Overexpression of Heat Shock Cognate Protein 71 kDa and Pyruvate Dehydrogenase in the Brain Tissue at the Early Stage of High Fat Diet Consumption

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ABSTRACT

High-fat diet (HFD) increases the risk of obese, while obesity increases the prevalence of metabolic syndrome and non-communicable diseases. Therefore, it will be interesting to evaluate the changes in metabolic parameters and brain profile upon the early consumption of HFD. In this study, a total of 12 Sprague-Dawley male rats were divided into two groups (n = 6), each group was fed with normal diet and HFD (39% of total calories from fats), respectively, for 6 weeks consecutively. The body weight, blood glucose, cholesterol and triglyceride values were measured. Furthermore, the brain and visceral adipose tissues were harvested at the end of the experiment. Protein was extracted from the brain tissue, and the protein extracts were separated by using two-dimensional gel electrophoresis and analyzed by liquid chromatography tandem mass spectrometric analysis (LC/MS/MS). In terms of food calorie, the rats fed with HFD consumed more energy than the rats fed with normal diet. Nevertheless, the blood triglyceride and cholesterol, and the visceral adipose tissue of both the HFD and normal diet fed rats were indifferent. At the molecular level, overexpression of stress proteins, namely heat shock cognate protein 71 kDa (Hsc70) and pyruvate dehydrogenase were detected in brain tissue of HFD group. These results suggest that HFD intake causing significant change in brain proteins profile at the early phase of its consumption when no clear metabolic changes were observed. This showed that the brain was affected by HFD.

1. Introduction

The prevalence of obesity and overweight is increasing worldwide. Death associated to obesity accounted for approximately 2.8 million yearly (Roberto et al. 2015). Obesity is recognised as a chronic disease that increases the global burden of non-communicable diseases such as cardiovascular disease, diabetes and cancer (Engin 2017).

Body weight will be increased if the energy intake exceeds the energy expenditure over a given period of time (Hill et al. 2012). Dietary fat is one of the major factors contributing to energy intake related to obesity, and adiposity increases with increasing fat content up to 60% (Hu et al. 2018). The acceptable macronutrient distribution range for total fat is 20–35% of total daily calories, diets that fall beyond this range are considered as HFD (Bothclett and Wu 2018). Donahoo et al. (2008) suggested that HFD increases the likelihood of excessive energy intake as fats are stored more efficiently than excess carbohydrates. Chronic HFD consumption could result in weight gain/obesity, increased systemic inflammation, oxidative stress and disruption of glucose homeostasis (Bothclett and Wu 2018b).

The brain is enriched with fat; lipids account for ~50% of brain’s dry weight, possibly because brain uses more energy than other organs (Chianese et al. 2017). Dietary fats transfer energy to the brain and therefore calories intake has an impact on brain health. Over-consumption of calories could cause the formation of free-radicals that surpass the buffering capacity of cellular anti-oxidant responses, which in turn reduce synaptic plasticity and lead to cell damage (Gomez-Pinilla 2008). Typically, diet contains mixture of different types of fatty acid including saturated and mono/polyunsaturated...
fatty acids. Dietary fats impact brain's processes by regulating the synaptic transmission, membrane fluidity and signal-transduction pathways (Chianese et al. 2017; Melo et al. 2019).

Saturated fatty acids (SFAs) were the main contributors of obesity (Figueiredo et al. 2017). Furthermore, an SFA, namely palmitic acid can induce the activation of Toll-like receptor 4 in hypothalamic microglia of the brain (Valdearcos et al. 2014), that potentially lead to brain inflammation. Oil palm based cooking oil used in this study was made up of 100% high grade pure palm olein, consisting of ≈ 40% of palmitic acid and ≈ 40% of oleic acid (Malaysia Palm Oil Board). Animals fed with high saturated fat diet was shown to have higher concentrations of protein amyloid-ß, a marker for Alzheimer's disease (Galloway et al. 2007), and compromised blood-brain barrier integrity (Farrall and Wardlaw 2009; Takechi et al. 2013).

