AMP-activated protein kinase mediates insulin-like and lipo-mobilising effects of β-glucan-rich polysaccharides isolated from Pleurotus sajor-caju (Fr.), Singer mushroom, in 3T3-L1 cells

G. Kanagasabapathy a, c, K.H. Chua a, c, S.N.A. Malek b, c, S. Vikineswary b, c, U.R. Kuppasamy a, c, *

a Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
b Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
c Mushroom Research Centre, University of Malaya, 50603 Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 22 January 2013
Received in revised form 25 June 2013
Accepted 13 August 2013
Available online 21 August 2013

Keywords:
Pleurotus sajor-caju Lipogenesis Lipolysis Polysaccharides Oxidative stress

ABSTRACT

Mushrooms have been used to treat various diseases for thousands of years. In the present study, the effects of Pleurotus sajor-caju mushroom on lipogenesis, lipolysis and oxidative stress in 3T3-L1 cells were investigated. The β-glucan-rich polysaccharides (GE) from P. sajor-caju stimulated lipogenesis and lipolysis but attenuated protein carbonyl and lipid hydroperoxide levels in 3T3-L1 cells. This extract caused an increase in the expression of 5-AMP-activated protein kinase subunit γ-2 (PKRAG2) and 5-AMP-activated protein kinase subunit γ-3 (PKRAG3) when compared to control (untreated) cells. Moreover, GE induced the expressions of hormone-sensitive lipase, adipose triglyceride lipase enzymes, leptin, adiponectin and glucose transporter-4 in 3T3-L1 cells which may have contributed to the lipolytic and insulin-like activities observed in this study. These findings suggest that GE is a novel AMPK activator that may be valuable in the formulation of nutraceuticals and functional food for the prevention and treatment of diabetes mellitus.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

The increasing worldwide incidence of diabetes mellitus (DM) in adults constitutes a global public health burden. The prevalence of DM was estimated to be 2.8% of the general population in the year 2000. It is predicted to be doubled by the year 2030 to about 380 million (Kanagasabapathy et al., 2012) and India, China and the United States will have the largest numbers of people with diabetes (Frode & Medeiros, 2008). Hence, there is a clear need for effective pharmaceutical intervention for treating DM (Umar, Ahmed, Muhammad, Dogarai, & Mat Soad, 2010). The most effective hypoglycemic drugs are insulin, sulphonylurea derivatives, biguanides, thiazolidinediones and alpha glucosidase inhibitors. However, these agents have undesirable side effects such as obesity, high blood pressure and heart disease (Slovecak, Pavlik, & Slovacova, 2008).

Adipose tissue (composed of adipocytes) plays a critical role in lipid metabolism, glucose homeostasis and energy balance. The primary role of adipose tissue is to store energy in the form of triglycerides when energy intake exceeds energy expenditure and to release it in the form of free fatty acids in starvation. Adipocyte differentiation, known as lipogenesis, is the anabolic process of fat cell development (Lee et al., 2010) while lipolysis is the catabolic process that causes the breakdown of triglycerides stored in fat cells to fatty acids and glycerol (Jeon et al., 2004). Insulin favours lipid storage through the activation of lipogenesis, lipoprotein lipase synthesis and export to the vascular endothelium and triglyceride esterification through the production of glycerol-phosphate from glucose. By contrast, adrenergic hormones (glucagon and epinephrine) activate lipolysis through binding to a β-adrenergic receptor and production of cellular cAMP. In adipose tissue, lipolysis (fasting and exercise) activates AMP-activated protein kinase (AMPK). AMPK acts as a fuel sensor and regulates glucose and lipid homeostasis in adipocytes (Kong, Kim, & Kim, 2009). Once activated, AMPK phosphorylates a number of proteins and modulates the transcription of genes implicated in the regulation of energy metabolism to switch on catabolic pathways that produce ATP and switch off anabolic pathways that consume ATP (Daval, Foufelle, & Ferre, 2006).

