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Journal of Nutritional Biochemistry 120 (2023) 109412

Journal of Nutritional Biochemistry

RESEARCH PAPER

The ketogenic diet promotes triacylglycerol recycling in white adipose tissue and uncoupled fat oxidation in brown adipose tissue, but does not reduce adiposity in rats

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Received 12 December 2022; received in revised form 6 June 2023; accepted 3 July 2023

Abstract

The purpose of this study was to determine whether the weight-reducing and fat burning effects of the ketogenic diet (KD) could be attributed to alterations in the energy dissipating pathways of brown adipose tissue (BAT) uncoupled oxidation, and white adipose tissue (WAT) browning and triacylglycerol (TAG) recycling. To investigate this, male Wistar rats were fed one of the following three diets for either 8 or 16 weeks: a standard chow (SC), a high-fat, sucrose-enriched (HFS) obesogenic diet, or a KD. At the end of the intervention, subcutaneous inguinal (Sc Ing) and epididymal (Epid) fat, and interscapular and aortic BAT (iBAT and aBAT, respectively) were extracted. These tissues were used for the analysis of proteins involved in WAT browning and thermogenesis. Isolated adipocytes from WAT were assayed for basal and isoproterenol (Iso)-stimulated lipolysis and basal and insulin-stimulated lipogenesis, and BAT adipocytes were assayed for the determination of coupled and uncoupled glucose and palmitate oxidation. Adiposity similarly increased in HFS- and KD-fed rats at weeks 8 and 16. However, in HFS-fed animals insulin-stimulated lipogenesis and Iso-stimulated lipolysis were impaired in WAT adipocytes, whereas in KD-fed animals these pathways remained intact. The KD also significantly elevated WAT glycerol kinase levels, and favored TAG recycling under conditions of enhanced lipolysis. In BAT, the KD significantly increased uncoupling protein-1 levels and uncoupled fat oxidation. In summary, the KD preserved insulin sensitivity and lipolytic capacity in WAT and also upregulated energy-dissipating pathways in BAT, but it was not sufficient to prevent an increase in adiposity.

Keywords: Lipolysis; UCP1; Glycerol kinase; Insulin-stimulated lipogenesis; PGC-1α.

1. Introduction

Functional adipose tissue (AT) is crucial for the prevention of insulin resistance and Type 2 diabetes [1]. There are generally two main forms of AT with distinct physiological roles: white and brown. White adipose tissue (WAT) stores energy substrate under conditions of energy surplus, and releases fatty acids and glycerol under conditions of energy deficit [2–4]. On the other hand, brown adipose tissue (BAT) burns glucose and fatty acids and releases the energy as heat [2,4]. Thermogenesis in BAT mainly occurs through uncoupling protein-1 (UCP1), which uncouples oxidative phosphorylation from ATP production [2]. Under conditions of obesity, WAT and BAT functions are altered differently. Diet-induced obesity enhances the thermogenic capacity by increasing UCP1 content and fatty acid oxidation in BAT [3,4]. This serves to counteract energy storage through a mechanism known as diet-induced thermogenesis [3,4]. However, this is not sufficient to prevent the excess storage of fat in white adipocytes and the development of insulin resistance in the WAT [1,5]. In fact, chronically elevated caloric intake overwhelms the hypertrophic capacity of the adipocyte, leading to cellular hypoxia and immune cell infiltration [1,5]. At the level of the WAT, inflammation inhibits insulin signaling, which ends up enhancing basal lipolysis [1], although catecholamine-stimulated lipolysis becomes severely limited under conditions of obesity [6,7]. The dysregulation in lipid storage and breakdown redirects lipids to ectopic tissues such as liver [1] and muscle [1,8]. This is detrimental to insulin sensitivity and metabolism in these tissues, and negatively impacts whole-body glucose homeostasis [1,8]. In this context, interventions that combat adiposity and restore AT function are of great interest for the prevention and treatment of obesity and insulin resistance.

A dietary intervention that exerts anti-obesity and insulin-sensitizing effects is the ketogenic diet (KD). The KD is typically low in carbohydrates and high in fat [9]. Despite being abundant

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https://doi.org/10.1016/j.jnutbio.2023.109412
0955-2863/

in fat, recent evidence shows that 3 months of KD-feeding significantly attenuated body weight (BW) and blood pressure, and improved glycaemia in diabetic women [10]. Furthermore, as little as 6 days of KD significantly reduced intra-hepatic triglycerides in overweight and obese subjects [11]. These promising health effects have prompted investigations into the effects of the KD on cellular metabolism. In BAT and WAT, the KD increases UCP1 content and UCP1 gene expression, respectively [12]. This elevation in WAT UCP1 is characteristic of a process known as browning, whereby WAT acquires structural and functional features that resemble BAT [2,4,13]. Additionally, by lowering circulating insulin levels, the KD favors WAT lipolysis and the release of non-esterified fatty acids (NEFAs) into the bloodstream [14]. NEFAs not undergoing oxidation return to the WAT where they are recycled back to triacylglycerol (TAG). This latter process creates an energy-consuming "futile cycle" that can also contribute to whole-body energy expenditure [2]. Thus, the combined effects of the KD on BAT uncoupled oxidation, WAT browning, and TAG cycling could potentially support the diet-induced shift from energy storage to dissipation in WAT and reduce adiposity. However, the effects of the KD on WAT TAG recycling and BAT uncoupled oxidative capacity, and whether these two mechanisms lead to reduced adiposity, is yet to be confirmed. Therefore, the purpose of our study was to assess KD-induced alterations in these energy-consuming pathways in brown and white adipocytes. We hypothesized that the KD would increase UCP1 content and enhance fat consumption in BAT, whereas in WAT the machinery for TAG recycling would be up-regulated, ultimately contributing to a reduction in adiposity. To test this hypothesis, rats were fed for up to 16 weeks either a high-fat, sucrose enriched (HFS) obesogenic diet or a KD to compare the effects of these diets on WAT and BAT metabolism. To test potential time-dependent effects of the KD on metabolic pathways involved in energy dissipation, we assessed, in BAT and WAT, the thermogenic and TAG recycling machineries at 8 and 16 weeks of the dietary interventions. Here, we show that KD-feeding promoted distinct TAG recycling effects in subcutaneous and visceral WAT and enhanced the capacity of BAT to consume fatty acids. However, the induction of these energy consuming pathways by the KD did not lead to a reduction in adiposity.

2. Materials and methods

2.1. Reagents

Type II collagenase, isoproterenol (Iso), fatty acid-free bovine serum albumin (BSA), palmitic acid, and the free glycerol determination kit were obtained from Sigma (St. Louis, MO, USA). Oligomycin (Oligo) was purchased from Cayman Chemical (Ann Arbor, MI, USA). [1-^{14}C] palmitic acid and D-[U-^{14}C] glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Protease (cOmplete Ultra Tablets) and phosphatase (PhosSTOP) inhibitors were obtained from Roche Diagnostics GmbH (Mannheim, Germany). The β-hydroxybutyrate (BHB; cat. no. ab83390) assay kit, and the UCP1 (ab23841) and glycerol kinase (GyK; cat. no. ab180525) antibodies were purchased from Abcam (Toronto, ON, Canada). β-actin (cat. no. 4967) and adipose triglyceride lipase (ATGL; cat. no. 2138) antibodies were purchased from Cell Signaling (Danvers, MA, USA). The peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α; cat. no. ab3242) was purchased from Millipore (Burlington, MA, USA). The β3-adrenergic receptor (β3AdR; sc-50436) antibody was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA).

Table 1
Fatty acid (FA) profile of the SC, HFS and KD expressed as % of the total fatty acids.

