UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Hyperpalatable diet and physical exercise affects the brain metabolism: role of the brain lactate shuttle

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Porto Alegre, Junho de 2013

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“A mente que se abre a uma idéia nova jamais voltará ao seu tamanho original.”

(Albert Eistein)

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Apresentação

Este trabalho encontra-se na forma de artigo científico, a fim de ser submetido à revista *NeuroMolecular Medicine*. O guia para os autores encontra-se anexado, ao final deste trabalho.

Hyperpalatable diet and physical exercise affects the brain metabolism: role of the brain lactate shuttle.

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Abstract

Diet rich in fat and sugar associated to sedentary habits are the main cause of obesity. Obesity is a risk factor for the development of insulin resistance and diabetes. Insulin receptors and signaling modulates brain energetic metabolism. However, brain insulin resistance is associated with cognitive deficits and neurodegeneration. Lactate is an important energetic substrate to brain in specific situations; it is transported by monocarboxylate transporters (MCTs), which are regulated by many factors including insulin signaling. Hyperpalatable diet (HP) impairs brain insulin signaling whereas physical exercise improves insulin signaling and cognition function. We evaluated the effects of four months of HP diet followed by one month of physical exercise plus HP diet in MCT expression, lactate levels on hippocampus and brain mitochondrial function. Male C57BL/J6 mice, 1 month old were divided in the following groups: control diet sedentary (CDS), control diet exercise (CDE), HP diet sedentary (HPS), and HP diet exercise (HPE) (n=15 per group). Lactate extracellular brain fluid was increased in HPE group after Y-maze task when compared to other groups. The MCT-1 and 4 levels increased in exercise and HP diet groups. The hydrogen peroxide (H_2O_2) production stimulated by succinate in homogenate of hippocampus was increased in HPS group. Incubation of insulin (0.1 ug/mL) reduced the H_2O_2 production in all groups. In summary, these results showed that a HP diet increases MCT expression, affects brain lactate shuttle and mitochondrial function. However, voluntary physical exercise revert the negative effects of HP diet in this brain metabolic outcomes.

Introduction

The prevalence of obesity is increasing substantially in the last decade and become the most prevalence syndrome in western society (Antunes et al., 2010). Environmental, behavioral and genetic factors are contributory factors for the obesity epidemics (Mokdad et al., 2003; Bains et al., 2004). The main behavioral factors implicated in obese phenotype are the excess energy intake, particularly food with high fat and sugar and sedentary habits (Newman et al., 2013; Snoek et al., 2004; Stranahan et al., 2008). Addindionally, obesity and overweight are important risk to the development of insulin resistance, diabetes, metabolic syndrome (Mokdad et al., 2003; Muller et al., 2008; Sandu et al., 2005) and neurodegenerative diseases (Kalmijn et al., 1997; Medhi and Chakrabarty, 2013).

Insulin resistance is associated to neurodegenerative diseases and cognitive deficits once insulin has a key role in the central system which involves energy metabolism and neurotrophic effects (Park, 2001). Moreover, insulin resistance on brain may causes cognitive damages and neuronal death (Lannert and Hoyer, 1998). Diets enriched in sugar/fat could impair brain insulin signaling (Battú et al., 2012; Muller et al., 2008). Brain intracelular insulin receptors (IR) are involved in important signaling cascades that, in last instance result in regulation of mithocondrial fucntion (Cheng et al., 2010) and learning and memory processess (Ghasemi et al., 2013).

The brain is an organ that rely on a lot of energy for its normal function, mostly provided by glucose (Pellerin and Magistretti, 2003). However, in conditions of high metabolic demands, like in injuries and normal cognitive processes, brain could be sustained by other energetic substrates like lactate and ketone bodies (Schelp and Burini, 1995). For example, lactate is essential to long term memory (Suzuki et al., 2011) and during the moderate or intense exercise, the adult brain is able to consume high amounts of lactate, at

the expense of glucose (Dalsgaard et al., 2004; Smith et al., 2003). Brain have high density of monocarboxylates transporters (MCTs) that exchange lactate and ketone bodies (MCT 1, 2 and 4) between neural cells (Pellerin et al., 2005). The expression of these MCTs depends on the cell type. Moreover, MCTs function can be modulated by many factors like insulin and the insulin like growth factor type-1 (IGF-1), which is dependent on the activation of PI3K pathway (Chenal and Pellerin, 2007; Chenal et al., 2008; Pellerin, 2010; Pierre et al., 2009; Robinet and Pellerin, 2010).