Mushroom polysaccharides have been studied extensively because they are known to possess many medicinal properties, such as antitumor, immune-modulating, antioxidant, anti-hypercholesterolemic (Oyetayo, 2008), antiviral, antibacterial, anti-inflammatory and anti-diabetic activities (Fukushima, Ohashi, Fujiwara, Sonoyama, & Nakano, 2001). In Malaysia, the widely
cultivated ‘edible fungal food’ is the *Pleurotus* mushroom, commonly referred to as oyster mushroom (cendawan tiram). Oyster mushrooms have been discovered to have definite nutritive and medicinal values with good quality proteins, carbohydrates, iron, vitamins and very little lipid or starch. Reports on medicinal properties of *P. sajor-caju* polysaccharides are still scant but chemical characterisations of the polysaccharides are well-known. Pramanik, Mondal, Chakraborthy, Rout, and Islam (2007) have reported that polysaccharides from hot water extract of *P. sajor-caju* mushrooms have been discovered to have definite nutritive and heteropolysaccharides. The polysaccharides are identified as (1→3),(1→6)-β-glucans.

Therefore, the present study was undertaken to investigate the effects of β-glucan-rich polysaccharides of *P. sajor-caju* (GE) on lipogenic and lipolytic activities in 3T3-L1 cells, as well as the oxidative stress level during these processes. In order to elucidate the underlying mechanism, the protein and gene expression studies were carried out using the adipocytes treated with GE and insulin as positive control. The findings of this study may be valuable in the formulation of nutraceuticals and functional food for the prevention or adjuvant therapy for diabetes mellitus.

2. Materials and methods

2.1. Mushroom samples

All necessary permits and permission for the collection of materials for the described field of study were obtained and the party involved is duly acknowledged. Fresh fruiting bodies of *P. sajor-caju* (10 kg) were grown and collected from a mushroom farm in Semenyih, Selangor Darul Ehsan, Malaysia. Authentication of *P. sajor-caju* was carried out by the Mushroom Research Centre (MRC), University of Malaya and a voucher material (KUM 50082) for this study was deposited at the MRC culture collection.

2.2. Isolation, purification and determination of β-glucan in polysaccharide (GE) from hot-aqueous extract of *P. sajor-caju*

The isolation and purification of polysaccharide were based on the method described by Roy, Maiti, Mondal, Das, and Islam (2008) except that, in this study, the polysaccharide was not further purified by gel permeation chromatography. The β-glucan level in GE was estimated using a β-glucan kit (specific for mushroom and yeast) purchased from Megazyme International (Ireland). The enzyme kit, contains exo-1,3-β-glucanase, β-glucosidase, amylglucosidase, plus invertase, glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-aminantipyrine) and glucose standard solution. To estimate the total glucan content, GE was hydrolysed with 37% hydrochloric acid (v/v) for 45 min at 30 °C and continued for 2 h at 100 °C. After neutralisation with 2 M potassium hydroxide, hydrolysis (onto glucose) was done using a mixture of exo-β-(1-3)-α-glucanase plus β-glucosidase in sodium acetate buffer (pH 5.0) for 1 h at 40 °C. To measure the total glucan content, glucose oxidase-peroxidase mixture was added to GE and incubated for 20 min at 40 °C. The absorbance was measured using a spectrophotometer (Bio-Tek Instruments Inc, USA) at 510 nm. The α-glucan content was estimated according to the same method as described above, after enzymatic hydrolysis with amylglucosidase and invertase. The β-glucan content was calculated by subtracting the α-glucan from the total glucan content.

2.3. Reagents and cells

3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin and insulin were purchased from Sigma Chemical Co. (USA). Dexamethasone and 1-methyl-3-isobutyl xanthine (IBMX) were purchased from ICN (USA). Amphotericin B (Fungizone®) was purchased from Flow Lab (Australia). Thiazoyl blue tetrazolium bromide (MTT) was purchased from Bioworld (USA). Isopropanol was purchased from Thermo Fisher Scientific Inc. (USA).