	SC	HFS	KD
Saturated FA	20.8	32.2	32.6
Monounsaturated FA	26.7	35.9	36.2
Polyunsaturated FA	52.5	31.9	31.1

2.2. Animals

Male albino rats from the Wistar strain (Envigo, Indianapolis, IN, USA) weighing 200–250 g (initial weight) were maintained in a constant-temperature (23 °C), with a fixed 12-h light/12-h dark cycle and fed for either 8 or 16 weeks *ad libitum* either a standard chow diet (SC, 27.0 %, 13.0 %, and 60.0 % of calories provided by protein, fat, and carbohydrates, respectively), a HF, sucrose-enriched (HFS) diet (20.0%, 60.0%, and 20.0% of calories provided by protein, fat, and carbohydrates [sucrose], respectively) or a KD (20%, 80%, and 0% of calories provided by protein, fat and carbohydrates, respectively). Caloric densities of the diets were 3.43-, 5.24- and 6.14 kcal/g for the SC, HFS and KD, respectively. The saturated, monounsaturated and polyunsatured fatty acid % contribution to the total fatty acids in each of the diets was 20.8, 26.7, and 52.5% for the SC, 32.2, 35.9, and 31.9% for the HFS diet and 32.6, 36.2, and 31.1 for the KD (Table 1). The SC (standard rat chow, catalog # 5012) was purchased from LabDiet (Richmond, IN, USA). The HFS and ketogenic diets (catalog # D12492 and D03022101, respectively) were purchased from Research Diets Inc. (New Brunswick, NJ, USA). At the end of the feeding period, animals were anesthetized under ketamine/xylazine anesthesia (90 mg and 10 mg/100 g BW, respectively) and the subcutaneous inguinal (Sc Ing), Epid, interscapular BAT (iBAT) and aortic BAT (aBAT) fat pads were extracted and weighed. A sample of each fat depot was immediately frozen in liquid nitrogen for subsequent experiments.

2.3. Ethics approval

The protocol containing all animal procedures described in this study was specifically approved by the Committee on the Ethics of Animal Experiments of York University (York University Animal Care Committee, YUACC, permit number: 2021-03) and performed strictly in accordance with the YUACC guidelines. All tissue extraction procedures were performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering [15]. All experiments in this study were carried out in compliance with the ARRIVE guidelines [16].

2.4. Measurement of blood glucose levels

Blood from all animals in the fed state was collected between 15:00 and 16:00 h by saphenous vein bleeding and used to determine plasma glucose; using the Contour Next one meter blood glucose monitoring system by Ascensia Diabetes Care US (Parsippany, NJ, USA).

2.5. Adipocyte isolation from Epid and Sc Ing fat depots

Adipocyte isolation from the Epid and Sc Ing fat pads was performed as previously described [2]. Briefly, the adipose tissue was minced in Krebs-Ringer bicarbonate HEPES buffer (KRBH) prepared fresh on the day of each experiment from stock solutions of salts

and buffers (stored at 4°C) to give the following final concentrations: 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM $NaHCO_3$, 30 mM HEPES, and type II collagenase (0.5 mg/mL). Before use, KRBH was gassed for 45 min with carbogen (95% O_2, 5% CO_2) and then BSA (3.5%) and glucose (5.5 mM) were added. pH of the buffer was adjusted to 7.4 with NaOH. Minced tissues were incubated at 37°C with gentle agitation (120 orbital strokes/ min) for ~25–30 min. The digested tissue was then strained using a nylon mesh, and cells were transferred to 50 mL tubes, washed three times, and resuspended in KRBH containing 3.5% fatty acid-free BSA (KRBH-3.5% BSA). To distribute an equal number of adipocytes in each treatment condition, cell diameters were measured and total cell numbers were determined [17].

2.6. Isolation of BAT adipocytes

iBAT and aBAT were extracted and carefully trimmed of any muscle, connective tissue, and white fat. The tissues were then used for adipocyte isolation as previously described [18]. To increase the yielding of brown adipocytes, iBAT and aBAT were combined. The tissues were initially incubated in KRBH-4% BSA supplemented with collagenase (0.83 mg/mL) at 37°C under orbital agitation (120 orbital strokes/min) for 5 min. Subsequently, the combined tissues were vortexed for 5 s, the contents of the vial were filtered, and the filtrate was discarded. The remaining tissues were finely minced and incubated in fresh KRBH-4% BSA containing collagenase (0.83 mg/mL) at 37°C under orbital agitation (120 orbital strokes/min) until complete digestion. The procedure then continued as described above for WAT adipocyte isolation.

2.7. Measurement of glucose and palmitate oxidation

Glucose and palmitate oxidation as measures of oxidative capacity were assessed by production of $^{14}CO_2$ in BAT adipocytes (5×10^5 cells), as previously described [2]. Briefly, cells were incubated in KRBH-3.5% BSA containing either 0.2 μCi/mL of [1-^{14}C]palmitic acid and 200 μM nonlabeled palmitate or 0.2 μCi/mL of D-[U-^{14}C]glucose and 5.5 mM nonlabelled D-glucose for 1h. To distinguish substrate utilization used for ATP synthesis (coupled oxidation) from proton leak (uncoupled oxidation), 15 min before incubation, adipocytes were treated with the ATP synthase inhibitor oligomycin (Oligo) [2]. Following 1 h of incubation, either in the absence or continuous presence of Oligo (100 μM), the media were acidified with 0.2 mL of H_2SO_4 (5 N), and the vials were maintained sealed at 37°C for an additional 1 h for the collection of $^{14}CO_2$ released from the cells and the media. The vials used for incubation had a centered isolated well containing a loosely folded piece of filter paper that was moistened with 0.2 mL of 2-phenylethylamine/methanol (1:1, vol:vol) for the capture of $^{14}CO_2$. At the end of the incubation, the filter paper was removed and transferred to a scintillation vial for radioactivity counting [2,6,19]. All oxidation values are expressed as fold change of Oligo-induced oxidation over basal oxidation.

2.8. Measurement of glucose incorporation into lipids

Glucose incorporation into lipids was measured in isolated adipocytes (5×10^5 cells) from the Epid and Sc Ing fat depots as previously described [19]. Briefly, cells were incubated in KRBH-3.5% BSA containing 0.2 μCi/mL of D-[U-^{14}C] glucose and 5.5 mM nonlabelled D-glucose for 1 h in the absence or presence of insulin (100 nM). Lipids were then extracted according to the method of Dole and Meinertz [20] and assessed for radioactivity [19]. Data are expressed as the fold change of insulin-stimulated glucose incorporation into lipids, relative to basal glucose incorporation into lipids.

2.9. Measurement of glycerol release

Lipolysis was measured in isolated Epid and Sc Ing adipocytes (6×10^5 cells). Adipocytes were incubated in the absence or presence of 100 nM Iso (β-nonspecific agonist) [21]. Each condition was assayed in triplicates and the samples were incubated for 75 min at 37°C with gentle agitation (80 orbital strokes/min). After incubation, a 200 μL aliquot of medium was taken from each vial for the determination of glycerol concentration. Data are expressed as Iso-stimulated glycerol release, over basal glycerol release.

2.10. Western blotting analysis of UCP1, PGC1α, β3AdR, ATGL and GyK in WAT and BAT

Sc Ing, Epid and iBAT samples were homogenized in a buffer containing 25 mM Tris-HCl, 25 mM NaCl (pH 7.4), 1 mM $MgCl_2$, 2.7 mM KCl, 1% Triton X-100 and protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged, the infranatant was collected, and an aliquot was used to measure protein by the Bradford method. Samples were diluted 1:1 (vol:vol) with 2x Laemmli sample buffer and heated to 95°C for 5 min. 25 μg of protein were loaded in each well. Samples were then subjected to SDS-PAGE, transferred to PVDF membrane, and probed for the proteins of interest. All primary antibodies were used at a dilution of 1:1,000. All densitometry analyses were performed using the ImageJ program.

2.11. Statistical analyses

Data were expressed as Mean ± SEM. Statistical analyses were performed by using mixed-model analyses and one-way ANOVAs with Bonferroni multiple comparison *post hoc* tests, as indicated in the figure legends. The GraphPad Prism software version 9.1.12 was used for all statistical analyses and for the preparation of all graphs. The level of significance was set to $P<.05$.