Interestingly, physical exercise is recognized as feasible strategy to improve the brain health and cognitive function (Nichol et al., 2007). The increment in the insulin signaling in hippocampus achieved through the PI3k pathway has been proposed to participated in these beneficial effects (Muller et al., 2011) .

The aim of this work was to evaluate the effects of HP diet and physical exercise in brain metabolic outcomes including MCT expression, lactate levels and mitochondrial function.

Materials and Methods

Animals, diet and exercise protocol

Male C57 BL/J6 mice, 1 month old, were obtained from Foundation for Health Science Research (FEPPS, Porto Alegre/RS, Brazil). Animals (4-5 per cage) were placed into a controlled temperature room (22 ºC) under a 12h light/12h dark cycle (lights on at 7 am) and had free access to food and water. Mice were maintained during 5 months, receiving hyperpalatable diet (HP) or control diet, at the end of the fourth month the animals were divided in four groups: control diet sedentary (CDS) (n=15), control diet exercise (CDE) (n=15), hyperpalatable diet sedentary (HPS) (n=15) and hyperpalatable diet exercise

(HPE) (n=15). The HP diet were composed by 57% carbohydrates (34% condensed milk, 15% in starch and 8% sucrose), 25,3%% protein, 10% fat (soybean oil), 5% salt, 2,7% fibers and vitamins (Dietrich et al., 2007). The animals were engaged in voluntary exercise so they had free access to a running wheel, which has a travelled distance recorder. All experiments were in agreement with the Committee on the Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Glucose tolerant test (GTT)

Animals were submitted to a glucose tolerant test after the experimental intervention (diet plus exercise). They received an intraperitoneal injection of glucose (2 mg/g body weight), which was performed in 12 h fasted mice. The blood was collected by a small puncture on the tail immediately before (0 min), 30, 60, and 120 minutes after the injection. At each time, glucose was measured by a glucosimeter (AccuChek Active, Roche Diagnostics®, USA).

Surgical procedure and microdialysis

A guide cannula of microdialysis was placed in dorsal hippocampus at 2 mm posterior to the bregma, 2 mm right of the midline and 1 mm hole made in the cranial bone. Seven days after surgery, the animals were submitted to microdialyses to collect extra cerebral fluid through BASi Brain Microdialysis Probes (ref MD-2200-BR2, USA). After one hour of habituation to microdialyses artificial extracellular fluid (aECF); (124 mM NaCl, 3 mM KCl_2, 1 mM $MgSO_4$ $7H_2O$, 26 $NaHCO_3$, 2 mM $CaCl_2$ H_2O, 1 mM glucose, buffered at pH 7.4) (Garrido et al., 2012), samples were collected in three blocks of 10 minutes to analyze basal levels. After that, the animals were submitted to Y-maze, during 10 minutes, and then, samples were collected in three blocks of 10 minutes to analyze the

animal's recovery. These samples were used to evaluate lactate levels through a commercially available colorimetric kit (Katal, Brazil).

Y-maze task

Memory performance by spontaneous alternation was evaluated using Y-maze test. The apparatus consists in a radial-arm maze of three identical arms (arms = 40 cm in length X 9 cm in width X 16 cm in wall height with an arm angle of 120º). The animals were placed in the intersection of the arms and chose randomly which arm to enter during 10 minutes. Arm entry was considered when all animal's legs were in the arm (arm entry required all 4 legs of the subject mouse to enter an arm). Perfect alternations were defined as exploration of all three arms sequentially given 3 opportunities independent of a right or left arm choice at initiation. Results were calculated as the percentage of the number of perfect alternations divided by total opportunities (considered 100%) (Kaczmarczyk et al., 2013).

Metabolic Parameters, blood biochemical evaluation and fat pad weight

The body weight of each mouse was measured during the four months of diet and also during the month of exercise. The animals were sacrificed by decapitation. Blood was collected and centrifuged at 5000 *x g*/10 min to obtain serum samples. Then, serum was stored at –20^{o}C until the day of analysis. Fat tissues from mesenteric and epididymal regions were dissected and weighted as previously described (Parekh et al., 1998).

