via tail nick (0 minutes), and animals were then injected with 2.5 g/kg of glucose. Blood glucose measurements were obtained via a drop of blood collected from the tail vein at 20, 40, 60, and 120 minutes post injection. Blood glucose measurements were performed using a Breeze 2 glucometer (Bayer, Parsippany, New Jersey).

Cell culture

Murine 3T3-L1 preadipocytes were grown in DMEM (Sigma-Aldrich, St. Louis, Missouri) with 10% bovine calf serum. Two days after confluence, the preadipocytes were induced to differentiate using a standard protocol and induction cocktail composed of MDI cocktail (3-isobutyl-1-methylxanthine [IBMX], dexamethasone, insulin) and 10% characterized fetal bovine serum (FBS) in DMEM. HyClone bovine calf serum and FBS were purchased from Thermo Scientific (Waltham, Massachusetts) or GE Healthcare Life Sciences (Marlborough, Massachusetts). The medium was changed every 48 to 72 hours during growth and differentiation.

Pharmacological inhibitor (UK-5099) treatments

3T3-L1 preadipocytes were trypsinized and seeded into six-well plates at a density of 5.8×10^5 cells/cm² in antibiotic-free 10% bovine calf/DMEM when approximately 70% confluent in 10-cm plates. 3T3-L1 preadipocytes were treated with 10 μM UK-5099 (Sigma-Aldrich; PZ0160) added to the medium upon seeding. Preadipocytes were induced to differentiate as previously described. Cells were fed with antibiotic-free media and treated with UK-5099 every 48 hours. Seven days after the induction of differentiation, the cell monolayers were harvested for protein in immunoprecipitation (IP) buffer containing 10mM Tris (pH 7.4), 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% Triton X-100, 0.5% IGEPAL CA-630 (octylphenoxypolyethoxyethanol), protease inhibitors (1mM phenylmethylsulfonyl fluoride [PMSF], 1 μM pepstatin, 50 trypsin inhibitory units of aprotinin, 10 μM leupeptin, and 1mM 1,10-phenanthroline), and phosphatase inhibitors (0.2mM sodium vanadate and 100 μM sodium fluoride) and were harvested for RNA in buffer provided in the RNeasy Mini Kit (Qiagen, Hilden, Germany) to assess knockdown efficiency. Three biological and technical replicates were analyzed for each dose. All reagents used to make the IP buffer were from Sigma-Aldrich.

Cytotoxicity and cell viability assays

The ToxiLight BioAssay Kit (Lonza, Cologne, Germany) was utilized according to the manufacturer's protocol. ToxiLight 100% lysis reagent (Lonza) was utilized as a positive control. Cell viability was assessed by counting trypan blue-stained cells using a hemacytometer.

Whole-cell extract preparation

Cell monolayers were rinsed once with phosphate-buffered saline (PBS) and then scraped into non-denaturing IP buffer. The whole-cell extracts were stored at -80°C before being thawed and passed through a 20-gauge needle five times. The whole-cell extract was clarified via centrifugation at 13,000g for 10 minutes at 4°C .

siRNA-mediated knockdown

3T3-L1 preadipocytes were trypsinized and replated in six-well plates at a density of 5.8×10^5 cells/cm² in antibiotic-free 10% bovine calf/DMEM when approximately 70% confluent in 10-cm plates. Using the protocol from Dharmacon, preadipocytes were transfected with 33nM siRNA (Dharmacon, Lafayette, Colorado; nontargeting siRNA Cat#: D-001810-10-50, siRNA targeting MPC1 Cat#: L-040908-01-0005) and

the DharmaFECT Duo transfection reagent (Dharmacon; Cat#: T-2010-03) in Opti-MEM reduced serum medium (Thermo Fisher, Waltham, Massachusetts; Cat#: 31985088). Nontargeting siRNA was used as a negative control. Cells were treated with the siRNA cocktail during initial plating and grown to confluence. Two days after confluence, cells were induced to differentiate with the MDI induction cocktail, as previously described, and transfected again with the siRNA cocktail. After 48 hours, the cells were treated with one-quarter normal dose of insulin and transfected once again with the siRNA cocktail. Cells were fed every 48 hours with antibiotic-free media throughout the entire knockdown process. Seven days after the induction of differentiation, the cell monolayers were harvested for protein in IP buffer and for RNA in buffer provided in the RNeasy Mini Kit to assess knockdown efficiency. Three biological and technical replicates were analyzed for each dose.