Diet consisting of HFD is the leading cause of obesity, while obesity is one of the major causes of non-communicable diseases. Although the impact of HFD on health was well documented, the bodily change due to early consumption of HFD is not well reported. Therefore, we aimed to study the impact of HFD on the metabolic parameters and brain protein profile of rats that were fed with HFD for six-week consecutively. The information may be useful for the intervention of obesity-related diseases.

2. Materials and Methods

2.1. Materials

Urea, trizma base, thiourea, CHAPS, acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), glycine, sodium chloride, Brilliant Blue R, Bromophenol blue, N,N,N',N'-tetramethylethylenediamine (TEMED), DL-dithiothreitol (DTT) and iodoacetamide were obtained from Sigma-Aldrich (Missouri, USA). The diet powder (#1820) was customized from Altromin (Lage, Germany). Sodium phosphate dibasic dihydrate (NaH₂PO₄·2H₂O), sodium phosphate monobasic dihydrate (NaH₂PO₄·H₂O) and acetic acid (glacial 100%) were purchased from Merck (Massachusetts, USA). A few solvents have been used including methanol (analytical grade, R and M chemical), MS grade acetonitrile and MS grade water (Fisher scientific, Hampton USA), and acetone (QRëC, New Zealand). Trichloroacetic acid was procured from Merck (New Jersey, USA). The RC DCTM protein assay kit, protein assay standard I (lyophilized bovine γ-globulin), ampholytes 3/10 and low melting temperature agarose were purchased from BioRad (California, USA). Mineral oil (light, white) and glycerol were obtained from Ameresco (Ohio, USA). The applied anaesthetic agents were ketamine (TROY Laboratories, Glendenning, Australia) and xylazine (Indian Immunologicallys, Jubilee Hills, India). Formic acid was purchased from Fisher scientific (Hampton, USA). Palm olein cooking oil was obtained from Buruh (Lamsoon, Malaysia).

2.2. Procedure for Animal Study

2.2.1. Animals Preparation

A total of 12 Sprague-Dawley male rats, aged 12 weeks and weighed between 280–300 g were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. The procedures of housing and handling used in this study conformed to the animal ethics guidelines of the USM Institutional Animal Care and Use Committee (USM IAUC, USM/Animal Ethics Approval/2016(717)). The rats were acclimatized individually (one rat in a cage) for 1 weeks prior to the experiment under controlled environmental conditions (12 h light/dark cycles, 22-24°C) to allow them habitually familiar to new environment. Finally, only the rats weighed between 300-350 g were used in this study.

2.2.2. Animal Feeding Program

After acclimatization, the rats were divided into two groups, each group consisted 6 animals (n = 6), and one rat is placed in a cage. The rats were further monitored for 4 weeks. After 4 weeks, one group was fed with normal diet food pellets while the other group was fed with HFD. The Calorie for normal diet was 3.61 kcal/g while for HFD, it was 4.56 kcal/g. Both types of food pellet were daily supplied to the rats at 25±1 g weight, together with water (ad libitum) for 6 weeks. Both normal diet and HFD pellets were self-prepared by mixing the custom-made diet powder with water at a ratio of 1:1 and baked in an oven at 42°C, overnight. The HFD pellets were made to contain extra oil by adding palm olein cooking oil at 22.4% (w/v). On each day, the uneaten food pellet that left was weighed and was replaced with fresh food pellets.

2.3. Procedure of Biochemical Analysis

2.3.1. Harvesting of Animal Specimens

At the end of experiment, all rats were euthanized using a cocktail of ketamine (75 mg/kg) and xylazine (8 mg/kg) followed by cardiac puncture using 27G×½” needle to collect blood into clean tubes (MiniCollect®, Greiner Bio-One, Austria). The blood was spun at 3000 × g, 4°C for 10 mins. After the centrifugation, the supernatant (plasma) was immediately transferred into the clean polypropylene tubes and stored at -80°C until further analysis. After cardiac puncture, the brain was harvested and rinsed 3 times with phosphate buffered saline (pH 7.4, 140°C).
mM sodium chloride, 10 mM phosphate buffer and 3 mM potassium chloride). The brain tissue was cut into two parts (left and right brain). The brain tissues were then ground into fine powder forms in liquid nitrogen and stored at -80°C.

