CHARACTERIZATION OF THE HYPOGLYCAEMIC EFFECT OF *BACILLUS AMYLOLIQUEFACIENS* EXOPOLYSACCHARIDES ON INSULIN-RESISTANT INTESTINAL EPITHELIAL CELLS

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摘要

學號　：M10618030

論文題目：探討液化澱粉芽孢桿菌之胞外多醣對胰島素抗性腸道上皮細胞的降血糖效果

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中文摘要：

第 2 型糖尿病佔全世界糖尿病病例 90%以上，是指對胰島素產生抗性所引發的高血糖症。因此，胰島素抗性是第二型糖尿病發展的關鍵因子，研發有助於降低胰島素抗性的藥物對於治療第二型糖尿病非常重要。微生物胞外多醣（EPS）是由微生物分泌的胞外醣類聚合物。在本實驗室先前的研究中發現，*Bacillus amyloliquefaciens* 的 EPS 能降低正常小鼠的血糖，因此本研究的目的是探討 *Bacillus amyloliquefaciens* 的 EPS 是否對胰島素抗性細胞也具有降血糖作用。方法是將腸上皮細胞株 IEC-18 細胞用大腸桿菌的脂多醣（LPS）處理以誘導胰島素抗性，然後再用 EPS 處理 IEC-18 細胞。結果發現，LPS 有效地導致 IEC-18 細胞產生胰島素抗性，胰島素誘導的葡萄糖攝取和 Akt 的活化受到抑制，而 EPS 明顯提升此抗性細胞的葡萄糖消耗和 Akt 活化。更進一步的分析發現 EPS 是一種胰島素增敏劑，在胰島素抗性細胞中，它能恢復胰島素刺激的 Akt 活化和葡萄糖攝取。這些結果表

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明 *Bacillus amyloliquefaciens* 的 EPS 作為胰島素增敏劑，能夠有效的促進胰島素對胰島素抗性細胞的作用。因此，EPS 在治療第二型糖尿病方面的潛力值得進一步探索。

關鍵詞：胰島素抵抗，胞外多醣，液化澱粉芽孢桿菌，2 型糖尿病

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ABSTRACT

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Title of Thesis : Characterization of the hypoglycaemic effect of *Bacillus amyloliquefaciens* exopolysaccharides on insulin-resistant intestinal epithelial cells

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Name of Student : Jazimatus Syarifah

Adviser :Hsueh-Ling Cheng, Ph.D.

The content of this abstract in this thesis :

Type 2 diabetes contributes to > 90 % diabetic cases worldwide and is referred to hyperglycemia initiated by resistance to insulin. Thus, insulin resistance is a key biomarker of the development of type II diabetes. Exploration of medicines that help lower blood glucose in insulin-resistant condition is very important in treating type II diabetes. Microbial exopolysaccharides (EPS) are extracellular carbohydrate polymers produced and secreted by microorganisms. The EPS from *Bacillus amyloliquefaciens* was found to lower blood glucose in non-diabetic mice in our previous study. The purpose of this study is to investigate whether the EPS of *B. amyloliquefaciens* has a hypoglycemic effect on insulin-resistant cells. The intestinal epithelial cell line, IEC-18 cells, were treated by lipopolysaccharides (LPS) of *E.coli* to induce insulin resistance, followed by treating with EPS. The results showed that LPS effectively

resulted in insulin resistance in IEC-18 cells that insulin-induced glucose uptake and the activation of Akt was inhibited, and EPS obviously enhanced the glucose consumption and Akt activation of the cells. Further investigation suggested that EPS acted as an insulin sensitizer, in that its recoved the insulin-stimulated Akt activation and glucose uptake. Together, these results suggested that the EPS of *B. amyloliquefaciens* worked as an insulin sensitizer to promote the effect of insulin on insulin-resistant cells. Therefore, the potential of EPS in treating type II diabetes deserves to be further explored.

Keywords : Insulin resistance, Exopolysaccharide, *Baciluus amyloliquefaciens,* Type 2 diabetes

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TABLE OF CONTENT

ABSTRACT..I
TABLE OF CONTENT... VI
LIST OF FIGURE.. VIII
1.1 Background..Error! Bookmark not defined.
1.2 The Purpose and Frame Work of this Study........Error! Bookmark not defined.
1.3 Future Impact..Error! Bookmark not defined.
CHAPTER II. LITERATURE REVIEW......................Error! Bookmark not defined.
2.1 Overview of Hyperglycemia and Diabetes.......... Error! Bookmark not defined.
2.2 The Insulin Signaling Pathway............................ Error! Bookmark not defined.
2.3 Insulin Resistance...Error! Bookmark not defined.
2.4 Treatment for Insulin Resistance........................ Error! Bookmark not defined.
2.5 Bacterial EPS...Error! Bookmark not defined.
CHAPTER III. MATERIALS AND METHODS.........Error! Bookmark not defined.
3.1 Materials... Error! Bookmark not defined.
3.1.1 Cell Line... Error! Bookmark not defined.
3.1.2 Media..Error! Bookmark not defined.
3.1.2.1 Dulbecco's Modified Eagle Medium (DMEM)Error! Bookmark not defined.
3.1.2.2 Modified Eagle Medium (MEM)................Error! Bookmark not defined.
3.1.3 *Bacillus amylolliquefaciens* exopolysaccharides (EPS)Error! Bookmark not defined.
3.1.4 Reagent for Cell Treatment............................Error! Bookmark not defined.
3.1.5 Reagent for Western Blotting Analysis.........Error! Bookmark not defined.
3.1.6 Other Chemical and Reagents........................Error! Bookmark not defined.
3.2.2 Instruments for Western Blotting and Protein AnalysisError! Bookmark not defined.
3.2.3 Instruments for Glucose Uptake Assay......... Error! Bookmark not defined.
3.2.4 Instruments for Western Blotting...................Error! Bookmark not defined.
3.3 Experimental Procedures................................. Error! Bookmark not defined.
3.3.1 Cell Culture.. Error! Bookmark not defined.