3. Results

3.1. Effect of diet on body weight (BW), plasma BHB levels, and blood glucose concentration

As expected, all groups of animals displayed a progressive increase in BW throughout the 16-week feeding period. At the end of the study, BW was slightly higher in HFS- and KD-fed rats than the SC-fed counterparts, however these differences were not statistically significant (Table 2). When expressed as percent increase in weight gain relative to baseline, the HFS and KD induced 1.2-fold increases in this parameter (Table 2). Plasma BHB was significantly elevated by the KD at weeks 4, 8, and 16 when compared to both the SC and HFS groups (Table 2). At week 8, BHB levels were significantly higher in HFS-fed rats than the SC-fed group; however, at week 16, BHB levels did not differ between these two groups (Table 2). Fed-state blood glucose levels were significantly higher in HFS- than SC-fed animals at weeks 4 and 16, whereas in KD-fed rats glycaemia was not significantly increased (Table 2).

3.2. Energy intake and energy efficiency in SC, HFS and KD-fed animals

Total energy intake during the 16-week feeding period was similar between the three groups (Table 3). However, energy efficiency at week 16 was significantly elevated in KD-fed animals when compared to the SC group (Table 3).

Table 2
The effects of a standard chow (SC), high-fat, sucrose-enriched (HFS) diet or a ketogenic diet (KD) on body weight, % increase in body weight relative to baseline, plasma β-hydroxybutyrate (BHB) levels and blood glucose concentration after 0, 4, 8, and 16 weeks of feeding.

		Duration of study (weeks)			
		0	4	8	16
Body weight (g)	SC	247.2 ± 4.7	403.8 ± 15.9	486.3 ± 17.8	553.9 ± 20.0
	HFS	232.7 ± 4.2	407 ± 14.7	504.7 ± 16.2	590.3 ± 24.3
	KD	245.3 ± 5.1	409.3 ± 9.2	511.4 ± 13.0	607.9 ± 19.1
Body weight increase relative to week 0 (%)	SC	-	64.2 ± 4.9	96.7 ± 5.9	124.1 ± 6.6
	HFS	-	76.3 ± 3.7	116.8 ± 5.4	153.2 ± 8.1*
	KD	-	70.3 ± 4.5	108.9 ± 5.7	148.1 ± 7.5*
Plasma BHB (mM)	SC	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
	HFS	0.16 ± 0.02	0.19 ± 0.03	0.24 ± 0.03*	0.15 ± 0.02
	KD	0.13 ± 0.02	0.30 ± 0.03†	0.38 ± 0.03†	0.29 ± 0.02†
Blood glucose (mM)	SC	7.4 ± 0.2	6.8 ± 0.3	6.8 ± 0.2	5.9 ± 0.3
	HFS	7.3 ± 0.1	7.3 ± 0.2*	7.2 ± 0.1	6.7 ± 0.3*
	KD	7.3 ± 0.1	7.2 ± 0.1	7.0 ± 0.1	6.6 ± 0.2

* $P<.05$ vs SC in the respective week;
† $P<.05$ vs all other groups. Data are expressed as mean ± SEM. Mixed-effects model analysis. n=6–9 for body weight and % increase in weight gain. n=6–7 for BHB. n=6–15 for blood glucose.

Table 3
Effect of diet on total energy intake for the 16-week intervention and energy efficiency.

	SC	HFS	KD
Total energy intake (kcal)	9554 ± 478.1	8814 ± 318.3	9010 ± 517.7
Energy efficiency (g/kcal)	$0.033 \pm 7.4 \times 10^{-4}$	$0.035 \pm 9.7 \times 10^{-4}$	0.039 ± 0.002*

* $P<.05$ vs SC. Data are expressed as mean ± SEM. One-way ANOVA. n=4.

3.3. Effect of diet on WAT and BAT masses

At week 8 of the intervention, Sc Ing fat mass was 2.6- and 2.1-fold higher in HFS and KD-fed animals than SC-fed rats, respectively (Fig. 1A). At week 16, Sc Ing mass increased significantly in all three groups in comparison to week 8, but HFS and KD-fed animals continued to display significantly higher Sc Ing fat masses than SC-fed rats (Fig. 1A). Similarly, at week 8, Epid fat mass was significantly elevated in HFS- (~2.7-fold) and KD-fed (~2-fold) rats when compared to SC-fed counterparts (Fig. 1B). At week 16, Epid fat masses of HFS- and KD-fed animals were 2- and 1.9-fold higher than the SC group, respectively (Fig. 1B). With respect to BAT, the HFS diet and the KD increased week 8 iBAT mass by 1.6- and 1.4-fold, respectively, relative to the SC animals (Fig. 1C). At week 16, the HFS diet and KD-induced elevation in iBAT mass was maintained (Fig. 1C). The aBAT was also found to be elevated by the HFS diet at weeks 8 and 16, and by the KD at week 16 (Fig. 1D), relative to the SC group. Therefore, despite possessing different macronutrient compositions, the HFS diet and KD induced similar alterations in WAT and BAT mass (Fig. 1).

3.4. Effects of diet on browning, lipolytic proteins, and β-adrenergic-stimulated lipolysis in Sc Ing fat

Given these diet-induced differences in fat mass, it was important to investigate how the interventions were influencing metabolism at the tissue and cellular levels. Firstly, Sc Ing UCP1 levels were measured to determine whether the KD induced browning in this tissue. We found that Sc Ing UCP1 was undetectable after either 8 or 16 weeks of any of the three dietary interventions (Fig. 2A). At week 8 of the intervention, Sc Ing β3AdR and ATGL levels were elevated 4.5- (Fig. 2B) and 4-fold (Fig. 2C), respectively, in KD-fed animals. Sc Ing ATGL was also increased by the HFS diet at week 8 (Fig. 2C). However, at week 16, no differences were detected in β3AdR levels among the three groups (Fig. 2D), although ATGL was 2.1-fold higher in HFS- than SC-fed rats (Fig. 2E). In line with these findings, β-adrenergic stimulated glycerol release was not significantly different between the three diet groups (Fig. 2F). Thus, the KD did not induce browning and although it promoted a transient increase in lipolytic capacity, these effects were not sustained at week 16.

3.5. Effects of diet on browning, lipolytic proteins and β-adrenergic-stimulated lipolysis in Epid fat

Similar to the Sc Ing fat, UCP1 was not detectable in the Epid fat depot with any of the three diets (Fig. 3A). At week 8, Epid β3AdR (Fig. 3B) and ATGL (Fig. 3C) levels were not significantly different between the three groups, although much lower levels of ATGL (P=.06 vs SC) were found in the Epid fat of HFS-fed rats. At week 16, Epid β3AdR levels were significantly elevated 3.6- and 4.7-fold by the HFS diet and KD, respectively (Fig. 3D), whereas ATGL content was not altered (Fig. 3E). Despite elevations in Epid β3AdR content, the HFS diet attenuated (~49%) Iso-stimulated lipolysis in comparison to the SC group (Fig. 3F). Conversely, in Epid adipocytes from KD-fed rats, Iso-stimulated lipolysis was maintained at the same levels as those of SC-fed counterparts (Fig. 3F).