Respirometry

Mitochondrial function was assessed by respirometry of intact cells using a Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, California). Mature 3T3-L1 adipocytes were seeded at 1.0×10^5 cells per well in an XF24 specialized cell culture microplate that was coated with 0.1% gelatin for 48 hours prior to seeding. Prior to measuring oxygen consumption, cells were incubated in XF base media, pH 7.4 (Agilent Technologies) supplemented with 25mM glucose and 2mM L-glutamine at 37°C without CO₂ for 1 hour. After basal oxygen consumption was measured for three cycles, 1mM sodium pyruvate $\pm$ 10 μ M UK-5099 was injected into each well. After three additional measurement cycles, 1 μ M oligomycin, 600nM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and 5 μ M rotenone/antimycin A were serially injected, and oxygen consumption was measured for three, six, and three cycles, respectively. After the assay was complete, cells were harvested in radioimmunoprecipitation assay (RIPA) buffer, and protein concentration was quantified using the bicinchoninic acid (BCA) assay kit (Sigma-Aldrich; Cat#: BCA1). Oxygen consumption rate was normalized to micrograms of protein.

Gel electrophoresis and immunoblotting

Protein content of cell extracts was quantified via BCA assay. Samples were separated on 7.5%, 12%, or 15% sodium dodecyl sulfate (SDS) polyacrylamide gels (acrylamide; National Diagnostics, Atlanta, Georgia; Cat#: EC-890) and transferred to nitrocellulose membranes (BioRad, Hercules, California; Cat#: 162-0115) in 25mM Tris, 192mM glycine, and 20% methanol. After the transfer, membrane strips were blocked in 4% nonfat milk for 1 hour at room temperature and washed with Tris-buffered saline - Tween 20 (TBS-T) before incubating with primary antibodies overnight at 4°C. Strips were washed with TBS-T and then incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania) for 1 hour. Strips were washed with TBS-T and visualized with enhanced chemiluminescence (Pierce/Thermo Scientific, Waltham, Massachusetts).

Antibodies

Anti-STAT (signal transducer and activator of transcription) 5A (L-20; sc-1081; rabbit polyclonal), anti-adipsin (M-120; sc-50419; rabbit polyclonal), anti-STAT3 (C-20; sc-482; rabbit polyclonal), and anti-ERK (extracellular signal-regulated kinase) 1/2 (C-16; sc-93; rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas). Anti-adiponectin (PA1-054; rabbit polyclonal) antibody was purchased from Thermo Scientific. Anti-MPC1 (14462S; rabbit monoclonal) and anti-MPC2 (D417G; rabbit monoclonal) antibodies were purchased from Cell Signaling Technology (Danvers, Massachusetts).

RNA analysis

Total RNA from tissue samples was extracted according to TRIzol manufacturer instructions (Ambion, Carlsbad, California; Cat#:15596018). RNA from tissue or adipocyte monolayers was purified using the RNeasy Mini Kit. Ten microliters of purified RNA was used for reverse transcription to generate complementary DNA (cDNA) according to the Applied Biosystems protocol (Applied Biosystems, Foster City, California; Cat#: 4368813). Complementary DNA was quantified using the real-time quantitative polymerase chain reaction (qPCR) method in a total volume of 10 μ L (2 μ L DNA and 8 μ L reaction master mix) using an Applied Biosystems 7900HT system with SDS 2.4 software. qPCR was performed using Takara SYBR Premix (Takara Bio USA Inc., Madison, Wisconsin) and primers from Integrated DNA Technologies (Skokie, Illinois). Thermal cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C; the dissociation stage was 15 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 95°C. Cyclophilin A (*Ppia*; also known as peptidylprolyl isomerase A) and non-POU domain containing octamer binding protein (*Nono*) were used as reference genes. The following mouse genes were examined by reverse transcription (RT)-qPCR: oxoglutarate dehydrogenase (*Ogdh*), succinate dehydrogenase complex iron sulfur subunit B (*Sdhb*), *Mpc1*, *Mpc2*, CCAAT/enhancer binding protein, alpha (*Cebpa*), peroxisome proliferator-activated receptor gamma (*Pparg*), adiponectin (*Adpn*), citrate synthase (*Cs*), fatty acid binding protein 4 (*Ap2*), fatty acid synthase (*Fas*; also known as TNF receptor superfamily member 6), and adipisin (*Cfd*). Primer sequences are shown in Table 1.