2.4. Assessment of viable cells

The cells were seeded at a density of 3 × 10^4 cells/ml. Preadipocyte suspension (90 μl) was transferred into sterile 96-well tissue culture plates and incubated for 24 h at 37 °C and 5% CO₂. Then, aliquots (10 μl) of different concentrations of GE were added to the culture wells to yield final concentrations of one to 1000 μg/ml and the plates incubated for 24 and 48 h, respectively. Milli-Q water was used as the negative control. The viability of preadipocytes was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The absorbance was measured at 560 nm and 700 nm (reference), using a spectrophotometer. The percentage of cell viability was calculated as stipulated below:

\[
\text{Cell viability} (\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{control}}} \times 100
\]

2.5. Differentiation and lipogenesis assay

For the differentiation process, cells were seeded at a density of 3 × 10^4 cells/ml in each well of a 24-well culture plate. Confluent preadipocytes were differentiated with part-1 differentiation media (DM1) (10% FBS, 2 mM fungizone, 2 mM penicillin–streptomycin, 1 μM dexamethasone, 0.5 mM IBMX, 10 μg/ml insulin and DMEM media) for two days at 37 °C and 5.0% CO₂. On day three, DM1 was removed and the cells were replenished with part-2 differentiation media (DM2) (10% FBS, 2 mM fungizone, 2 mM penicillin–streptomycin, 10 μg/ml insulin and DMEM media). On day five, the cells were replenished with growth media (GM) (FBS, 2 mM fungizone, 2 mM penicillin–streptomycin and DMEM media). Each concentration of GE (0.01–1000 μg/ml) was tested in triplicate. After incubating for 48 h, the adhering cells were washed with PBS and fixed with formalin, rinsed with 60% isopropanol, stained with Oil Red O working solution (0.3% in 60% isopropanol) and incubated in the dark for 2 h. Subsequently, the cells were washed with phosphate buffered saline (PBS). The cells were then dissolved in 100% isopropanol and the absorbance was measured at 510 nm, using a spectrophotometer. Insulin was used as the positive control while Milli-Q water was used as the negative control. The percentages of lipogenic activity were calculated as stipulated below:

\[
\text{Lipogenic viability} (\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{control}}} \times 100
\]

2.6. Lipolysis assay

Fully differentiated adipocytes were treated with various concentrations (1–1000 μg/ml) of GE. After 4 h of treatment, lipolysis assay was performed according to the protocol provided in the lipolysis kit purchased from ZenBio (USA). This method is based on a coupled reaction, which measures non-esterified fatty acids and glycerol released by adipocytes. The glycerol and free fatty acids were quantified by an enzyme-based reaction, which yields a proportional quantity of hydrogen peroxide (H₂O₂), which is measurable once modified into chromogenic compounds. Isoproterenol
was used as positive control and Milli-Q water was used as negative control. Two parameters were measured, namely glycerol and free fatty acids (FFA). The fold of induction of glycerol and fatty acids was calculated, based on the standard curves of glycerol and fatty acid, respectively.

2.7. Protein carbonyl content assay (AOPP)

The level of AOPP present was determined using the method previously described (Kuppusamy et al., 2005). Fully differentiated adipocytes were treated with various concentrations of GE. After 48 h of treatment, the AOPP level in the culture media was measured. Chloramine-T solution of known concentrations (0–500 μM) was used as a standard for the estimation of AOPP concentration and the results were expressed as μmol chloramine-T/g.

2.8. Lipid hydroperoxide assay

The assay was carried out using the method described by Esterbauer and Cheeseman (1990) with modifications. Fully differentiated adipocytes were treated with various concentrations of GE. After 48 h, the lipid hydroperoxide level in the culture media was measured. 1,1,3,3-Tetraethoxypropane (TEP) solution of known concentration (2.5–20 μM) was used as a standard for quantification and the lipid hydroperoxide level was expressed as μmol TEP/g.