3.6. Effect of diet on glucose incorporation into lipids and GyK content in Sc Ing and Epid fat

In Sc Ing adipocytes from HFS-fed rats, insulin-stimulated lipogenesis was 82.6% lower than the SC group (Fig. 4A), whereas in adipocytes from KD-fed rats this variable did not differ from the SC group (Fig. 4A). This indicates that the KD preserved insulin sensitivity and lipid deposition in Sc Ing adipocytes. In Epid adipocytes, insulin-stimulated lipogenesis was not altered by diet

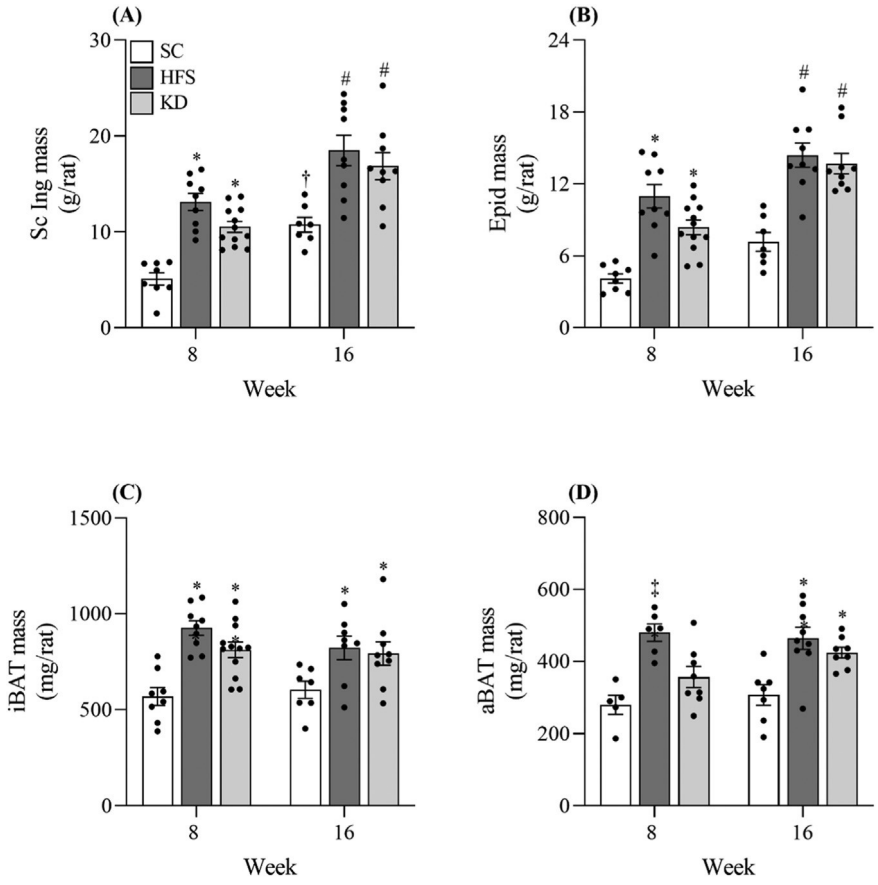

Fig. 1. HFS and KD-feeding significantly elevated subcutaneous inguinal (Sc Ing) (A) and epididymal (Epid) (B) fat masses. Sc Ing mass increased from week 8 to week 16 with all diets (A); however, only the HFS and KDs significantly enhanced Epid mass over this time (B). Interscapular BAT (iBAT) (C) and aortic BAT (aBAT) (D) masses were elevated in both the HFS and KD-fed animals at week 16 of the intervention. However, at week 8, only the HFS diet caused aBAT growth (D). *P<.05 vs SC, within the respective week; #P<.05 vs week 8, within the respective group, and week 16 SC; †P<.05 vs week 8, within group; ‡P<.05 vs week 8 SC and week 8 KD. Bars represent mean ± SEM. Two-way ANOVA. n=5–12.

(Fig. 4B), showing fat-depot specific differences in response to dietary intervention. Furthermore, in Sc Ing and Epid adipocytes, the KD increased GyK levels by 7- and 5.5-fold, respectively (Fig. 4C and D). Thus, the KD preserved the ability of adipocytes to promote lipogenesis in response to insulin and to also recycle TAG by increasing GyK levels.

3.7. *Effects of diet on BAT uncoupled oxidation*

At week 16, uncoupled palmitate oxidation was elevated 1.9-fold in brown adipocytes from KD-fed animals when compared to HFS-fed animals (Fig. 5A), whereas glucose oxidation was not altered by diet (Fig. 5B). UCP1 levels did not significantly increase in BAT of HFS-fed animals (Fig. 5C); however, in BAT adipocytes from KD-fed rats UCP1 levels increased 4.9- and 2.1-fold, when compared to the SC and HFS groups, respectively (Fig. 5C). Other thermogenic proteins such as PGC1α (Fig. 5D), β3AdR (Fig. 5E), and ATGL (Fig. 5F) were not changed by any of the diets, but GyK levels increased 4.2- and 6.5-fold in BAT of HFS and KD-fed animals, respectively (Fig. 5G).

4. Discussion

In this study, we show that a high-fat KD, devoid of carbohydrate, increased BAT uncoupled FA oxidative capacity and the machinery involved in lipolysis and TAG recycling in WAT. This was characterized by elevated UCP1 levels and uncoupled fatty acid oxidation in BAT, and increased GyK levels in WAT. Despite these adaptive responses being typically associated with dissipation as opposed to energy storage [14], the WAT mass of KD-fed animals increased to a level similar to the HFS diet. However, a major difference between the KD and the HFS diet was that the former sustained insulin-stimulated lipogenesis, whereas the latter impaired it. Thus, contrary to the obesogenic HFS diet, the KD-induced increase in adiposity did not impair the classical physiological functions of the WAT. Besides maintaining insulin-stimulated lipogenesis and Iso-stimulated lipolysis, the KD also upregulated a pathway by which white adipocytes can recycle TAG via GyK-mediated glycerol phosphorylation. These adaptive responses in WAT are compatible with an enhanced ability of this tissue to handle an abundant supply of food-derived fat and of NEFAs that escape oxidation in peripheral tissues and return to the WAT.

Neither the Sc Ing nor the Epid fat depots had detectable levels of UCP1, which indicates that the KD did not induce browning of the WAT. Previous studies have proposed that the KD causes browning in Sc Ing [12] and Epid [22] WAT. However, these studies measured the gene expression of UCP1 [12,22] and other markers of browning [12]. Evidently, this does not necessarily translate to KD-induced alterations in protein levels, indicating that