Lipid accumulation measurement

Seven days after the induction of differentiation, cells were fixed and stained with Oil Red O (Sigma-Aldrich) as described previously (16).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8; La Jolla, California). Differences between groups were calculated using *t* tests and two-way ANOVA. Area under the curve (AUC) calculations and standard linear regression analyses were performed to determine correlations between MPC protein expression and adiposity or GTT AUC. Results from studies of cultured adipocytes are shown as mean $\pm$ SEM. Results were considered statistically significant when $P < 0.05$.

Results

Loss of MPC1 has no effect on *in vitro* adipogenesis

Prior to determining the potential requirement for MPC1 expression for adipocyte differentiation, we examined the protein expression of MPC1 and MPC2 over a 7-day adipogenesis time course in 3T3-L1 cells. As shown in Figure 1, both MPC1 and MPC2 protein expressions were highly induced during differentiation. The increased expression was apparent 4 days after the induction of adipogenesis (Figure 1A-1B). To assess the requirement of MPCs on adipogenesis, we used a potent pharmacological inhibitor of MPCs, UK-5099, which acts by binding to MPCs and modifying a thiol group to prevent the formation of the MPC1 and MPC2 heterodimer, thus reducing pyruvate transport into the mitochondrial matrix (17,18). Preadipocytes were induced to differentiate in the presence of UK-5099 at various doses. As shown in Figure 2, a range of inhibitor doses did not inhibit adipocyte differentiation as judged by lipid accumulation with Oil Red O staining (Figure 2A) or adipocyte marker gene expression (Figure 2B). Three adipogenic markers (*Adpn*, *Ap2*, *Fas*) were examined to demonstrate adipocyte development.

TABLE 1 qPCR primer sequences

Gene	Primer 1, 5'-3'	Primer 2, 5'-3'
Cyclophilin A (<i>Ppia</i>)	CCACTGTGCTTTTCGCCGC	TGCAACAGCTCGAAGGAGACGC
Non-POU domain containing octamer binding protein (<i>Nono</i>)	CATCATCAGCATACCACCA	TCTTCAGGTCAATAGTCAAGCC
Oxoglutarate dehydrogenase (<i>Ogdh</i>)	ATGGGAAGACCAAGCTGA	CCATGCAGCAGGATAGACAT
Succinate dehydrogenase complex, subunit B, iron sulfur (<i>Sdhb</i>)	GGAGGGCAAGCAACAGTATC	GCACACAGGATGCACTCGTA
Mitochondrial pyruvate carrier 1 (<i>Mpc1</i>)	CAAGGACTTCGGGACTATC	CATCCGCCCACTGATAATCTC
Mitochondrial pyruvate carrier 1 (<i>Mpc2</i>)	CCGACTCATGGATAAAGTGGAG	CTAGTCCAGCACACACCAAT
Adiponectin (<i>Adpn</i>)	AAAAGGGCTCAGGATGCTACTG	TGGGCAGGATTAAGAGGAACA
Citrate synthase (<i>Cs</i>)	AGAACTCATCCTGCCTCGT	CCTGCTCCTTAGGTATCAGATTG
Fatty acid binding protein 4 (<i>Ap2</i>)	CCCTCCTGTGCTGCAGCCTTT	GTGGCAAAGCCCACTCCCATT
Fatty acid synthase (<i>Fas</i>)	GGCATATTGGGCACTCCTT	ACCACCAGCTGCCATGGATC
Adipsin (<i>Cfd</i>)	CGAGGCCGATTCTGGG	GAGTCGTATCCGTCACCTCC
CCAAT/enhancer binding protein, alpha (<i>Cebpa</i>)	ACAAGAACAGCAACGAGTACC	TCATTGTCACTGGTCAACTCC
Peroxisome proliferator-activated receptor gamma (<i>Pparg</i>)	CGAGTGGTCTTCATCACGG	TCACAAGAAATTACCATGGTTGAC

Figure 1 MPC1 and MPC2 expression are induced during adipogenesis in 3T3-L1 cells. 3T3-L1 adipocytes were induced to differentiate using the 3-isobutyl-methylxanthine, dexamethasone, insulin (MDI) cocktail, and whole-cell extracts were harvested at the indicated time points to assess MPC1 protein expression over a time course of adipocyte differentiation. (A) Whole-cell extracts (75 μ g of protein per lane) were subjected to Western blot analysis. Adiponectin was utilized as a positive control for adipogenesis. (B) Densitometry quantification of MPC1 and MPC2 band intensities at the indicated time points. MPC1 and MPC2 band intensities were normalized to ERK 1/2 ($n=3$ pooled 10-cm plates per time point). Experiments were repeated three times on independent batches of cells.

