การศึกษาโปรตีนจากเซลล์ไขมันที่หลั่งเพิ่มขึ้นตามระยะเวลาที่ถูกกระตุ้นด้วยกรดไขมันปาล์มิติก

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2ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ จังหวัดปทุมธานี

บทคัดย่อ

ในช่วง 20 ปีที่ผ่านมาความชุกของโรคเบาหวานชนิดที่ 2 ได้เพิ่มขึ้นอย่างรวดเร็ว โดยมีสาเหตุหลักมาจากภาวะดื้ออินซูลิน การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการแสดงออกของโปรตีนที่หลั่งจากเซลล์ไขมันในสภาวะที่ถูกกระตุ้นด้วยกรดไขมันปาล์มิติกเปรียบเทียบกับในสภาวะปกติ ด้วยเทคนิคทางโปรตีโอมิกส์แบบ GeLC-MS/MS โดยการกระตุ้นเซลล์แพะเลี้ยง 3T3-L1 adipocytes ด้วย ImM palmitic acid เป็นเวลา 0 (กลุ่มควบคุม) 15 30 60 120 และ 240 นาที ผลที่ได้กำหนดเครื่องปริมาณด้วยโปรแกรม DecyderMS และโปรแกรมชนิดของโปรตีนด้วยโปรแกรม Mascot พบโปรตีนที่มีการแสดงออกแตกต่างอย่างมีนัยสำคัญทางสถิติจำนวน 236 ชนิด (p < 0.05) ซึ่งมีหน้าที่เกี่ยวกับการแบ่งกลุ่มการควบคุมโปรตีนชัดเจนและมีเป็นตัวบ่งชี้การดื้ออินซูลินมีความเกี่ยวข้องกับการควบคุมการก่อตัวโปรตีน และมีความเกี่ยวข้องกับการก่อตัวโปรตีนในกระบวนการต่าง ๆ ได้แก่ cell differentiation, cell adhesion, cell division, cytoskeleton arrangement, DNA replication, DNA repair, immune response, insulin metabolism, metabolic processes, cellular respiration, signal transduction, stress response, transcription, translation และ transport จากการศึกษาแนวโน้มการแสดงออกของโปรตีนด้วยโปรแกรม MEV พบว่ามีกลุ่มโปรตีน 23 ชนิดที่มีการแสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ตามระยะเวลาการกระตุ้นที่เพิ่มขึ้น และพบว่าโปรตีนเหล่านี้มีความเกี่ยวข้องกับการดื้ออินซูลินหรือเกี่ยวกับการเกิดโรคเบาหวาน ซึ่งมีความน่าสนใจที่จะนำมาศึกษาความเป็นไปได้ในการใช้เป็นตัวบ่งชี้ภาวะดื้ออินซูลิน เพื่อช่วยป้องกันการพัฒนาเป็นโรคมะเร็งในอนาคตได้

คำสำคัญ: เซลล์ไขมันชนิด 3T3-L1 การกระตุ้นด้วยกรดไขมันปาล์มิติก โปรตีโอมิกส์ เทคนิค GeLC-MS/MS

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Secretome Analysis of Palmitic Acid-Induced 3T3-L1 Adipocyte: A Focus on Time-Regulated Release

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Abstract

The prevalence of type 2 diabetes has increased dramatically within the last 20 years and the major cause was identified as insulin resistance. The objective of this study was to examine the expression of proteins secreted from palmitic acid induced adipocytes as compared to those from the control by using the proteomics technique. The 3T3-L1 adipocytes were induced with 1 mM palmitic acid for 0 (control), 15, 30, 60, 120 and 240 min. The secreted proteins were analyzed by GeLC-MS/MS. The raw MS/MS data were quantified using DecyderMS and identified by Mascot software. The 236 proteins secreted from palmitic acid-induced 3T3-L1 adipocytes showed significant differences from those of the control \((p < 0.05)\). These proteins are known to be involved in various processes, including cell differentiation, cell adhesion and cell division, cytoskeleton arrangement, DNA replication and repair, immune response, insulin metabolism, metabolic processes, cellular respiration, signal transduction, stress response, transcription, translation and transport. Analysis of the protein expression using MEV software demonstrated that the expression of 23 proteins tend to increase with increasing incubation periods. These 23 proteins may play important roles in type 2 diabetes, therefore, they are possible candidates for biomarkers of insulin resistance, which can help prevent the development of diabetes in the future.