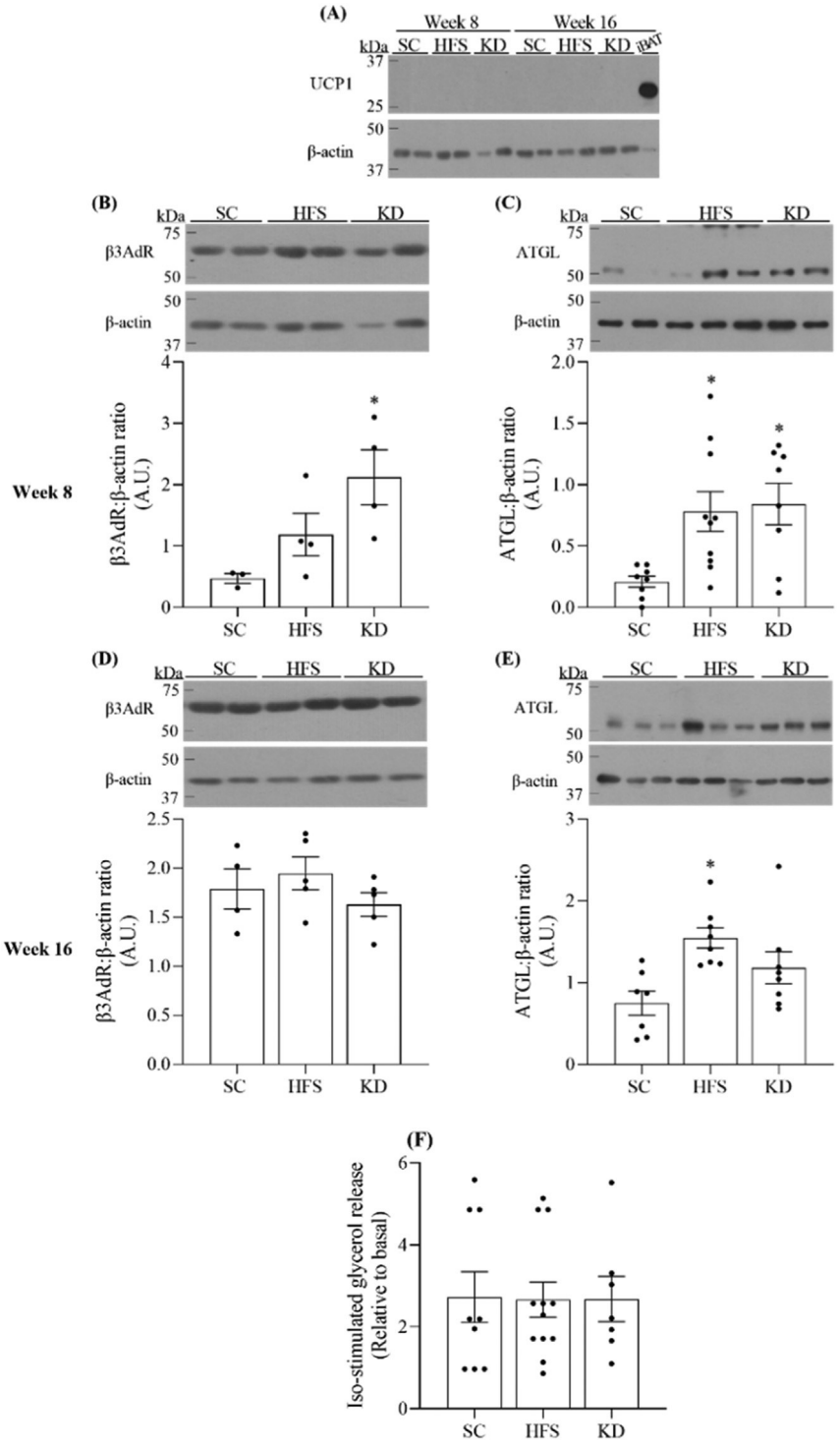

Fig. 2. Uncoupling protein-1 (UCP1) levels were undetectable in Sc Ing fat either at week 8 or 16 (A). At week 8 of the intervention, β3-adrenergic receptor (β3AdR) levels were increased by the KD (B), and adipose triglyceride lipase (ATGL) was significantly elevated by HFS and KD (C). At week 16, β3AdR (D) and ATGL (E) protein levels diminished, where β3AdR was not altered by any of the diets and ATGL was only increased by the HFS diet (D and E, respectively). Isoproterenol (Iso)-stimulated lipolysis was not significantly different between the three groups (F). *P<.05 vs SC. Bars represent mean ± SEM. One-way ANOVA. n=3–12.

UCP1-mediated thermogenesis was not enhanced by KD in WAT. However, the WAT of KD-fed rats displayed time-dependent and depot-specific responses to different diets with regards to levels of lipolytic proteins and how each fat depot responded to β-adrenergic-induced lipolysis. This was evident for β3-AdR and ATGL levels that increased in the Sc Ing fat depot at week 8 of KD and HFS feeding, whereas at week 16 only ATGL was elevated in HFS-fed rats. Conversely, the Epid fat of KD- and HFS-fed rats had unchanged levels of β3-AdR and ATGL at week 8, but at week 16 both diets elevated β3-AdR levels in the tissue. Furthermore, rates of Iso-stimulated lipolysis were not affected by the dietary interventions in Sc Ing adipocytes, whereas in Epid fat adipocytes the

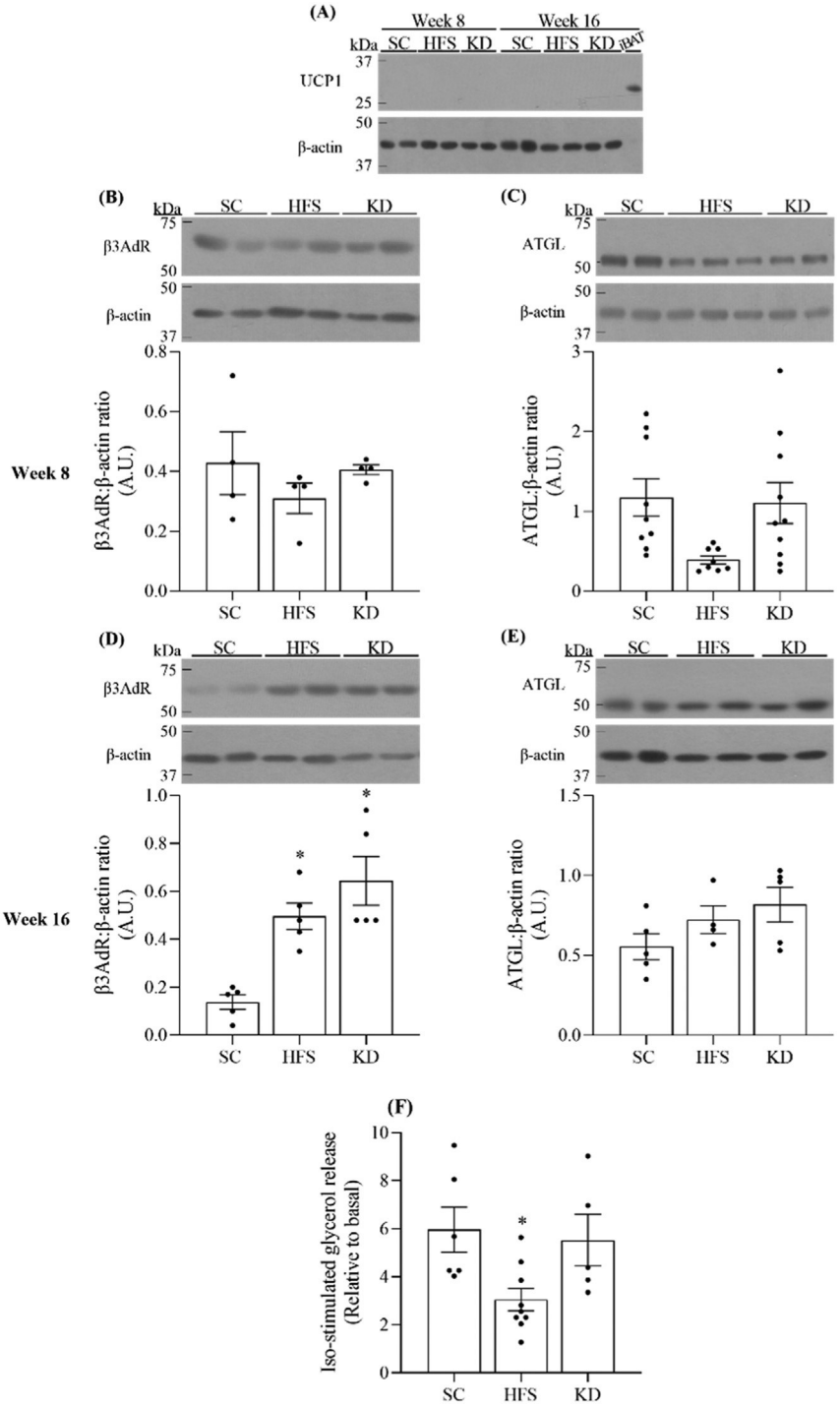

Fig. 3. In Epid fat, UCP1 levels (A) were undetectable and β3AdR (B) and ATGL (C) levels were not altered at week 8, although, Epid ATGL did show a trend to decrease with the HFS diet (C). At week 16, β3AdR levels were increased in the Epid fat of HFS and KD-fed animals (D). ATGL did not differ among the groups (E). The HFS diet significantly attenuated isoproterenol (Iso)-stimulated lipolysis in Epid adipocytes (F). *P<.05 vs SC. Bars represent mean ± SEM. One-way ANOVA. n=4–10.

HFS diet markedly reduced it. This impairment in lipolytic capacity is supported by existing literature reporting that obesity causes catecholamine resistance and blunts β-adrenergic-stimulated lipolysis [7]. Along the same lines, the HFS diet-induced elevation in Epid β3AdR was not sufficient to reverse the attenuation in β-adrenergic signaling. These fat-depot specific patterns of adaptations in WAT indicate that fat accumulation was favored in visceral over Sc WAT, which is metabolically detrimental [1]. Additionally, because adiposity similarly increased in HFS- and KD-fed rats at weeks 8 and 16, time and fat depot-specific effects of the KD do not seem to dictate accrual of fat mass. However, by preserving the lipolytic and lipogenic responses of the WAT, the KD likely exerted a protective effect against the potentially harmful effects of diet-induced obesity.