To examine the efficacy of UK-5099, respirometry experiments were performed on 3T3-L1 cells to measure oxygen consumption in the presence of pyruvate with or without the addition of the inhibitor. There were no differences in oxygen consumption observed during baseline readings after the addition of pyruvate $\pm$ UK-5099 or oligomycin. After the addition of FCCP, the maximal oxygen consumption rates in 3T3-L1 cells treated with the inhibitor were significantly reduced compared with the control (1.167 vs. 3.411 pmol/min/ μ g protein) (Figure 2C-2D). Oxygen consumption rates were reduced by UK-5099 in the presence of the mitochondrial uncoupler, FCCP, likely because of a reduction of pyruvate substrate entry into the mitochondria. Cell viability and cytotoxicity assays show that UK-5099-treated 3T3-L1 cells had no changes in cytotoxicity (Figure 2E) or cell viability (Figure 2F) in comparison with dimethyl sulfoxide (DMSO), which indicates that UK-5099 has no toxic effects on the cells.

An independent approach using siRNA-mediated knockdown of MPC1 was also performed to examine the role of MPCs on adipogenesis in

3T3-L1 cells. Despite substantial loss of MPC1 protein expression (Figure 3A-3B), there were no observed changes in adiponectin protein expression or lipid accumulation (Figure 3A-3C). Though *Mpc1* gene expression was significantly reduced, mRNA expression of *Mpc2* and adipogenic markers, such as *Adpn*, *Ap2*, and *Pparg*, was not affected (Figure 3D). Although *Cebpa* gene expression increased in the absence of MPC1, this increase did not affect adipogenesis. These data confirm that loss of MPC1 expression does not have any significant effects on adipogenesis of 3T3-L1 cells.

MPCs are regulated in adipose tissue of mice during high-fat feeding

To further study MPCs, we examined MPC1 and MPC2 protein expression in several adipose tissue depots and compared it with other tissues in mice. As shown in Figure 4, MPC1 and MPC2 were highly expressed in BAT, heart, liver, and kidneys in C57BL/6J mice. When directly compared, it was apparent that there were differences in protein

Figure 2 UK-5099, a pharmacological inhibitor of MPC1, does not inhibit adipogenesis of 3T3-L1 cells. **(A,B)** 3T3-L1 preadipocytes were induced to differentiate either untreated or treated with DMSO vehicle or indicated dose of UK-5099 at the time of induction and every 2 days during differentiation. **(A)** Seven days post induction, cells were fixed and stained with Oil Red O. Photographs are from the same experiment, and wells ($n=3$ wells per treatment) were treated at the same time. **(B)** Seven days post induction, RNA was isolated, purified, and subjected to RT-qPCR to measure gene expression of *Mpc1*, adipogenic markers (adiponectin [*Adpn*], fatty acid binding protein 4 [*Ap2*], and fatty acid synthase [*Fas*]), and the mitochondrial marker citrate synthase (*Cs*) ($n=3$ wells per treatment). Target gene expression was normalized to the reference gene, *Nono*, and data are plotted as fold change over the DMSO control. **(C,D)** Respirometry experiments were performed to demonstrate the efficacy of the MPC1 inhibitor in mature 3T3-L1 adipocytes. After introducing 1mM pyruvate $\pm$ 10 μ M UK-5099, the oxygen consumption rate (OCR) was measured using a Seahorse XF analyzer for three cycles prior to performing the mitochondrial stress test using 1 μ M oligomycin (Oligo), 600nM FCCP, and 5 μ M rotenone (Rot)/antimycin A (AA). **(C)** Full respiration profile showing the mean OCR normalized by the protein content $\pm$ SEM for each well ($n=10$ wells per treatment). **(D)** Maximal respiration after the addition of 600nM FCCP ($n=10$ wells per treatment). Data shown are mean OCR of cells treated with pyruvate (P) or pyruvate and UK-5099 (P+I). Panels C and D were analyzed by unpaired t test. **** $P < 0.0001$ vs. pyruvate control. Experiments were repeated at least twice on independent batches of cells. **(E,F)** Seven days post induction, UK-5099-treated 3T3-L1 cells were either **(E)** subjected to cytotoxicity assay via ToxiLight BioAssay Kit or **(F)** stained with trypan blue and counted to assess cell viability at indicated doses ($n=3$ wells per treatment for a single batch of cells).