Mitchocondrial function

The mitochondrial production of hydrogen peroxide (H_2O_2) was assessed by the Amplex Red oxidation method. The animal's hippocampus was rapidly removed and homogenized in 'Sucrose Medium' (320 mM sucrose, 1mM EDTA, 0.25 mM dithiothreitol, pH 7.4). As a strategy to improve the capacity of the homogenate in generating H_2O_2, a

standard respiration buffer (100 mM KCI, 75 mM mannitol, 25 mM sucrose, 5 mM phosphate, 0.05 mM EDTA, and 10 mM Tris-HC1, pH 7.4) (Sims and Blass, 1986) supplemented with 10 µM Amplex Red and 2 units/mL horseradish peroxidase was used. The basal H_2O_2 level was considered without the presence of substrate in the incubation medium, while succinate was used as substrate to stimulate mitochondrial respiration. Insulin to 0.1 at 1.0µg/ml was incubated in the standard respiration buffer plus succinate to evaluate the effect of insulin on H_2O_2 production. The fluorescence was monitored at excitation (563 nm) and emission wavelengths (587 nm) in Spectra Max M5 microplate reader (Molecular Devices, USA) (Muller et al., 2012)

Western blotting

For western blot analysis, 30µg of protein from the hippocampus homogenates were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to PVDF membranes as previously reported (Muller et al., 2011). Antibodies against MCT1 (provided by laboratory of Dr. Pellerin and characterized in (Pierre et al., 2000) 1:500), MCT4 (Santa Cruz 1:200) and tubulin (Cell Signaling Technology, 1:500) were used.

Hippocampal IL-1β and TNF-α levels

After decapitation, the hippocampus was dissected and homogenized in PIK buffer (1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I and II; Sigma-Aldrich, USA). The homogenate was centrifuged, and the supernatant was collected. The total protein content was measured (Peterson, 1977). The homogenates were stored at -70 ºC until analysis. The IL-1 and TNF-α assays were performed using commercially available ELISA kits (refs. DY401 and DY410).

The protocol was conducted according to the instructions of the manufacturer (R&D systems, USA).

Statistical Analysis

The results were calculated and expressed as the means ± S.E.M. To analyze the differences between groups, we used one-way ANOVA followed by a post-hoc Tukey test and a Student's t-test between groups. The differences were considered statistically significant at $p < 0.05$.

Results

Effects of diet and exercise in MCT expression, lactate levels and Y-maze task

Microdialysis experiments showed that lactate levels increased in HPE group compared to other groups 10 min after exposure to the Y-maze task (* HPE > other groups, $p < 0.05$) (Figure 1A). Furthermore, the western blot analysis showed that the levels of MCT1 were increased in HP diet compared to their control groups. Also, exercise increased MCT1 levels in the HP diet group (* CDS < other groups; & HPE > HPS, $p < 0.05$) (Figure 1B). The MCT4 levels of diet groups and CDE group were significantly higher than CDS group (* CDS < other groups, $p < 0.05$) (Figure 1C). The memory performance evaluated through the Y-maze task showed no significant differences between groups (Figure 1D).

Mitchocondrial function evaluation

The H_2O_2 production induced by succinate was increased in HPS group when compared to the CDS group (* HPS > CDS, $p < 0.05$). The addition of the other substrate nucleotide ADP and FCCP (carbonylcyanide-ptrifluoromethoxyphenylhydrazone a reversible inhibitor of mitochondrial oxidative phosphorylation) decreased the H_2O_2

production (Figure 2A). Insulin incubation at dose of 0.1ug/mL in hippocampal homogenates reduced the H_2O_2 production in all groups ($p < 0.05$) (Figure 2B).

Exercise data and effects of diet on body weight, body fat and GTT

After 4 weeks with of free access to the running wheel, each mouse in the diet control group traveled an average 3947m ± 307.4 (Mean ± SEM) of distance and the HP diet group traveled an average 3185m ± 264.1 (Mean ± SEM) of distance. There is no significant difference in distance traveled between groups (Figure 3A). The group HPS showed an increase in body weight compared to CDS group. The exercised groups showed similar body weight to the CDS group (* HPS > CDS, $p < 0.05$) (Figure 3B).