2.9. Protein extraction, iTRAQ labelling and identification using LCMS/MS

The fully differentiated adipocytes were treated with GE (100 μg/ml final concentration) and Milli-Q water was used as negative control. After 48 h of incubation, the protein was extracted from the adipocytes, using the ITSIPrep™ kit (ITSIbiosciences, USA). The quantitation of protein concentration was done using the 2D Quant Kit (GE Healthcare, USA). Bovine serum albumin was used as a standard and the absorbance was read at 480 nm, using a nanophotometer (IMPLEN GmbH, Germany). The iTRAQ labelling and identification, using LCMS/MS, was sent and performed by ITSIbiosciences, USA (Project Code: ITSI BIO 2012-40). Briefly, protein at a concentration of 28 μg (for both control and treated samples) was dissolved in water. A ToPA™ kit (ITSIbiosciences, USA) for protein precipitation was used to remove interfering compounds in the samples. Precipitated proteins were then dissolved in iTRAQ dissolution buffer containing denaturant (2% SDS). The dissolved protein was reduced and alkylated according to the manufacturer’s protocol and 5% trypsin was used for overnight digestion. Trypsinized proteins were labelled with iTRAQ reagents – 116 for treated and 114 for the control samples, respectively. After 2 h of labelling with iTRAQ reagents, the sample was mixed and diluted 10× with SCX loading buffer. The diluted sample was cleaned, using a strong cation-exchange cartridge to remove excess iTRAQ reagents. Eluents were dried, using a speed vac and reconstituted in 5% acetonitrile with 0.1% formic acid. Samples were loaded onto a PicoFrit™ C18 Nanospray column (Thermo-Scientific, USA).

2.10. Mass-spectrometry conditions

A linear acetonitrile gradient was used to separate the tryptic peptides with the following conditions: acetonitrile concentration at 0 min was 5% and was increased gradually to 40% until 210 min with a flow rate of 300 nl/min. A LTQ XL mass-spectrometer (Thermo-Scientific, USA) was used for peptide sequencing with the following parameters: spray voltage was 1.8 kV, ion transfer capillary was set at 180 °C, and a data-dependent Top 6 method was used where a full MS scan from m/z 400–1500 was followed by sequential CID and PCD scans. Raw data files were searched against a subset database for Mus musculus from the Uniprot Database, using Proteome Discoverer 1.3 (Thermo Scientific).

2.11. Gene expression using real time RT-PCR

Fully differentiated adipocytes were treated with GE (100 μg/ml final concentration) or insulin (10 μg/ml final concentration), which was used as positive control. Milli-Q water was used as negative control. The total RNA was isolated from the adipocytes, using an Ambion- RNAqueous Micro™ kit (Applied Biosystems, USA). The purity of recovered total RNA was then estimated by calculating the ratio of absorbance readings 260 nm and 280 nm. The integrity of RNA was estimated using an Agilent 2100 Bioanalyzer (Applied Biosystem, USA). RNA samples with RIN values of 8–10 were used in this study. Purified RNA was then used to synthesise complementary DNA (cDNA) by the PCR method. High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA), which contains all reagents (RT buffer, dNTP mix, random primers, Multiscribe reverse™ transcriptase enzyme and nuclease free water) needed for reverse transcription (RT) of total RNA to single-stranded cDNA, was used in this study. Generally, 10 μl of RNA sample was mixed with 10 μl of high capacity cDNA (reverse transcription). The mixture was loaded into a thermal cycler (Eppendorf, USA) and PCR was carried out according to optimised thermal cycling conditions. The genes used in this study were: adiponectin (assay ID: Mm 00456425_m1), leptin (assay ID: Mm 00434759_m1), glucose transporter-4 (GLUT-4) (assay ID: Mm 00436615_m1), hormone sensitive lipase (HSL) (assay ID: Mm 00495359_m1) and adipose triglyceride lipase (ATGL) (assay ID: Mm 00503040_m1). Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan® (Applied Biosystems, USA) probe mix. Endogenous control used was eukaryotic 18S rRNA. All TaqMan® probes used in this investigation were labelled with FAM™ reporter dye at the 5′ end and a MGB quencher at the 3′ end. The quantification approach used was the comparative Ct method, also known as 2−ΔΔCt method (Livak & Schmittgen, 2001).

2.12. Statistical analysis

Data are shown as means ± SD. One-way analysis of variance (ANOVA) and Duncan’s multiple range test (DMRT) were used to determine the significant differences between groups. STATGRAPHICS Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA) was used for all statistical analyses. Statistical significance was accepted at p < 0.05. All Figures were drawn using GraphPad Prism 5 (GraphPad Software Inc., California, USA).