expression of MPC1 and MPC2 in some tissues. MPC1 had a much higher protein expression than MPC2 in the skeletal muscle, in both the extensor digitorum longus and the gastrocnemius, and in the brain (Figure 4). Also, the levels of MPC1 and MPC2 expression were low in all of the WAT depots (Figure 4).

To determine whether MPC expression was regulated by DIO, mice were placed on either LFD or HFD for 12 weeks. BAT, inguinal WAT (iWAT), and gonadal/epididymal WAT (gWAT) were harvested and analyzed in both male and female mice on LFD or HFD. In both male and female mice, *Mpc1* mRNA expression was significantly decreased in the BAT, iWAT, and gWAT during high-fat feeding of male and female mice, while *Mpc2* mRNA expression was decreased in the gWAT of males and the BAT and gWAT of females during high-fat feeding (Figure 5). We also observed an expected decrease in *Fas* gene expression during high-fat feeding (19). Protein expression of both

MPC1 and MPC2 in BAT was significantly reduced in male mice on an HFD (Figure 6A-6B). This significant decline in MPC1 and MPC2 levels was not observed in the female mice fed an HFD (Figure 6C-6D). In iWAT, MPC1 protein expression was extremely low, near the detection limit, and no changes in protein levels were observed as a result of HFD feeding (data not shown). These data indicate that *MPC* gene expression is downregulated in a depot and sex-specific manner by high-fat feeding.

Because female C57BL/6 mice are typically less susceptible to metabolic dysfunction during HFD-induced obesity than their male counterparts (20-22), we examined the relationships between MPC1 or MPC2 protein expression and adiposity or glucose tolerance. Protein expression of MPC1 and MPC2 for males significantly correlated with adiposity and GTT AUC for male, but not female, mice (Figure 7). For the male mice, the linear fits of MPC1 or MPC2 versus

Figure 3 Knockdown of *Mpc1* gene expression with siRNA does not inhibit adipogenesis of 3T3-L1 cells. 3T3-L1 preadipocytes were transfected with nontargeting (NT) siRNA or MPC1 siRNA upon plating and every 48 hours following induction of differentiation with MDI cocktail until end point assessments. Protein and gene expression assessments as well as Oil Red O staining were conducted on the cells at 7 days post MDI. (A) Whole-cell extracts were isolated and 50 μ g of protein per lane was analyzed by Western blot analysis ($n=3$ replicate wells per treatment). Adiponectin was utilized as a differentiated adipocyte marker. (B) MPC1 band intensities were quantified by densitometry and normalized to ERK 1/2 for each treatment. (C) Cells were fixed and stained with Oil Red O to examine lipid accumulation. (D) RNA was isolated, purified, and subjected to RT-qPCR to show gene expression of *Mpc1*, *Mpc2*, adipogenic markers (adiponectin [*Adpn*], fatty acid binding protein 4 [*Ap2*], CCAAT/enhancer binding protein, alpha [*Cebpa*], and peroxisome proliferator-activated receptor gamma [*Pparg*]), and the mitochondrial marker (citrate synthase [*Cs*], oxoglutarate dehydrogenase [*Ogdh*], and succinate dehydrogenase complex, subunit B, iron sulfur [*Sdhb*]). Data were analyzed by two-way ANOVA. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. NT control ($n=3$ wells per treatment).

Figure 4 MPC1 and MPC2 proteins are highly expressed in brown adipose tissue, liver, and heart. Select tissues were harvested from a 27-week-old C57BL/6J mouse to assess the tissue distribution of MPC1 and MPC2 proteins. (A) Tissue samples were homogenized, and 100 μ g of total protein per lane was analyzed by Western blotting. (B) MPC1 and MPC2 band intensities were quantified by densitometry and normalized to respective ERK 1/2 intensities for each tissue depot. BAT, brown adipose tissue; EDL, extensor digitorum longus; gastroc, gastrocnemius; gWAT, gonadal white adipose tissue (WAT); iWAT, inguinal WAT; mWAT, mesenteric WAT; rpWAT, retroperitoneal WAT.

adiposity or GTT AUC had an R^2 value of greater than 0.5, and the slopes were significantly nonzero ($P<0.01$), whereas female mice R^2 values were less than 0.4, and none of the slopes was significantly

nonzero. These data show that decreased levels of MPC proteins in male mice were associated with increased adiposity and decreased glucose tolerance.