Key words: 3T3-L1 adipocytes, Palmitic acid, Insulin resistance, Proteomics, GeLC-MS/MS

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1. Introduction

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia as a result of defects in insulin secretion, action, or both. Changes in human behavior and lifestyle result in a dramatic increase in the incidence of diabetes and the prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030.\(^{(1)}\) From 2010 to 2030, approximately 69% and 20% increase in number of adults with diabetes were expected to be found in developing and developed countries, respectively.\(^{(2)}\)

The occurrence of insulin resistance is a primary cause and an important part of developing diabetes. Supporting evidences come from the reports showing that the presence of insulin resistance occurs 10-20 years earlier than the onset of the disease.\(^{(3-4)}\)

The hyperinsulinaemic euglycemic clamp is the reference method for quantifying insulin resistance. The main limitations of the glucose clamp approach are that it is time consuming, labor intensive, expensive, and requires an experienced operator.\(^{(5-6)}\). Therefore, the current diagnosis of diabetes is generally based on fasting plasma glucose (FPG), random plasma glucose, oral glucose tolerance test (OGTT) and HbA1C as suggested by the American Diabetes Association (ADA) or WHO.\(^{(7-8)}\) When insulin resistance is detected a prevention program of diabetes is recommended. Eighty percent of type 2 diabetes can be prevented if the occurrence of insulin resistance is detected in the early stage.\(^{(9)}\) Therefore, identification of biomarkers that are sensitive and specific for insulin resistance is important for more effective screening and prevention of type 2 diabetes.

In our previous study, 120 proteins were found secreted from 3T3-L1 adipocytes induced by using 1 mM palmitic for 2 h.\(^{(10)}\) However 1 mM palmitic acid was also shown to reduce glucose uptake at 0.5, 2, 4, 6, 12, 24 and 48 h \((p < 0.05)\) when compared with the control (100% uptake).\(^{(11)}\) Therefore, the aim of this study was to examine more closely the proteins secreted from 3T3-L1 adipocytes treated with 1mM palmitic acid at shorter time intervals of 0 (as control), 15, 30, 60, 120 and 240 min by GeLC-MS/MS.

2. Materials and methods

2.1 3T3-L1 cell culture and differentiation

The 3T3-L1 fibroblasts (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (25 mM glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, 200 kU/L) in 5% CO\(_2\) at 37 °C. The cells were differentiated into adipocytes by initiation with 5 µg/mL insulin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine (IBM) in DMEM supplemented with 10% fetal bovine serum. After 48 h (Day 2), the culture medium was replaced with DMEM supplemented with
10% fetal bovine serum. Insulin at 5 \( \mu g/mL \) was added and the cells were then fed every 2 days with DMEM containing 10% fetal bovine serum. Cytoplasmic triglyceride droplets were visible by Day 4, and cells were fully differentiated by Day 8.

**2.2 Fatty acid-induced insulin resistance in adipocytes**

Adipocytes were treated with palmitic acid to induce insulin resistance.\(^{(12)}\) They were incubated with 1 mM palmitic acid/1% fatty acid free BSA in DMEM with 10% FBS, antibiotics, and 5 \( \mu g/mL \) insulin, in 5% CO\(_2\), at 37 \(^\circ\)C for 0 (as control), 15, 30, 60, 120 and 240 min. Control cells were similarly treated under the same conditions except the addition of palmitic acid. Glucose uptake was determined by using 2-NBDG [2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] uptake assay.\(^{(13)}\)

**2.3 Protein preparation**

The medium was centrifuged at 1800 xg for 10 min to remove the cell debris and the supernatant filtered using a 0.22 \( \mu m \) syringe driven filter to remove the floating adipose cells. The clear supernatant was then concentrated and determined the protein concentration by Lowry protein assay.\(^{(14)}\) Sample protein (50 \( \mu g \)) was loaded and separated on a 12.5% SDS-PAGE gel, stained with silver stain and each lane cut into 1×1×1 mm\(^3\) size gel plugs.

**2.4 In-gel digestion**

The gel plugs were destained with 5% H\(_2\)O\(_2\) until clear. Samples were dehydrated by the addition of 200 \( \mu L \)/well of 100% acetonitrile (ACN) and allowed to dry at room temperature for 10 min. Proteins were reduced by the addition of 20 \( \mu L \)/well of 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate (NH\(_4\)HCO\(_3\)) and incubated at 56\(^\circ\)C for 1 hr. The reduced proteins were then alkylated by the addition of 30 \( \mu L \)/well of 100 mM IAA in 10 mM NH\(_4\)HCO\(_3\) and further incubated at room temperature in the dark for 1 h followed by washing twice with 100% ACN. The proteins were digested by the addition of 20 ng of trypsin in 10 mM NH\(_4\)HCO\(_3\) and incubated at room temperature for 20 min followed by incubation at 37\(^\circ\)C for 3 h. The protein extract was incubated at 40 \(^\circ\)C until dry and samples were kept at -80\(^\circ\)C until analysis by LC-MS/MS.