In addition to the WAT-specific effects, the KD also altered metabolism in BAT. Similar to previous studies, we found that the

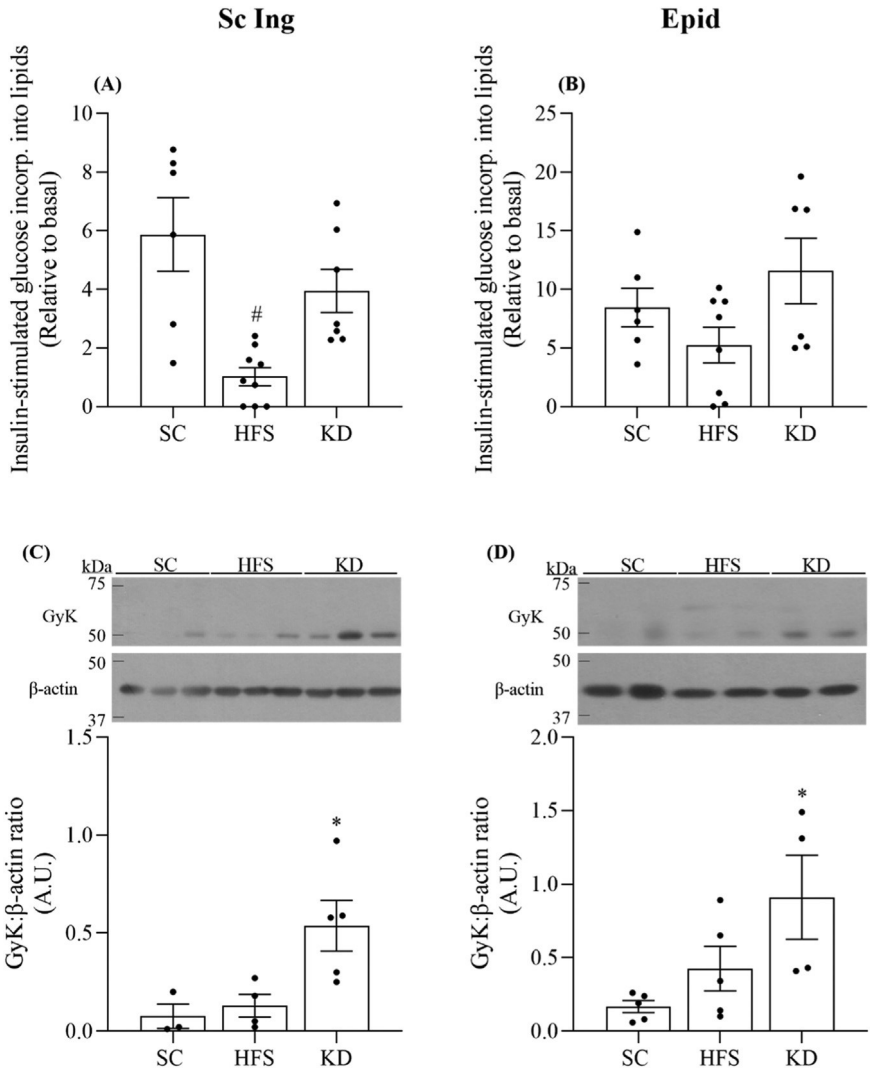

Fig. 4. HFS-feeding significantly impaired insulin-stimulated glucose incorporation into lipids (A) in Sc Ing adipocytes, but not in Epid adipocytes (B). The KD significantly elevated glycerol kinase (GyK) levels in Sc Ing (C) and Epid (D) fat pads. $^{*}P<.05$ vs SC; $\#P<.05$ vs all other groups. Bars represent mean ± SEM. One-way ANOVA. $n=3$–9.

KD increased BAT UCP1 content [12,23]. However, to our knowledge, the effects of the KD on palmitate and glucose oxidation in brown adipocytes have not been investigated. We measured Oligo-induced vs basal oxidation as a measure of uncoupled oxidation, where the ratio between Oligo-induced and basal oxidation represents the capacity for proton leak and, thus, uncoupling capacity in brown adipocytes [2]. Here, we show that the KD enhanced the Oligo-induced oxidation of fatty acids in BAT, relative to basal, when compared to the HFS diet group. In line with these findings, the KD also significantly elevated BAT GyK content. In BAT, GyK mediates the replenishment of the TAG supply for thermogenesis [24]. In fact, it has been shown that cold exposure significantly increased BAT GyK activity and expression in rats [24]. Thus, the observed elevations in UCP1, GyK and palmitate oxidation suggest that KD-feeding could potentially enhance BAT thermogenesis. These effects were consistent with the enhancement in BAT mass in the KD-fed animals. On the other hand, the HFS-induced elevation in iBAT mass could be attributed to the increased GyK content that was not met with an increase in fat consumption, leading to lipid accumulation and BAT growth.

The KD-induced increase in body weight and adiposity was contrary to our original hypothesis and contradicts what has been reported in studies that show that KD attenuates body weight, body mass index [10], and fat mass [11]. However, in these studies, energy intake was either not reported [10] or the KD was hypocaloric [11]. This is relevant because it did not discriminate whether the observed effects were caused by reduced energy intake or due to altered macronutrient composition of the diet. In fact, studies that use a KD that is isocaloric to the control diet generally show that fat mass is not significantly reduced [23,25], and may even be elevated under KD conditions [22]. Similarly, we also observed a KD-induced increase in fat mass under isocaloric conditions, which was likely supported by the enhanced energy efficiency observed in these animals, and a reduction in energy expenditure [14]. In this context, the KD-induced elevation in BAT thermogenic capacity was likely not sufficient to account for the increased dietary fat intake under chronic KD conditions. Furthermore, TAG recycling may not have increased energy expenditure enough to induce fat loss [26]. This limited capacity to oxidize fat could have led to the diversion of fatty acids for storage in the WAT, driving the increase in adiposity. Nevertheless, TAG recycling remains a crucial mechanism under KD conditions because it allowed the WAT to cope with the abundance of dietary fat [2,27], which likely facilitated the preservation of catecholamine and