Figure 5 Steady-state mRNA expression of *Mpc1* is substantially decreased in adipose tissue after high-fat feeding in male and female C57BL/6J mice. Six-week-old C57BL/6J mice were fed either an HFD or LFD (low fat, LF) for 12 weeks ad libitum before euthanizing and collecting tissue. Brown adipose tissue (BAT), inguinal white adipose tissue (iWAT), and gonadal white adipose tissue (gWAT) samples were homogenized, and RNA was isolated and purified from each tissue depot for (A) male and (B) female mice. Each sample was subjected to RT-qPCR to show gene expression of *Mpc1*, *Mpc2*, adipogenic markers (*Adpn*, *Ap2*, and *Fas*), adipokine (*Adipsin*), and the mitochondrial marker (*Cs*). Data were analyzed by unpaired *t* tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 vs. LFD (*n* = 6 per condition). Each sample was run in duplicate. Target gene expression was normalized to the reference gene, *Ppia*, and data are plotted as fold change over the LF control.

Figure 6 MPC1 and MPC2 expression is significantly decreased in the BAT of male mice, but not female, following high-fat feeding. Six-week-old male and female C57BL/6J mice were fed either a high-fat diet (HFD) or low-fat diet (LFD) for 12 weeks ad libitum before euthanizing and collecting BAT. Tissue samples were homogenized, and 30 μ g of total protein per lane was subjected to Western blot analysis (*n* = 6 per condition) for (A) males and (C) females. MPC1 and MPC2 band intensities were quantified by densitometry and normalized to respective STAT3 intensities for each sample; (B) males and (D) females. Data were analyzed by two-way ANOVA. ***P* < 0.01 vs. LFD.

Figure 7 Decreased MPC1 and MPC2 protein expression in BAT during DIO correlates with increased adiposity and decreased metabolic health in male, but not female, mice. (A,B) MPC protein expression from the immunoblot quantification in Figure 6 was correlated with percent fat mass (adiposity) for male and female mice on LFD and HFD. Protein extracts were collected and adiposity measured at 19 weeks of age (12 weeks on diet). (C,D) MPC protein expression values for male and female mice were also plotted against area under the curve (AUC) of glucose tolerance tests (GTT) performed on LFD- and HFD-fed mice at 17 weeks of age (10 weeks on diet) for male and female mice. Linear regression analyses were performed and are shown. R^2 values indicate goodness of the linear fit for each data set, and whether the slope is significantly differently from zero is denoted by P value or marked “ns” (not significant).

Discussion

Our novel data demonstrate the dispensability of MPC1 during adipogenesis *in vitro* as well as show the expression and modulation of MPCs in BAT *in vivo* during DIO. The observed increase in protein expressions of both MPC1 and MPC2 during adipocyte differentiation of 3T3-L1 cells (Figure 1) has not been previously reported in 3T3-L1 cells, but MPC1 and MPC2 expression was shown to increase upon differentiation of leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5)+ intestinal stem cells (23). The increase in MPC1 and MPC2 expression in adipocytes is likely due to increased mitochondrial density and gene expression that occurs to generate energy needed to accommodate the enhanced metabolic requirements during adipogenesis (9,12,13). Because of enhanced protein expression of MPCs during differentiation, we hypothesized that MPCs were necessary for the differentiation of 3T3-L1 cells.

Contrary to our premise, two independent approaches using both pharmacological inhibition with UK-5099 (Figure 2) and siRNA-mediated