**2.5 LC-MS/MS**

The dried samples were dissolved in 0.1% formic acid in LC-MS grade water and transferred to the vials for injection. Nanoscale LC separation of tryptic peptides was performed with an Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker, Germany)) with electrospray at a flow rate of 300 nL/min to a nanocolumn (Onyx monolithic HDC18, 0.2 mm i.d. × 150 mm). Mobile phase A was 0.1% formic acid in water and mobile phase B was 80% acetonitrile in 0.1% formic acid. A multistep gradient was used to elute peptides: a linear increase from 10%-70% B for 13 min, 90% B at 13-15 min...
followed by a decrease to 10% B at 15-20 min. Peptide fragment mass spectra were acquired in data-dependent AutoMS mode with a scan range of 300-1500 m/z, 3 averages, and up to 5 precursor ions selected from the MS scan 50-3000 m/z.

2.6 Protein quantitation and identification

DeCyder MS Differential Analysis software was used for protein quantitation. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted for a database search using the Mascot software (Matrix Science, London, UK). The data was searched against the NCBI database for protein identification. Proteins considered as identified proteins had at least one peptide with an individual mascot score corresponding to \( p < 0.05 \).

Database interrogation was; taxonomy (Human or Eucaryote or rodents); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages (1). Proteins considered as identified proteins had at least two peptides with an individual mascot score corresponding to \( p < 0.05 \).

Gene ontology annotation was performed using Software Tool for Rapid Annotation of Proteins (STRAP). STRAP allows collection and annotation of information about the proteins in a data set. It compiles all of the protein annotation information and displays it in a Gene ontology term that includes biological process, cellular component and molecular function, respectively. The final distribution pie charts were generated using Microsoft Excel.

Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using Multi Experiment Viewer (Mev) software. Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. They performed statistical tests of variance of differences (ANOVA) for these data sets with statistical significance at the \( \alpha = 0.05 \) level.

Analysis of protein-interactions, the expressed proteins were analyzed by using STRING database to understand the relationship among differentially expressed proteins with other proteins and chemicals.

3. Results

The in vitro insulin resistance model was induced in 3T3-L1 adipocytes using 1 mM palmitic acid. The insulin response was determined by using 2-NBDG uptake assay.
Palmitic acid inhibited glucose uptake in 3T3-L1 adipocytes. The decrease in insulin response was observed as shown in Fig. 1.

Protein expression was analyzed by using GeLC-MS/MS at different incubation time of 15, 30, 60, 120 and 240 min compared with the control group. DeCyder MS Differential Analysis software was used for protein quantitation. Data showed different levels of protein expression of 1,582 proteins in the cell culture supernatants of the 6 experimental groups. Quantitative proteome analysis revealed that the expression of 236 proteins in the fatty acid-induced groups were significantly different from those of the control group ($p < 0.05$). These proteins were categorized to play important roles in several different biological processes including cell differentiation, cell adhesion, cell division, cytoskeleton arrangement, DNA repair, DNA replication, immune response, insulin metabolism, metabolic processes, cellular respiration, signal transduction, stress response, transcription, translation, transport and unknown processes. However, the majority of the proteins are involved in metabolic processes, signal transduction and transcription as shown in Fig. 2.
The protein expression was analyzed by using MEV software and 23 proteins were found to show a significant trend to increase with increasing incubation periods as shown in Fig. 3. These proteins are involved in metabolic processes, transport, transcription, signal transduction, cell adhesion, cell division, immune response, and unknown functions (Table 1).

To understand the relationship among protein-interactions, the expressed proteins were analyzed by using STRING database. Protein-protein interaction and protein-chemical interaction of the 23 proteins were then categorized into three main groups. The first group consists of jumonji domain containing 2A (KDM4A), 3-hydroxy-3-methyl glutaryl-coenzyme A reductase (LDLCQ3), von Willebrand Factor (VWF), and platelet glycoprotein V (GP5) which are associated with lovastatin, simvastatin, cholesterol, palmitate, and calcium. The second group is related to cartilage associated protein and leucine proline-enriched proteoglycan and the last group includes protein SDA1 homolog, transmembrane protein 71, PWPP domain-containing protein MUM1L1, serine/threonine-protein kinase LMTK2 precursor, structural maintenance of chromosomes protein 1B, N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2, Kinesin family member C3, serine palmitoyltransferase 3, lysosomal
alpha-glucosidase precursor, protein FAM76A isoform A and a trinucleotide repeat-containing gene 6C protein whose association with any chemical has never been described before as shown in Fig. 4.