2.3.2. Measuring of Blood Biochemical Parameters and Key Health Factors

The body weight and fasting blood glucose (FBG) level of each rat were monitored and recorded weekly. The triglycerides and cholesterol analysis were carried out according to the manufacturer protocol (Thermo scientific, Netherlands). The plasma was thawed to room temperature. A volume of 300 µl Infinity™ Cholesterol Liquid Stable Reagent or Infinity™ Triglycerides Liquid Stable Reagent, respectively was pipetted into 96 well plates. Then, 3 µl of plasma was added to the wells (Sample: Reagent ratio is 1:100). The plate was left at room temperature for 15 minutes and readings was measured using a microplate reader at 500 nm wavelength. Chemistry Calibrator (Pointe Scientific, USA) was used as the calibrators. The values of cholesterol and triglycerides were calculated by comparing the absorbance values to the calibrators absorbance values. The mean values from each treatment groups were presented.

2.4. Procedure for Protein Analysis

2.4.1. 2D-gel Electrophoresis and In-gel Digestion

Prior to 2D gel electrophoresis, the proteins were extracted from ground brain powder, purified using trichloroacetic acid (TCA)/acetone precipitation, then the concentration was measured using the RC DCTM protein assay kit. A 2D gel electrophoresis using IPG strip (pH 4–7) was employed. The IPG strips were rehydrated with 600 µg of protein for at least 15 hours at 20°C before the isoelectric focusing. The PROTEAN®i12™ system (Bio-Rad, USA) was used to perform the focusing at 20°C. The applied voltage was from 0–150 V (rapid climb) for 1 h, 150–200 V (linear, 1 h), 200–500 V (linear, 1 hr), 500–4,000 V (linear, 2 h) and the exposure of 10,000 Vhr. The focused IPG strips were then equilibrated twice in equilibrium buffer [6M urea, 0.375M Tris-HCl (pH 8.8), 2% (w/v) SDS, 20% (v/v) glycerol], first with 2% (w/v) DTT and then with 2.5% (w/v) iodoacetamide in dark for 30 minutes each. Subsequently, the protein loaded strips were subjected to SDS-PAGE (10% polyacrylamide) using a Mini-PROTEAN tetra cell (Bio-Rad, USA). The IPG strips were laid on top of the polyacrylamide gel and fixed with agarose gel [1% (w/v) agarose, 25 mM trizma base, 192 mM glycine, 0.1% (w/v) SDS, trace amount of bromophenol blue and run with a constant voltage supply of 120 V until the dye-front reached the bottom of the gel. The gels were then stained with Coomassie blue and the image was captured. The detected protein spots were analysed using PDQuest software (Ver. 7.3). Gel images were cropped, and the protein spots were matched and compared statistically. A representative image containing the common spots from every image within the same group was displayed. The protein spots of interest were defined as differentially expressed when p <0.05 and/or ≥2-fold expression change in spot intensity was identified between two groups. Differentially expressed spots were excised from the gels and subjected to trypsin in-gel digestion.

2.4.2. In Gel Digestion

In brief, trypsin in-gel digestion involved gel distaining, followed by reduction, alkylation and digestion of protein in the gel using trypsin. The targeted spots in the gel was cut and sliced into gel pieces. The gel pieces was distained and dehydrated using ammonium bicarbonate (NH₄HCO₃) (100 mM) and 100% acetonitrile respectively. The distained gel spots were then vacuum dried. The protein in the gel pieces was reduced in NH₄HCO₃ (100 mM) containing DTT (10 mM) for 1 hour at 56°C. The DTT solution was then discarded and replaced by iodoacetamide (55 mM) in NH₄HCO₃ (100 mM) and incubated in dark at room temperature for 45 minutes for alkylation. Next, iodoacetamide residues was removed. The gel pieces was washed by repeating the hydration and dehydration steps, vacuum dried and then proceeded to trypsin digestion. The gel pieces were immersed in digestion buffer (5 mM calcium bicarbonate, 50 mM NH₄HCO₃ containing trypsin (15 ng/µl) for 1 hour on ice. After that, the trypsin digestion buffer was discarded, replaced with fresh digestion buffer without trypsin and incubated overnight at 37°C. On next day, the digestion buffer was collected into a clean polypropylene tube. The digested peptides were further extracted from the gel pieces by using 20 mM NH₄HCO₃ and 70% acetonitrile with 5% formic acid. The obtained solutions were pooled together, dried under nitrogen stream and stored at -80°C prior to LC-MS/MS analysis.