The exercise protocol was capable to reduce the effects of HP diet in epididymal fat pad at levels of the CDS group (* CDE < CDS and ** HPE < HPS, $p < 0.05$) (Figure 3C). Similar effect was observed in the mesenteric fat pad (* HPE < HPS > CDS = CDE, $p < 0.05$) (Figure 3D). The GTT test profile was similar between groups (Figure 3E). However, the area under the curve showed increased glucose levels in the HP diet sedentary group compared to other groups (* HPS > other groups, $p < 0.05$) (Figure 3F).

IL-1 and TNF-α

IL-1 and TNF-α were measured in blood and hippocampus. There were no significant differences between groups (Table 1).

Discussion

The present work showed that HP diet affects the brain lactate shuttle and voluntary exercise affects brain metabolism in animals exposed to HP diet. Moreover, HP diet promotes brain insulin resistance expressed by mechanisms involving the mitochondrial

H_2O_2 production. The intake of HP diet caused undesirable effects in some metabolic outcomes whereas the voluntary physical exercise was able to improve it.

HP diet may impair the brain metabolism decreasing the entrance of insulin into the brain areas associated to learning and memory formation (Kaiyala et al., 2000) and thus, impairs insulin signaling and its neuromodulatory effects (Battú et al., 2012; Muller et al., 2008). The brain insulin resistance is putatively implicated in the development of neurogenerative diseases, cognitive deficits and impaired brain metabolism (Prada et al., 2005; Stranahan et al., 2008). Our results showed an increased on blood glucose levels and brain mitochondrial metabolism induced by HP diet which was attenuated by exercise. The insulin resistance is one of the particular metabolic situations in which alternative substrates like lactate and ketone bodies are predominantly used by brain. Consequently it is possible that altered insulin sensitivity caused by the long-term intake of high fat diet could be responsible for the increased in MCT levels (Pierre et al., 2007). It has been demonstrated that HP/high fat diet exposure may cause hormonal and metabolic alterations that may contribute to deficits in insulin sensitivity (Woods et al., 2004). Here, we showed that MCT-1 and 4 levels were increased by HP diet and exercise interventions. The increased on MCT-1 and MCT-4 induced by exercise was already reported in skeletal muscle (Yoshida et al., 2004), however up to our knowledge, this is the first time that this exercise improved MCT-1 and 4 expression on hippocampus. The modulatory effects exerted by physical after a HP diet exposure in brain lactate metabolism deserve additional attention.

The brain lactate shuttle is important in period of high metabolic demands as injury and cognition process (Pellerin and Magistretti, 2012). Cognitive tasks, high fat diet and insulin administration could modulate lactate levels on brain (McNay et al., 2010). We showed that lactate levels were increased after the Y maze task exposure in all groups. In

addition, HPE group showed a bigger increase on lactate levels, which was not observed in HPS. However, this modification on brain metabolism did not result on cognitive deficits. It's important to consider evidences suggesting that prior to the appearance of cognitive symptoms, there is a long, silent, cumulative, and dynamic combination of neurochemical and morphological alterations that under influence of brain insulin resistance could mediate cognitive dysfunctions (Talbot et al., 2012). Thus we can't rule out a possible harmful effect on cognition in other tasks or after more prolonged time of HP diet exposition as already showed by others (McNay et al., 2010; Molteni et al., 2002; Stranahan et al., 2008).

The normal mitochondrial function as well as normal glucose availability are important contributors for a satisfactory brain metabolism and cognitive function (Adeghate et al., 2013; Chen et al., 2006; Hauptmann et al., 2009). Insulin is able to decrease the H_2O_2 production in synaptosomes (Muller et al., 2013) and regulate brain mitochondrial function (Cheng et al., 2010). The HP diet increases the H_2O_2 production induced by succinate on hippocampus homogenate and the voluntary exercise avoids this increment (HPE group). Moreover, insulin incubation (0.1µg/ml) decreases H_2O_2 production on homogenate from hippocampus in all groups. At the dose of 1.0 µg/ml in CDS, CDE and HPE groups exist a tendency to decrease the H_2O_2 production, however this effect was abolished in HPS group.