4. Discussions

This study focuses on the analysis of proteins secreted from 3T3-L1 adipocytes treated with 1 mM palmitic acid at various incubation times. The experimental design is an imitation of the actual conditions in diabetes patients about 80% of whom have the association with obesity and high level of free fatty acid which consequently leads to insulin resistance.\(^{17}\)

The 3T3-L1 cell line is a well-established and extensively used in vitro model for studying of adipose tissue. These cells are sensitive to lipogenic and lipolytic hormones and drugs, including epinephrine, isoproterenol, and insulin.\(^{18}\) In 1997 Knutson and Balba used 3T3-L1 adipocyte as a cell culture model of insulin resistance\(^{19}\) while in 1994 Hunnicutt JW, \textit{et al.} described saturated fatty acid - induced insulin resistance in isolated rat
Table 1 Secreted proteins of insulin resistance induced 3T3-L1 adipocytes

The proteins were identified after SDS-PAGE fractionation and tryptic digestion analysis by LC-MS/MS. The data in Table 1 shows secreted proteins that were up-regulated depending on the incubation time of palmitic acid induced 3T3-L1 adipocytes with no expression in the control group.

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adipocytes\(^\text{(20)}\). In a previous study in 2013, Piumngam K \textit{et al.} reported insulin resistance in 3T3-L1 adipocytes treated with palmitic acid.\(^\text{(11)}\) More recently the secretome analysis of insulin resistance 3T3-L1 adipocyte induced by using 1 mM palmitic acid for 2 h demonstrated 120 expressed proteins most of which were associated with metabolic processes, cellular process and regulation.\(^\text{(10)}\) However, the analysis of protein expression was only carried out at 2h incubation whereas the reduction in glucose uptake was also observed at other incubation times. Therefore, in the current study analysis of the secreted proteins was performed at intervals from 0 (control), 15, 30, 60, 120 and 240 min of incubation time.

According to the findings in the current study, 236 expressed proteins were identified by using the protein database which ensured that they are secreted proteins. For example, Von Willebrand Factor has been reported to be a protein secreted from cells. In 2001, Gaede \textit{et al.} and in 2008, Frankel DS \textit{et al.}, reported that detecting an increased level of von Willebrand in the plasma together with microalbuminuria is more efficient to predict high risks of cardiovascular disease and

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After adipocytes were treated with palmitic acid, only 23 secreted proteins were significantly up-regulated with increasing incubation time. These proteins are involved in biological processes, including metabolic processes, transport, transcription, signal transduction, cell adhesion, cell division, immune response, and some other unknown functions. Only 16 out of these 23 proteins were found to interact with other proteins or chemicals on STRING database. Interestingly, all 16 proteins were found to be related to insulin resistance. Moreover, 4 proteins [jumonji domain containing 2A (KDM4A), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (LDLCQ3), von Willebrand Factor (VWF), and platelet glycoprotein V (GP5)] have been reported to be associated with lovastatin, simvastatin, cholesterol, palmitate, and calcium.
3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG Co-A reductase) is localized in the endoplasmic reticulum and functions as the rate-controlling enzyme in metabolic process of the mevalonate pathway. It is an important enzyme involved in cholesterol production. This enzyme is regulated by insulin which reduces the level of cAMP and consequently increases the production of cholesterol. It has been reported that drugs in a class of statin can be used to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase to lower cholesterol levels.\(^\text{(23-25)}\)

Von Willebrand Factor in a monomer form consists of 2,050 amino acid residues. It is a glycoprotein in blood plasma and each monomer is composed of a number of specific domains with a specific function. In 1996, Mansfield et al. and in 2010, Soares AL et al., showed an increase in factor VII coagulant activity (FVII:C) and fibrinogen in diabetes patients who had the abnormality of homeostatic factor.\(^\text{(26-27)}\) These factors are associated with insulin resistance and increased risks of contributing cardiovascular disease in diabetes patients.

5. Conclusion

Therefore, there is a potential for these secreted proteins to be used as biomarkers of insulin resistance. However, this study has been performed in cell culture which may be different from studying in whole animals or humans where biological systems are extremely complex. Future studies are still needed to accumulate evidence and knowledge for understanding the complication of insulin resistance-induced production of these secreted biomarkers.

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Conflicts of interest

The authors have no conflicts of interest to declare.

References