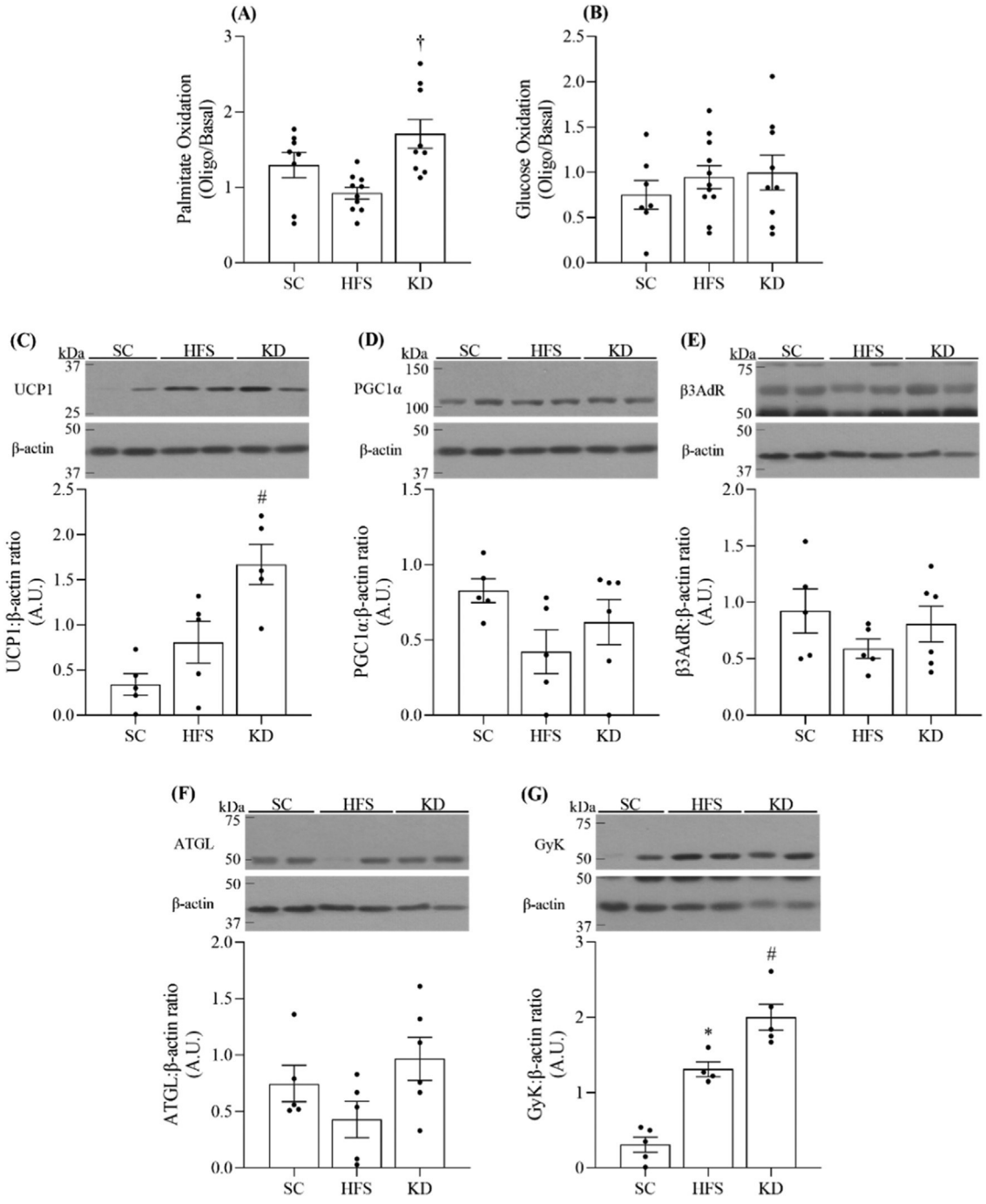

Fig. 5. The KD increased uncoupled oxidation of palmitate (A) but not glucose oxidation (B) in brown adipocytes. The KD also increased UCP1 levels in BAT (C), whereas peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (D), β3AdR (E), and ATGL (F) did not change with dietary intervention. However, GyK (G) was significantly elevated by HFS and KD, reaching higher levels in the latter . $^{*}P<.05$ vs SC; #$P<.05$ vs all other groups; †$P<.05$ vs HFS. Bars represent mean ± SEM. One-way ANOVA. n=4–11.

insulin sensitivity in the white adipocytes [1,6]. In turn, it is plausible that the maintenance of insulin-stimulated lipogenesis and β-adrenergic stimulated lipolysis prevented hyperglycemia in these animals, despite the robust increase in fat mass [1]. Altogether, our data support the notion that adiposity is not necessarily the cause of disease [1]. Rather, it is the dysfunctional storage and breakdown of fat that triggers the onset of metabolic disorders [1].

It is important to consider that the protein contents of the HFS and KDs differed from that of the SC diet, where the two former diets had 20% kcal from protein and the latter was 27%. Because total energy intake was not different, this means that kcal from protein was lower in the HFS and KDs. Importantly, a low protein diet has been shown to increase sympathetic flux to BAT and induce thermogenesis in this tissue [28]. Therefore, we cannot discard the possibility that lower dietary protein content in KD- and HFS-fed rats could have induced BAT activation and potentially impacted BAT uncoupled oxidation, when compared to the SC group. However, despite equal protein contents in the HFS and

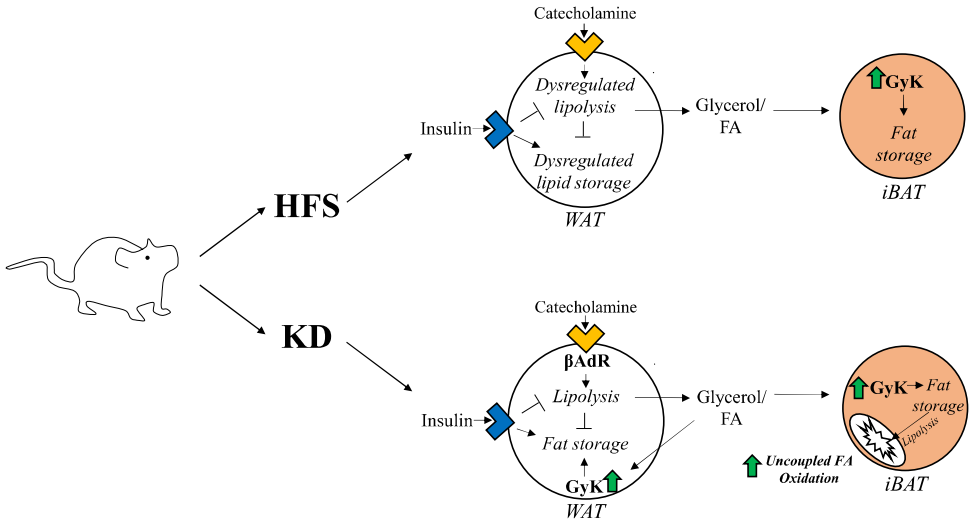

Fig. 6. The HFS diet impaired insulin-stimulated glucose incorporation into lipids and Iso-stimulated lipolysis in WAT. The HFS diet also decreased uncoupled FA oxidation and upregulated GyK in BAT. These metabolic adaptations likely enhanced the lipid storage in BAT as lipogenesis in WAT was impaired. In contrast, the KD preserved lipolysis and lipogenesis in the white adipocytes and increased GyK levels. These KD effects enabled the WAT to recycle glycerol and resynthesize lipids under conditions of enhanced lipolysis. Furthermore, the BAT of KD-fed animals had increased levels of UCP1 and enhanced uncoupled fat oxidation. The fat supply for uncoupled oxidation in BAT was maintained by the elevation in GyK. These energy-dissipating mechanisms allowed the WAT and BAT from KD-fed rats to better cope with the abundance of fat in the diet. Abbreviation: BAT, brown adipose tissue; KD, ketogenic diet; WAT, white adipose tissue; UCP1, Uncoupling protein-1.

KD groups, we observed an elevation in uncoupled oxidative capacity in the BAT of KD-fed animals, relative to HFS. Additionally, GyK was measured as an indication of fat-storing capacity and was proposed to enhance the supply for uncoupled FA oxidation in BAT from KD-fed animals. However, glycerol 3-phosphate may also be used by mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) to provide reducing equivalents for the electron transport chain [29]. This points to an alternative fate for glycerol 3-phosphate that contributes to thermogenesis in BAT [29]. However, mGPDH activity was not assessed in the present study and warrants future investigations into the potential effects of the KD on the glycerol 3-phosphate shuttle and mGPDH-mediated thermogenesis.

In conclusion, the KD enhanced uncoupled FA oxidation in BAT, increased the machinery for TAG recycling in white adipocytes, and preserved insulin sensitivity and lipolytic capacity in visceral and Sc WAT (Fig. 6). Despite upregulating these energy-dissipating pathways, this was not sufficient to overcome the abundance of dietary fat, leading to increased storage in the white fat depots. However, the induction of these pathways was crucial to prevent metabolic dysregulation and preserve insulin-stimulated lipogenesis [6] in a condition of increased adiposity in KD-fed rats. Together, these effects-maintained WAT function and could prevent ectopic lipid accumulation, which is of great importance to counteract obesity-induced dysfunctional metabolic alterations in WAT [1,8].

Authors Contributions

DD was involved in the conception and design of the study, conducted experiments, analyzed the results, prepared figures, drafted the article, and revised the manuscript. SJ conducted experiments, analyzed the results, and revised the manuscript. MS conducted experiments, analyzed the results, and revised the manuscript. RBC was involved in the conception and design of the study, conducted experiments, drafted the article, and revised the manuscript. All authors gave final approval of the revised version submitted and agreed to be accountable for all aspects of the work in ensuring that questions that may arise related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declarations of Competing Interest

The authors declare that there are no conflicts of interest.