There is a clear association between the accumulation of fat in body tissues and the development of insulin resistance (Saltiel and Kahn, 2001) and diabetes mellitus with the consequent activation of the inflammatory response through IL-1, IL-6, and TNF-α (Greenfield and Campbell, 2006) (Shankar et al., 2012). In contrast, the voluntary running exercise is proposed to reduce the effects of HP-diet induced adiposity (Yan et al., 2012). Also, physical exercise increases the peripheral insulin sensitivity (Muller et al., 2011). In this work, the exercise protocol reduce the accumulation of fat in mesenteric and epididymal

tissues caused by the HP diet, as well as improve the peripheral glucose metabolism. However, neither HP diet nor exercise affected the levels of pro-inflammatory cytokines in blood and brain.

In summary, this work showed that HP diet increases monocarboxylate transporters in hippocampus, the brain lactate shuttle and mitochondrial function. Furthermore, physical exercise mitigated the negative effects of HP diet in these brain metabolic outcomes.

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Legend to figures:

Figure 1: Effects of diet and exercise in MCT expression, lactate levels and Y-maze task. A) Lactate levels were increased in HPE group compared to other groups 10 min after the Y-maze task (* HPE > other groups, $p < 0.05$). B and C) HP diet increased MCT expression in hippocampus. Exercise increased MCT-1 levels in control diet groups. Exercise, also, increased those levels in the HP diet groups (* CDS < other groups; & HPE > HPS, $p < 0.05$). Exercise was capable to increase MCT-4 levels in control diet group (* CDS < other groups, $p < 0.05$). D) There is no difference between the groups in the Y-maze task.

Figure 2: Mitochondrial function data. A) The H_2O_2 production increased in the HPS group compared to the CDS group (* HPS > CDS, $p < 0.05$). The addition of the others substrates ADP and FCCP made the H_2O_2 production decreased the H_2O_2 production. B) Insulin incubation (0.1ug/mL) reduced the H_2O_2 production in hippocampus of all groups ($p < 0.05$).

Figure 3: Exercise data and effects of diet on body weight, body fat and GTT. A) Total daily distance traveled (m)/mouse, diet control group traveled an average 3947m ± 307.4 of distance and the HP diet group traveled an average 3185m ± 264.1 (Mean ± SEM). There is no difference between groups. B) HPS increased the body weight compared with the other groups (* HPS > other groups, $p < 0.05$). Exercise revert these effects. C) Exercise reduced epididymal fat pad in the HP diet group at levels of the CDS group (* CDE < CDS and ** HPE < HPS, $p < 0.05$). D) Exercise reduced mesenteric fat pad at levels of CDS group (* HPE < HPS > CDS = CDE, $p < 0.05$). E) There was no difference between groups in GTT test. F) The area under the curve was increased in the HPS group compared to others groups (* HPS > other groups, $p < 0.05$).