knockdown (Figure 3) of MPCs did not affect adipocyte development. Even at doses as low as 50nM, UK-5099 has been able to inhibit formation of the MPC1 and MPC2 heterodimer, thus inhibiting pyruvate transport via this complex (4,17). In BAT progenitor cells, the addition of UK-5099 inhibited ^{13}C -glucose incorporation into acetyl CoA (17), suggesting that the pyruvate produced from glycolysis of ^{13}C -glucose was not utilized for acetyl CoA production because of impaired pyruvate uptake by the MPC heterodimer via UK-5099 inhibition. Liver-specific loss of MPC2 in C57BL/6J mice resulted in compromised, but not eliminated, pyruvate metabolism and suggested that mechanisms such as pyruvate-alanine cycling were activated to compensate for the loss of the MPC complex functionality (24). A robust decline (~80%) in MPC1 expression via siRNA knockdown also had no effect on adipocyte development (Figure 3). Our data further support the current literature. Our observations suggest that although pyruvate is the primary substrate for energy production in the mitochondria, other substrates, such as alanine, glutamine (2,24,25), or branched chain amino acids (26), might bypass the MPC1/2 complex and enter into the mitochondrial matrix to compensate for the lack of pyruvate transport under

conditions in which MPC1 or MPC2 is not present or functional. A clear limitation of our adipogenesis studies is that they are solely *in vitro* observations. Although our findings show that the loss of MPC expression *in vitro* has no effect on adipocyte development, other *in vivo* studies have found that C57BL/6 mice with an adipocyte-specific loss of MPC1 had increased fatty acid oxidation, increased levels of triglycerides in circulation, deficiencies in storage of triglycerides, and mitochondrial damage. It also has been observed that heterozygous MPC1 knockdown mice had decreased body weight, activity, and fat accumulation and low body shell temperatures during cold exposure (8,27).

An analysis of several mouse tissue samples revealed that MPCs were highly expressed in BAT, heart, lung, and skeletal muscle (Figure 4). This is an expected finding because these are all mitochondria-rich tissues. However, it has not been reported that MPC1 and MPC2 are more highly enriched in BAT than other mitochondria-rich tissues (Figure 4). Because MPCs are highly enriched in BAT depots, but not in WAT depots, we assessed whether MPCs were regulated in adipose tissue depots during DIO. Although MPC1 was downregulated in BAT and WAT during high-fat feeding at the mRNA level (Figure 5), MPC1 protein expression was barely detectable and not obviously changed in the WAT of either male or female mice during high-fat feeding (data not shown). The differences in gene and protein expression could be due to a variety of factors, including translational rates, protein degradation rates, or a combination of both (28). However, it is a novel finding that MPC gene and protein expression is downregulated in BAT of male mice, but not female, during DIO (Figures 5,6). It was reported that reductions in diet-induced thermogenesis contributed to weight gain after consumption of high-fat meals (29). In addition, glucose oxidation rates were significantly decreased in individuals after consuming high-fat meals (29). This literature suggests that the decreased MPC expression correlated with decreased diet-induced thermogenesis that occurs with prolonged high-fat feeding. Also, the sex-specific regulation of MPCs (Figure 6) is likely attributed to sex-specific metabolic responses to high-fat feeding in C57BL/6J mice (21). Unlike male mice, previous studies have shown that female mice were more metabolically healthy, had reduced inflammation, and were more insulin sensitive despite having obesity while on an HFD (20-22). Accordingly, in our study the female mice on an HFD had 27% higher adiposity than the male HFD-fed mice but improved glucose metabolism during an intraperitoneal GTT. Data in Figure 7 demonstrate that decreased MPC protein levels correlate with increased adiposity and glucose intolerance in male, but not female, mice. We speculate that the variations in expression of MPC1 and MPC2 between male and female mice are due to differences in metabolic health and that MPCs are specifically regulated in BAT, but not WAT, depots in conditions of metabolic dysfunction.

In summary, our findings demonstrate that (1) the expression of MPCs is induced during adipocyte development but not required for adipogenesis; (2) a loss of MPC expression or activity during adipogenesis likely promotes the activation of alternative mechanisms to compensate for the loss of pyruvate transport; and (3) MPC expression is highly enriched in BAT but decreased as a result of high-fat feeding or DIO. Future studies will be needed to determine which substrates can be utilized to compensate for the loss or dysfunction of MPCs. In addition, it is necessary to determine whether adipogenesis in BAT is dependent on MPCs. Also, future studies will be necessary to determine whether the observed *in vitro* effects on adipogenesis are similar to *in vivo* models. **O**

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Author contributions: JAB and JMS conceived and designed experiments; JAB, WTK, and AJR performed experiments and analyzed data; JAB, AJR, and JMS interpreted results; JAB prepared figures; JAB and JMS drafted the manuscript; JAB, WTK, AJR, and JMS edited and revised the manuscript; all authors approved the final version of the manuscript.

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