3. Results and discussion

3.1. Weight and estimation of β-glucan concentration in GE

Fresh P. sajor-caju (5.5 kg) was boiled for 8 h to obtain 12.31 g (0.22%) of GE. The concentration of total glucan in GE was 86.0% (w/w) and the concentrations of α-glucan and β-glucan were 5.4% (w/w) and 80.6% (w/w), respectively. The concentrations of β-glucan in dried and fresh mushroom were 1.5% and 0.18%, respectively and the concentrations of α-glucan in dried and fresh mushrooms were 0.1% and 0.01%, respectively (Kanagasabapathy et al., 2012).
3.2. Effects of GE on proliferation of 3T3-L1 cells

In this study, the preadipocytes were treated with various concentrations of GE. The MTT assay was performed on treated and untreated preadipocytes after 24 and 48 h, respectively. GE exerted a dose-dependent increase in proliferation of preadipocytes, suggesting that GE was not cytotoxic towards 3T3-L1 adipocytes. A similar pattern was observed after 48 h of treatment with GE, as shown in Fig. 1(a).

3.3. Effects of GE on lipogenesis in 3T3-L1 cells

In this study, differentiating adipocytes were treated with various concentrations of GE (0.01–1000 µg/ml). After 48 h of incubation, the adipocytes were subjected to Oil Red O assay to determine the lipid content in the cells. Fig. 1(b) shows the lipogenic effects of GE and insulin on adipocytes. As expected, insulin induced a dose-dependent increase in lipogenesis. GE also induced lipogenesis and the effect was significantly higher than that of insulin in the dose range of 0.01–10 µg/ml.

3.4. Effects of GE on lipolysis in 3T3-L1 cells

GE stimulated lipolytic activity in fully differentiated adipocytes and this effect was dose-dependent, as shown in Fig. 2. The lipolytic activity was highest at 1000 µg/ml, whereas, at lower concentrations, no significant lipolytic effect was observed. Isoproterenol was used as positive control because it is a non-specific β-adrenergic agonist which activates the lipolytic pathway (Kuppusamy & Das, 1994). In this study, GE exhibited the same level of lipolytic activity as did isoproterenol.

3.5. Effects of GE on oxidative stress indices – lipid hydroperoxide and protein carbonyl levels

During lipogenesis and lipolysis, adipocytes are susceptible to oxidative stress either caused by the process of fat accumulation or overproduction of free fatty acids (due to lipolysis) during starvation. Besides that, oxidative stress may have also been caused by dexamethasone (a glucocorticoid) which was added in DM1 (Yan, Zhao, Suo, Liu, & Zhao, 2012) and the high concentration of glucose (4500 mg/l) in the DMEM media. This excessive oxidation can trigger cytotoxic chain reactions that are damaging to membrane lipids, proteins, nucleic acids, and carbohydrates (Garcia-Bailo et al., 2011). Since oxidative stress in adipocytes is known to play a central role in insulin resistance, products of oxidative damage, such as lipid hydroperoxides and protein carbonyls, were assessed. Table 1 shows the oxidative stress measurement in media after treating the 3T3-L1 cells with various concentrations of GE. The concentrations of protein carbonyl and lipid hydroperoxide were significantly reduced in GE-treated cultures as compared to the untreated cultures.
3.6. Effect of GE on AMP-activated protein kinase (AMPK)

AMPK is a heterotrimer composed of a catalytic subunit (α) and two regulatory subunits (β and γ). Table 2 shows the fold increase of AMPK activation in the GE-treated compared to control sample. The GE-treated sample increased the expression of 5'-AMP-activated protein kinase subunit γ-2 (PKRAG2) and 5'-AMP-activated protein kinase subunit γ-3 (PKRAG3). High confidence peptides, in 5'-AMP activated protein kinase, showed 1.4-times up-regulation on the phosphorylated peptide. AMPK proteins can be detected in skeletal and adipose tissues and almost 80% of total AMPK activity is associated with the γ isoforms (Cheung, Salt, Davies, Hardie, & Carling, 2000). The activation of AMPK plays an important role in regulating key enzymes involved in lipid and glucose metabolism by generating cellular events, such as glucose uptake and lipid oxidation, to produce energy, while turning off energy-consuming processes such as glucose and lipid production to restore energy balance (Chaves, Frasson, & Kawashita, 2011). AMPK activation leads to numerous metabolic changes that would be attractive targets in treatment of metabolic disorders, such as obesity, type-2 diabetes and metabolic syndrome.