Figure 1

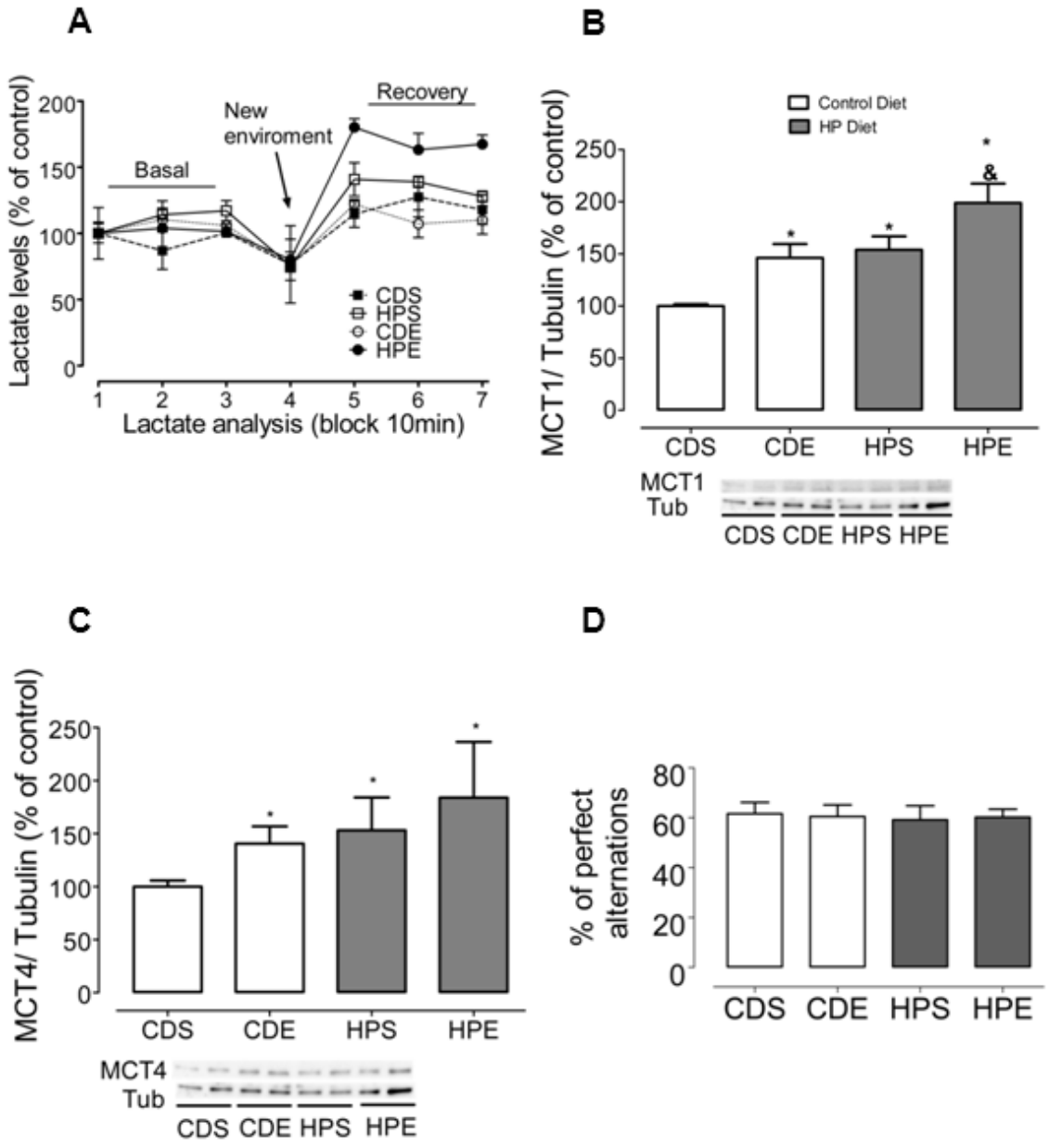
A
Lactate levels (% of control)
200
150
100
50
0
Basal
New enviroment
Recovery
CDS
HPS
CDE
HPE
1 2 3 4 5 6 7
Lactate analysis (block 10min)
B
MCT1/ Tubulin (% of control)
Control Diet
HP Diet
250
200
150
100
50
0
*
*
*
&
CDS CDE HPS HPE
MCT1
Tub
CDS CDE HPS HPE
C
MCT4/ Tubulin (% of control)
250
200
150
100
50
0
*
*
*
CDS CDE HPS HPE
MCT4
Tub
CDS CDE HPS HPE
D
% of perfect alternations
80
60
40
20
0
CDS CDE HPS HPE