3.3.2 Western Blot Analysis.................................Error! Bookmark not defined.
3.3.2.1 Cell Preparation...Error! Bookmark not defined.
3.3.2.2 Cell Lysis for Total Protein Extraction.......Error! Bookmark not defined.
3.3.2.3 Protein Quantification................................Error! Bookmark not defined.
3.3.2.4 Electrophoresis of Proteins by SDS-PAGE Error! Bookmark not defined.
3.3.2.5 Blotting of Proteins onto PVDF Membrane Error! Bookmark not defined.
3.3.2.6 Antibody Detection....................................Error! Bookmark not defined.
3.3.2.7 Visualization of Protein.............................Error! Bookmark not defined.
3.3.3 Glucose Uptake Assay.................................Error! Bookmark not defined.
3.3.4 WST-8 Assay..Error! Bookmark not defined.
3.3.5 Cytotoxicity Assay..Error! Bookmark not defined.
3.3.6 Statistic Analysis...Error! Bookmark not defined.
CHAPTER IV. RESULTS..Error! Bookmark not defined.
4.1 The Induction of Insulin Resistance by LPS......Error! Bookmark not defined.
4.2 The Effect of EPS on Insulin-Resistant IEC-18 Cells Error! Bookmark not defined.
CHAPTER V. DISCUSSION......................................Error! Bookmark not defined.
CHAPTER VI. CONCLUSION...................................Error! Bookmark not defined.
REFERENCES..Error! Bookmark not defined.

LIST OF FIGURE

Figure 4.1. The citotoxicity of LPS on IEC-18 cells ... **Error! Bookmark not defined.**
Figure 4.2. Induction of insulin resistance by LPS ...... **Error! Bookmark not defined.**
Figure 4.3. LPS inhibited insulin-induced activation of Akt **Error! Bookmark not defined.**
Figure 4.4. The citotoxicity of EPS on IEC-18 cells ... **Error! Bookmark not defined.**
Figure 4.5. The effect of EPS on the glucose uptake of insulin-resistant IEC-18 cells .... **Error! Bookmark not defined.**
Figure 4.6. EPS activated Akt in insulin-resistant IEC-18 cells **Error! Bookmark not defined.**
Figure 4 7 EPS worked as an insulin sensitizer in insulin-resistant IEC-18 cells .... **Error! Bookmark not defined.**
Figure 4.8. Effect of EPS on Akt in insulin-resistant IEC-18 cells **Error! Bookmark not defined.**

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CHAPTER I. INTRODUCTION

1.1 Background

Chronic hyperglycemia will lead to the metabolic syndrome and diabetes mellitus (Shoelson et.al, 2007). Diabetes mellitus is caused due to deficiency in insulin secretion, insulin action or both in combination. People with diabetes either don't produce enough insulin to rebalance their blood sugar typically in type 1 diabetes, or type 2 diabetes.

Type 2 diabetes is a progressive disease in which the risks of myocardial infarction, stroke, microvascular events, and mortality are all strongly associated with hyperglycemia. The disease course is primarily characterized by a decline in *β-cell* function and worsening of insulin resistance (Fonseca, 2009). Insulin resistance ia a state in which cells do not respond to insulin appropriately, so glucose in the blood are not absorbed by cells. Insulin resistance results in decreased suppression of hepatic glucose production and decreased glucose uptake by skeletal muscle and adipose tissue. Therefore, treatment of insulin resistance is a key strategy to prevent from type 2 diabetes.

Insulin resistance is closely related to lowgrade inflammation. In type 2 diabetes, a subclinical inflammation can have a central role in the induction of insulin resistance. Development of insulin resistance is mainly associated with low grade tissue specific inflammatory responses induced by various pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α (Feve and Bastard, 2009), and inflammatory factors such as lipopolysaccharides (LPS). Hagiwara et.al (2011) determined the effect of insulin therapy on cardiac function in a rat model

of systemic inflammation with treated LPS. In systemic inflammatory conditions, diabetes increases various proinflammatory mediators and inhibits cardiac function. Meanwhile, LPS also could play a role in the pathogenesis of insulin resistance. LPS directly inhibits insulin signaling and glucose transport in human muscle cells (Liang et.al, 2013).