Funding

This research was funded by a Natural Science and Engineering Research Council of Canada Discovery Grant (RBC, #311818-2011) and infrastructure grants from the Canada Foundation for Innovation and the Ontario Research Fund awarded to RBC. DD was supported by the Natural Sciences and Engineering Research Council of Canada Alexander Graham Bell Canada Graduate Scholarship-Doctoral.

References

[1] Roden M, Shulman GI. The integrative biology of type 2 diabetes. Nature 2019;576:51–60. doi:10.1038/s41586-019-1797-8.
[2] Sepa-Kishi DM, Jani S, Da Eira D, Ceddia RB. Cold acclimation enhances UCP1 content, lipolysis, and triacylglycerol resynthesis, but not mitochondrial uncoupling and fat oxidation, in rat white adipocytes. Am J Physiol - Cell Physiol 2019;316:C365–76. doi:10.1152/ajpcell.00122.2018.
[3] So M, Gaidhu MP, Maghdoori B, Ceddia RB. Analysis of time-dependent adaptations in whole-body energy balance in obesity induced by high-fat diet in rats. Lipids Health Dis 2011;10:1–12. doi:10.1186/1476-511X-10-99.
[4] Wu MV, Bikopoulos G, Hung S, Ceddia RB. Thermogenic capacity is antagonistically regulated in classical brown and white subcutaneous fat depots by high fat diet and endurance training in rats: Impact on whole-body energy expenditure. J Biol Chem 2014;289:34129–40. doi:10.1074/jbc.M114.591008.
[5] Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 2008;9:367–77. doi:10.1038/nrm2391.Adipocyte.
[6] Gaidhu MP, Anthony NM, Patel P, Hawke TJ, Ceddia RB. Dysregulation of lipolysis and lipid metabolism in visceral and subcutaneous adipocytes by high-fat diet: Role of ATGL, HSL, and AMPK. Am J Physiol - Cell Physiol 2010;298:961–71. doi:10.1152/ajpcell.00547.2009.
[7] Mowers J, Uhm M, Reilly SM, Simon J, Leto D, Chiang SH, et al. Inflammation produces catecholamine resistance in obesity via activation of PDE3B by the protein kinases IKKε and TBK1. Elife 2013;132:1–18. doi:10.7554/eLife.01119.
[8] Jani S, Da Eira D, Hadday I, Bikopoulos G, Mohasses A, de Pinho RA, et al. Distinct mechanisms involving diacylglycerol, ceramides, and inflammation un-

derlie insulin resistance in oxidative and glycolytic muscles from high fat-fed rats. Sci Rep 2021;11:19160. doi:10.1038/s41598-021-98819-7.
[9] Longo R, Peri C, Cricr D, Coppi L, Caruso D, Mitro N, et al. Ketogenic diet : a new light shining on old but gold biochemistry. Nutrients 2019;11:1–22.
[10] Walton CM, Perry K, Hart RH, Berry SL, Bikman BT. Improvement in glycemic and lipid profiles in type 2 diabetics with a 90-day ketogenic diet. J Diabetes Res 2019;2019:1–6. doi:10.1155/2019/8681959.
[11] Luukkonen PK, Dufour S, Lyu K, Zhang XM, Hakkarainen A, Lehtimäki TE, et al. Effect of a ketogenic diet on hepatic steatosis and hepatic mitochondrial metabolism in nonalcoholic fatty liver disease. Proc Natl Acad Sci U S A 2020;117:7347–54. doi:10.1073/pnas.1922344117.
[12] Douris N, Desai BN, Fisher M, Cisu T, Fowler AJ, Zarebidaki E, et al. Beta-adrenergic receptors are critical for weight loss but not for other metabolic adaptations to the consumption of a ketogenic diet in male mice. Mol Metab 2017;6:854–62. doi:10.1016/j.molmet.2017.05.017.
[13] Sepa-Kishi DM, Ceddia RB. Exercise-mediated effects on white and brown adipose tissue plasticity and metabolism. Exerc Sport Sci Rev 2016;44:37–44. doi:10.1249/JES.0000000000000068.
[14] Basolo A, Magno S, Santini F, Ceccarini G. Ketogenic diet and weight loss: is there an effect on energy expenditure? Nutrients 2022;14. doi:10.3390/NU14091814.
[15] Pinho RA, Sepa-Kishi DM, Bikopoulos G, Wu MV, Uthayakumar A, Mohasses A, et al. High-fat diet induces skeletal muscle oxidative stress in a fiber type-dependent manner in rats. Free Radic Biol Med 2017;110:381–9. doi:10.1016/j.freeradbiomed.2017.07.005.
[16] du Sert NP, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, et al. Reporting animal research: Explanation and elaboration for the arrive guidelines 2.0. PLoS Biol 2020;18:e3000411. doi:10.1371/journal.pbio.3000411.
[17] Fine JB, DiGirolamo M. A simple method to predict cellular density in adipocyte metabolic incubations. Int J Obes Relat Metab Disord 1997;21:764–768.
[18] Cannon B, Nedergaard J. Respiratory and thermogenic capacities of cells and mitochondria from brown and white adipose tissue. In: Ailhaud G, editor. Adipose Tissue Protoc. New Jersey: Humana Press Inc.; 2001. p. 295–304.
[19] Gaidhu MP, Fediuc S, Anthony NM, So M, Mirpourian M, Perry RLS, et al. Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. J Lipid Res 2009;50:704–15. doi:10.1194/jlr.M800480-JLR200.
[20] Dole VP, Meinertz H. Microdetermination of long-chain fatty acids in plasma and tissues. J Biol Chem 1960;235:2595–9. doi:10.1016/s0021-9258(19)76920-8.
[21] Ahlquist RP. Present state of alpha- and beta-adrenergic drugs I. The adrenergic receptor. Am Heart J 1976;92:661–4.
[22] Asrih M, Altirriba J, Rohner-Jeanrenaud F, Jornayvaz FR. Ketogenic diet impairs FGF21 signaling and promotes differential inflammatory responses in the liver and white adipose tissue. PLoS One 2015;10:1–14. doi:10.1371/journal.pone.0126364.
[23] Srivastava S, Baxa U, Niu G, Chen X, Veech RL. A Ketogenic diet increases brown adipose tissue mitochondrial proteins and UCP1 levels in Mice. IUBMB Life 2013;65:58–66.
[24] Festuccia WTL, Guerra-Sá R, Kawashita NH, Garófalo MAR, Evangelista EA, Rodrigues V, et al. Expression of glycerokinase in brown adipose tissue is stimulated by the sympathetic nervous system. Am J Physiol - Regul Integr Comp Physiol 2003;284:1536–41. doi:10.1152/ajpregu.00764.2002.
[25] Hall KD, Chen KY, Guo J, Lam YY, Leibel RL, Mayer LES, et al. Energy expenditure and body composition changes after an isocaloric ketogenic diet in overweight and obese men. Am J Clin Nutr 2016;104:324–33. doi:10.1371/journal.pone.0222971.
[26] Elia M, Zed C, Neale G, Livesey G. The energy cost of triglyceride-fatty acid recycling in nonobese subjects after an overnight fast and four days of starvation. Metabolism 1987;36:251–5. doi:10.1016/0026-0495(87)90184-3.
[27] Guan HP, Li Y, Jensen MV, Newgard CB, Steppan CM, Lazar MA. A futile metabolic cycle activated in adipocytes by antidiabetic agents. Nat Med 2002;8:1122–8. doi:10.1038/nm780.
[28] Pezeshki A, Chelikani PK. Low protein diets and energy balance: mechanisms of action on energy intake and expenditure. Front Nutr 2021;8:1–8. doi:10.3389/fnut.2021.655833.
[29] Mráček T, Drahota Z, Houštěk J. The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues. Biochim Biophys Acta - Bioenerg 2013;1827:401–10. doi:10.1016/j.bbabio.2012.11.014.