Figure 2

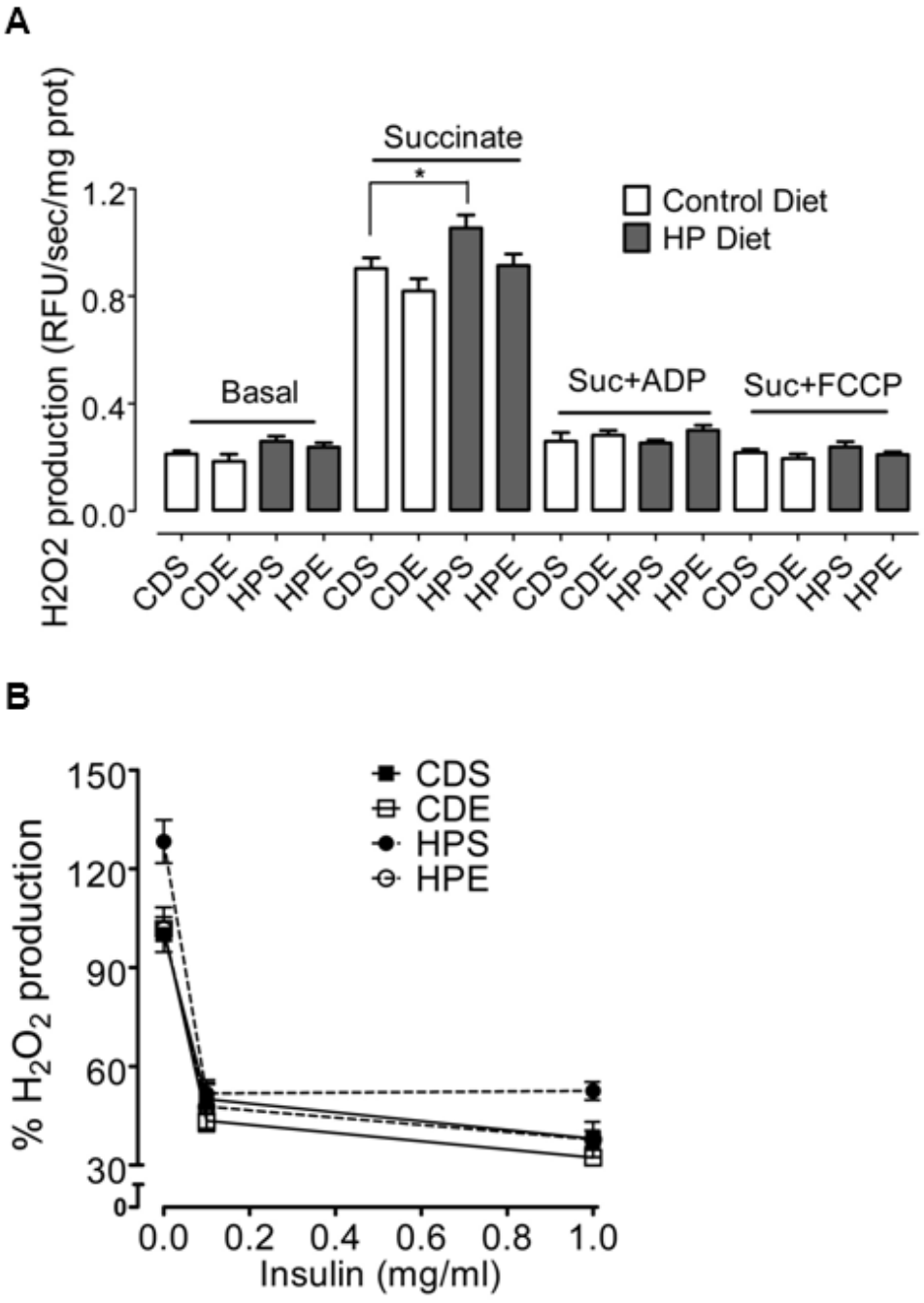
A
H2O2 production (RFU/sec/mg prot)
1.2
0.8
0.4
0.0
Succinate
*
Control Diet
HP Diet
Basal
Suc+ADP
Suc+FCCP
CDS CDE HPS HPE CDS CDE HPS HPE CDS CDE HPS HPE CDS CDE HPS HPE
B
% H2O2 production
150
120
90
60
30
0
CDS
CDE
HPS
HPE
0.0 0.2 0.4 0.6 0.8 1.0
Insulin (mg/ml)