3.7. Effect of GE on gene expression

The most important hormones produced by adipose tissue are leptin and adiponectin. Leptin and adiponectin are able to activate AMPK in adipose tissue. Leptin is a cytokine produced in proportions of adipose tissue and which acts to stimulate fatty acid oxidation through AMP-activated protein kinase activation (Minokoshi et al., 2002). Insulin and GE showed elevated levels of leptin (8.93-fold and 4.66-fold, respectively) as shown in Fig. 3. The possibility that leptin pathways act, in concert with insulin, to control glucose and lipid metabolism is well documented (Zou & Shao, 2008) where insulin and leptin play complementary roles in regulating the consumption, uptake, oxidation and storage of nutrients (Morrison, Huypen, Stewart, & Gettys, 2009). In contrast to leptin, adiponectin secretion and plasma concentration are inversely related to adiposity. Adiponectin is considered to be an insulin-sensitising hormone since it activates muscle glucose utilisation, and induces muscle and hepatic fatty acid oxidation (Tsao, Lodish, & Fruebis, 2002). Insulin caused 3.84-fold increase of adiponectin expression in the adipocytes, whereas GE induced 1.39-fold increase. Besides that, Chen et al. (2012) have reported that subjects

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oxidative indices measurement in media after treating with GE extract in 3T3-L1 adipocytes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts</td>
<td>Lipid hydroperoxides (µmol/g)</td>
</tr>
<tr>
<td>GE</td>
<td>1.29 ± 0.45*</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>18.6 ± 3.85</td>
</tr>
</tbody>
</table>

* p < 0.05 is the significance between means of treated (GE) and untreated samples. GE is polysaccharide.
suffering from metabolic syndrome have higher levels of oxidative stress markers, which significantly correlated with lower levels of adiponectin. In this study, the up-regulation of adiponectin by GE corresponded with decreased lipid hydroperoxide and protein carbonyl levels (Table 1) in the cells.

Insulin plays a key regulatory role in stimulating the transport of blood glucose into peripheral tissues through the GLUT-4 transporter in muscles and adipocytes. In insulin-treated adipocytes, insulin stimulated the expression of GLUT-4 transporter (4.56-fold) which induces the translocation and redistribution of glucose transport from specific intracellular compartments to the plasma membrane where it facilitates glucose uptake. AMPK activation also stimulates glucose transport, through increased GLUT4 translocation, by a mechanism independent of insulin signalling. A study performed in primary rat adipocytes has shown that adiponectin activates AMPK and increases glucose uptake (Daval et al., 2006). Adipocytes treated with GE also showed elevated (2.03-fold) expression of GLUT-4 gene (Fig. 3) and hence able to enhance glucose uptake in the cells.

The present study demonstrated that GE caused up-regulation of HSL and ATGL gene expression by 2.99-fold and 2.28-fold, respectively, while insulin down-regulated the expression of these genes by 4.22-fold and 4.20-fold, respectively. HSL is a substrate of AMPK. The activation of these genes could lead to mobilisation of triacylglycerol (TAG) to form glycerol and fatty acids; HSL mainly breaks down TAG to form diacylglycerol (DAG) whilst ATGL breaks down DAG to form monoaoylglycerol (MAG) and monoacylglycerol lipase (MGL) breakdown MAG to form free fatty acids and glycerol. The regulation of these genes by GE was similar to that of isoproterenol (a known β-adrenergic agent) (Robidoux, Martin, & Collins, 2004). In contrast, insulin binds to its insulin receptor substrates (IRS) in adipocytes and initiates a signalling event via phosphorylation and activation of phosphodiesterase 3B (PDE3B) protein, which decreases cAMP level and ultimately inhibits lipolysis (Dallas, Gerbi, Tenca, Juchaux, & Bernard, 2008). The importance of PDE3B is to suppress lipolysis in adipocytes and this correlates with the down-regulation of HSL and ATGL genes by insulin. From this study, GE did not correspond to the same signalling pathway as insulin (PDE3B); however, GE still enhanced the expression of insulin-like genes (adiponectin, leptin and GLUT-4) by a mechanism independent of the insulin signalling pathway. The activation of AMPK by GE may have induced the expression of these genes, as well as the lipolysis genes (HSL and ATGL). Hence this extract was able to stimulate lipogenesis in differentiating cells and lipolysis in differentiated cells.