2.4.3. Identification of Protein Identity using LC-MS/MS Analysis

Protein analysis was performed using Easy-nLC II nano liquid chromatography system (Thermo Scientific, USA) coupled with Finngan LTQ linear ion trap mass spectrometer (Thermo Fisher, USA). The pre-column, Easy-column C18 (20 × 0.10 mm i.d., 5 µm, Thermo Scientific, USA) was equilibrated with 15 µl at 3 µl/min while the analytical column.
Meanwhile, Easy-Column C18 (100 × 0.75 mm i.d., 3 µm, Thermo Scientific, USA) was equilibrated with 4 µl at 0.3 µl/min. A gradient mode was set from 5% to 100% of buffer B in 80 mins: Buffer (A) 0.1% (v/v) formic acid in MS grade water and (B) 0.1% formic acid in 100% acetonitrile. The parameters of mass spectrometry were set at full scan mass analysis from m/z 300-2,000 at a resolving power (FWHM) of 60,000 at m/z and acquisition time of 1 sec at 220°C with a source voltage of 2.1 kV. The data-dependent MS/MS analyses triggered by the 8 most abundant ions from the parent mass list. Singly or unassigned charged peptides were rejected. The fragmentation was done by collision induced dissociation (CID) with collision energy of 35 V. The obtained mass spectra were internally calibrated with trypsin peptides. The masses were then used to search in PEAK® studio version 7.0 software (Bioinformatic solution, Canada) against the SwissProt2019 database for identified proteins. Carbamidomethylation and oxidation (M) were set as variable post-translational modifications (PTM) with maximum missed cleavage per peptide at 3. The maximum variable PTM per peptide was set at 3.

2.4.4. Statistical Analysis

The statistical analysis including student t-test and Two-way ANOVA of the collected data was performed using GraphPad Prism ver.8. All the statistic results were presented in mean ± standard deviation.

3. Results

3.1. Effect of HFD Intake on Key Health Markers

The daily intake of HFD pellets (17.5±1.1 g) by rats was significantly less than those fed with normal diet pellets (19.6±1.1 g) although the same amount of food pellets (25±1 g) were supplied to the rats daily. The calorie intake for the rats fed with HFD (70.8±7.3 kcal/day) was insignificantly higher than for those of normal diet (61.5±3.3 kcal/day). The metabolic efficiency, which was defined as calorie per gram of weight gain in 6 weeks

Table 1. The food intake, biochemical parameters of rats fed normal diet and high fat diet (HFD)

|                          | Normal diet | HFD       | T-test p value |
|--------------------------|------------|-----------|---------------|
| Food intake (g/day)      | 19.6±1.1   | 17.5±1.1  | 0.015         |
| Calories intake (kcal/day)| 61.5±3.3  | 70.8±7.3  | 0.078         |
| Serum cholesterol (mmol/l)| 1.9±0.2   | 2.1±0.3   | 0.296         |
| Visceral adipose tissue (g) | 6.1±1.7   | 8.2±2.7   | 0.138         |
| Visceral adipose tissue (% of body weight) | 1.5±0.4   | 2.2±0.6   | 0.058         |
| Metabolic efficiency (kcal/g) | 249.8±91.6 | 143.9±69.3 | 0.377         |

Data are presented as mean ± SD (n = 6 rats), p <0.05 is statistically significant. Metabolic efficiency is defined by calories intake divided by weight gain in 6 weeks.

Figure 1. NC (normal diet), HFD (high fat diet). Effect of high fat diet on (A) body weight and (B) fasting blood glucose of rats over a 6-week time course. Data are presented as mean ± SD (n = 6-7 rats).
groups were subjected to mass spectrometry analysis. Two spots (spot 6702) and (spot 5201) were up-regulated at significant levels (p <0.05), these protein spots were identified as heat shock cognate 71 kDa protein and pyruvate dehydrogenase by LCMS/MS analysis and protein database search (Table 2). The identified heat shock protein was characterized by oxidation of methionine residues.