Figure 3

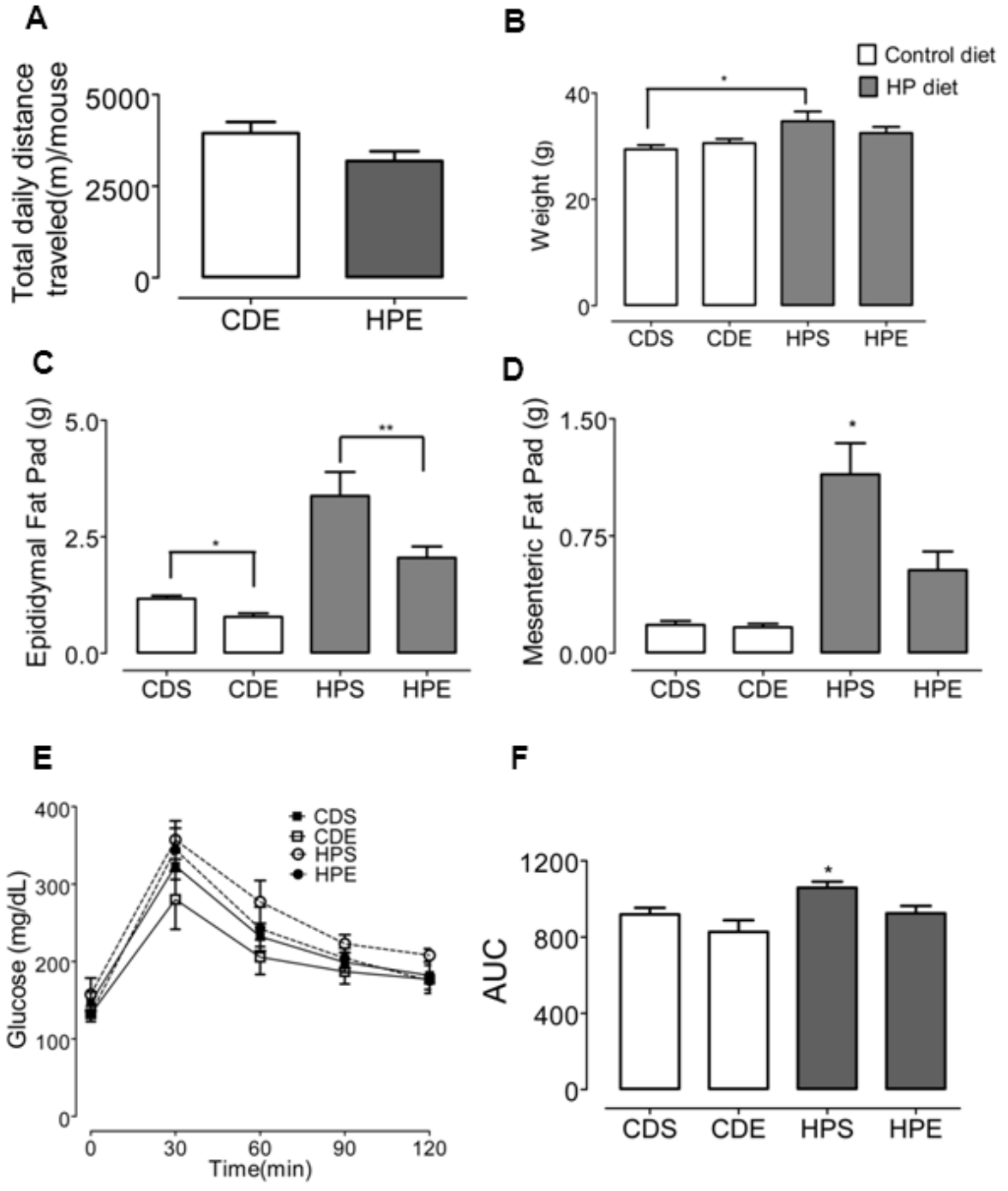
A
Total daily distance traveled(m)/mouse
5000
2500
0
CDE
HPE
B
Control diet
HP diet
Weight (g)
40
20
0
*
CDS
CDE
HPS
HPE
C
Epididymal Fat Pad (g)
5.0
2.5
0.0
*
**
CDS
CDE
HPS
HPE
D
Mesenteric Fat Pad (g)
1.50
0.75
0.00
*
CDS
CDE
HPS
HPE
E
Glucose (mg/dL)
400
300
200
100
0
CDS
CDE
HPS
HPE
0
30
60
90
120
Time(min)
F
AUC
1200
800
400
0
*
CDS
CDE
HPS
HPE

Table 1: IL-1 and TNF-α

		CDS	CDE	HPS	HPE
IL-1	Serum	0,274 ± 0,011	0,256 ± 0,023	0,258 ± 0,015	0,237 ± 0,042
	Hippocampus	0,059 ± 0,012	0,063 ± 0,009	0,064 ± 0,023	0,062 ± 0,005
TNF-α	Serum	0,162 ± 0,020	0,150 ± 0,020	0,163 ± 0,021	0,127 ± 0,022
	Hippocampus	0,039 ± 0,007	0,043 ± 0,004	0,042 ± 0,016	0,039 ± 0,003

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