In order to investigate the insulin-like and lipo-mobilising activities by GE, the concentration of interest was 100 μg/ml because, at this concentration, GE also showed a significant lipid mobilisation (lipolysis) in the differentiated adipocytes. The insulin-like activity of GE was evident in the lipid accumulation of differentiating 3T3-L1 cells, which concurred with the expression of GLUT-4, adiponectin and leptin. Interestingly, GE (at 100 μg/ml) was able to induce significant lipolysis in fully differentiated 3T3-L1 adipocytes and this concurred with the increased expression of HSL and ATGL through AMPK activation. This response was similar to the β-adrenergic receptor-mediated action of epinephrine or isoproterenol. It is noteworthy that, in the in vivo system, hormonal regulation of lipogenesis (by insulin) and lipolysis (by epinephrine or glucagon) do not occur concurrently so as to avoid a ‘futile metabolic cycle’. The GE extract appears to have the potential to adapt to the level of lipid present in the adipocytes in stimulating lipid accumulation and glucose uptake just like insulin, as well as stimulate lipid mobilisation when adequate lipid is present in the cells.

4. Conclusion

In summary, β-glucan-rich polysaccharides from P. sajor-caju (GE) induce lipogenesis in differentiating 3T3-L1 cells and lipolysis in differentiated 3T3-L1 adipocytes. This mechanism may, at least

---

Table 2
Expression of AMP-kinase (AMPK) protein in GE-treated cells over untreated cells (control) in 3T3-L1 cells.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein Description</th>
<th>Score</th>
<th>Calculated pI</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q91WC5</td>
<td>5'-AMP-activated protein kinase subunit gamma-2 (PRKAG2)</td>
<td>7.84</td>
<td>1.40</td>
<td>Regulation of fatty acid metabolic process, cellular lipid metabolic process, regulation of fatty acid oxidation, regulation of glucose import, regulation of glycolysis and glycogen metabolic process</td>
</tr>
<tr>
<td>Q8RGM7</td>
<td>5'-AMP-activated protein kinase subunit gamma-3 (PRKAG3)</td>
<td>2.53</td>
<td>1.02</td>
<td>Regulation of de novo biosynthesis of fatty acid and cholesterol</td>
</tr>
</tbody>
</table>

---

Fig. 3. Results are expressed as -fold variation over the carrier control group. Fold variations less than 0 were expressed as negative numbers (e.g., a -fold variation of 0.50 is expressed as –2.00). Values expressed are means ± SD of triplicate measurements. Statistical significance was calculated, based on the mean ΔCt values by DMRT. For the same gene with various treatment groups, means in different bars with superscripts (a–b) were significantly different (p < 0.05); HSL is highly sensitive lipase; ATGL is adipose triglyceride lipase; Adipoq is adiponectin; Lep is leptin; GLUT-4 is glucose transporter 4 and GE is polysaccharide.
in part, depend on the ability of GE to up-regulate the gene expression of adiponectin, GLUT-4, leptin HSL and ATGL through the activation of the AMPK-signalling pathway. Our findings suggest that GE is a novel AMPK activator that may aid in the prevention and treatment of diabetes mellitus.

Acknowledgements

Authors are grateful to the University of Malaya and Ministry of Higher Education, Malaysia for providing the following grants: RG083/09AFR and HIR F00002-21001.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.08.051.

References