3.2. Effect of HFD on Brain Protein Expression

Mass spectrometry analysis was applied to identify the identity of the proteins of interest. The number of protein spots detected in HFD group was almost identical to normal diet group (=145 spots) (Figure 2). Table 3 shows the protein spots’ images, respectively from the HFD and normal diet fed groups. Of these protein spots, the protein spots with >2 fold difference in intensity between HFD and normal diet groups were subjected to mass spectrometry analysis.

Table 2. The identified proteins from LC-MS/MS

| Spot number | Identified protein                      | Uniprot database accession no. | % coverage | Theoretical mW/pi | Matched peptides |
|-------------|----------------------------------------|---------------------------------|------------|-------------------|-----------------|
| 6702        | Heat Shock cognate protein (71 kDa)    | P63018                          | 28         | 70.739/5.37       | 18              |
| 5201        | Pyruvate dehydrogenase                 | P49432                          | 29         | 35.841/5.29       | 11              |

Table 3. The replicates gel images for spot 6702 and spot 5201 by normal diet and HFD fed rats

| Type of feed | Spot 6702 | Fold-change in HFD group | P value |
|--------------|-----------|--------------------------|---------|
| Normal diet  |           | ≥2-fold                  | P <0.02 |
| HFD          |           |                          |         |

| Type of feed | Spot 5201 | Fold-change in HFD group | P value |
|--------------|-----------|--------------------------|---------|
| Normal diet  |           | ≥2-fold                  | P <0.05 |
| HFD          |           |                          |         |
4. Discussion

Palm-oil based cooking oil has been used commonly in preparation of food. The health guideline for healthy adults recommended that daily calorie intake contributed by fat to consist ≤30% of total calories intake (Marshall and Besselsen 2002). In this study, we have carried out a comparison on two different values of fat contributing calories, namely HFD (39%) and normal diet (12%), respectively and evaluated the impact of these food on rats in a 6 weeks continual feeding program. Palm-oil based cooking oil has been used to enrich the fat content in HFD.

Metabolic efficiency is defined as the quantity of energy required to cause an increase in one gram of body weight (kcal/g). The higher value of metabolic efficiency by normal diet fed rats compared to HFD fed rats showed that more food (resulted in higher energy) was required by normal diet fed rats to gain a gram of body weight. This explained that although normal diet fed rats consumed significantly more quantity of food, the final body weight of the two groups of animals were insignificantly different after the feeding program (6 weeks). The visceral adipose tissue (VAT) of normal diet fed rats and HFD fed rats were 6.1 g and 8.2 g, respectively (Table 1). These values indicated that the calorie gained by HFD was stored as VAT, although it did not cause a significant VAT gain at this early stage of feeding program, on a long run, this accumulation of VAT by HFD can lead to obesity. Visceral adipose tissue (VAT) is known to be a pathogenic fat depot due to its association with various pathological conditions such as metabolic syndrome and cancers (Wagenknecht et al. 2003; Carr et al. 2004; Freedland 2004; Shuster et al. 2012) and causes ectopic lipid accumulation and lipotoxicity (Hardy et al. 2012). As for the six week feeding program, the deposition of VAT in rats between HFD and normal diet rats was not significantly different although a higher and more intense deposition of VAT was seen in HFD group (data not shown). Prolonged deposition of VAT is expected to increase VAT deposition.

Obesity or accumulation of excessive body fat is reported to increase the risk of developing insulin resistance and T2DM (Kahn et al. 2006). Mean total cholesterol and triglycerides concentrations are higher in obese persons in comparison to normal weight subjects, which increases the risk of coronary heart disease (Szczygiel ska et al. 2003). In conjunction with this, the serum cholesterol level of both feeding groups were within a normal range. The palm oil-based HFD in this study contained only traces amount of cholesterol. Usually, serum cholesterol could have resulted from the uptake dietary cholesterol via intestine adsorption and/or endogenously produced by the body (Arnold and Kwiterovich 2003). Hence, our data suggested the palm oil based HFD did not cause elevation in the serum cholesterol level and could possibly be due to the low amount of cholesterol in the diet.

Although there was no significant difference in the metabolic parameters and body weight of the rats after 6 weeks feeding program with HFD, at the molecular level, HFD was found to cause changes in the protein expression in brain tissues. The 2D gel electrophoresis analysis of rats fed HFD and normal diet revealed the number of protein spots detected was relatively similar (Figure 2). In this study, only the common protein spots that were found in all the rats within the same groups were considered for comparison study, this is to exclude protein spots that were expressed heterogeneously by individual animals and therefore cannot represent changes that due to diets treatment. HFD feed rats showed significant elevation of Hsc70 and pyruvate dehydrogenase expression by at least 2-fold in the brain tissue of rats fed on HFD compared to those fed on normal diet (Table 2). At the early stage of continually feeding of HFD may seems to produce tolerable effect to the physical body function of the rats as no significant difference in the metabolic parameters nor body weight increase were detected. However, the brain may experience the impact of HFD much earlier than the body. When the impact is prolonged, it can lead to mental health disorder (Lupien et al. 2018). Both the elevation of Hsc70 and pyruvate dehydrogenase expression could also correlate to HFD inducing oxidative stress (Freeman et al. 2013; Tan et al. 2018). This suggests that HFD possibly induced an early response to development of underlying health diseases.

Heat shock cognate protein 71 kDa is also known as Hsc70, Hspa8 and Hsp73. Hsc70 is a constitutively expressed member of the Hsp70 chaperone protein family that protects the proteome from stress and binds to nascent polypeptides to facilitate correct folding in an ADP/ATP-dependent manner (Stricher et al. 2013). Hsp70 is a common stress diagnostic marker for central nervous system (Fink et al. 1997; Beaucamp et al. 1998; Lu et al. 2014). The detected Hsc70 spot in rats fed HFD was characterised by post-translational oxidation of methionine residues. We have previously observed the upregulation of un-oxidised Hsc70 in diabetic rats on HFD treated with metformin. Oxidation of proteins can be used as a marker for oxidative damage and cellular stress (Shacter 2000). Hence, the up-regulated Hsc70 might
serve as an early indication of redox stress caused by HFD. Hsc70 is constitutively expressed and performs functions related to normal cellular processes, such as protein ubiquititation and degradation (Goldfarb et al. 2006), which can lead to a wide impairment in the function of an entire repertoire of proteins. Overexpression of Hsc70 was reported in the Parkinson disease (Mandel et al. 2005).

Pyruvate dehydrogenase (E1) was also differentially expressed in rats on HFD compared to those fed a normal diet. Pyruvate dehydrogenase is involved in pyruvate oxidation, converting pyruvate into acetyl-CoA and linking glycolysis with the citric acid cycle. The three-enzyme pyruvate dehydrogenase complex, of which E1 is a component, is an important target of oxidative stress. For example, hippocampal pyruvate dehydrogenase complex E1α subunit immunostaining is reduced 90% in animal models resuscitated on 100% O₂ (Martin et al. 2005). The differences in expression of pyruvate dehydrogenase suggest an alteration of glucose metabolism and possibly oxidative stress in the brain by HFD. Pyruvate dehydrogenase is an enzyme that catalyses the reaction of pyruvate, the overexpression of pyruvate dehydrogenase may also indicate the accumulation of pyruvate, which has been associated to the treatment of obesity (O‘Mathuna 1999). Pyruvate contribute to weight loss by increasing the breakdown of fat (Stanko et al. 1992). Therefore, we believed that pyruvate has been induced as the feedback mechanism to counteract the increased calories due to fat intake, which implies in the weight indeterminacy between the rats fed with HFD and NC despite of higher calories diet by HFD. The increased expression of pyruvate dehydrogenase may lead to the reduction in pyruvate quantity and causes the weight increase thereafter by continual consumption of HFD.

In conclusion, the fat content of a diet is an important determinant of energy intake. Upon six weeks of continual intake of HFD, no significant difference in the determined metabolic parameters and body weight of rats fed with HFD compared to those fed with normal diet. Nevertheless, significant overexpression of two chronic stress related proteins, namely Hsc70 and pyruvate dehydrogenase were detected. The upregulation of these two proteins indicate that the brain was experiencing oxidative stress and alteration of glucose metabolism as the result of HFD at its early stage of consumption. These data indicate that a short period of excessive fat intake has an impact on the brain at the molecular level much earlier than the onset of any standard clinical indications.

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