During the last decade, bacterial EPSs have been reported with different biological activities, such as lowering blood cholesterol level, and antioxidation, anticancer, and immunoregulation effects (Sasikumar et.al, 2017). In our previous study, the exopolysaccharides (EPS) of *B. amyloliquefaciens* were found to lower blood glucose in normal mice, and promote glucose uptake of normal cells. However, its effect on diabetic animals cells is not clear. Nonetheless, the EPS also exhibited an anti-inflammatory effect in cell-based assays as well as in animal models in our previous study. Thus, it is reasonable to speculate that EPS may have a hypoglycemic effect on inflammatory factor-induced insulin-resistant subjects.

1.2 The Purpose and Frame Work of this Study

The purpose of this study is to investigate the effect of the EPS from *B. amyloliquefaciens* on insulin-resistant intestinal cells, aiming to explore the potential application of EPS on treating type 2 diabetes. Therefore, the experimental design of this study was framed as follows :

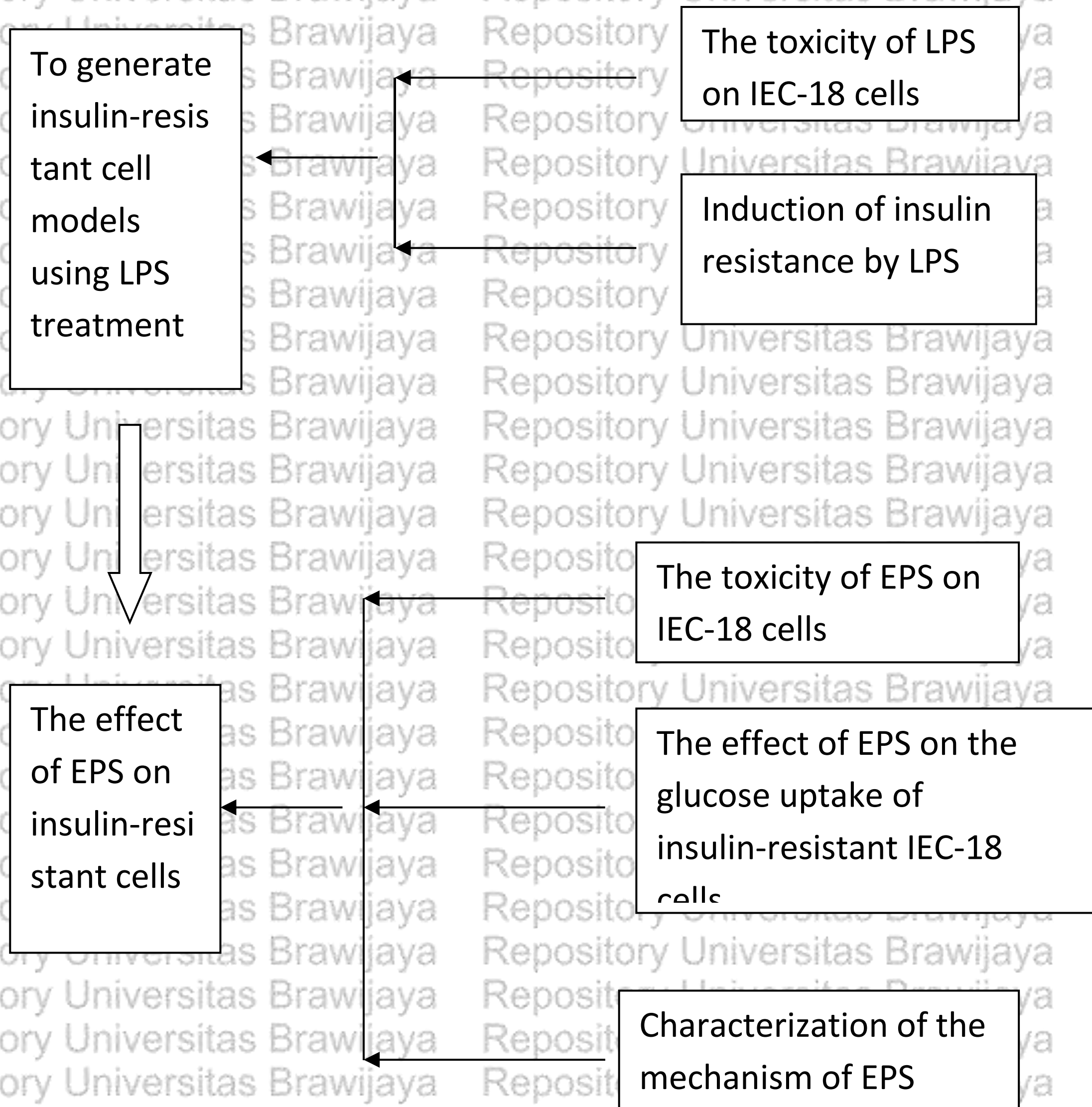

Figure 1.1 The frame work of this study

1.3 Future Impact

The results of this study can answer whether EPS works on insulin-resistant cells. It will provide important information for the potential of EPS on treating diabetes

CHAPTER II. LITERATURE REVIEW

2.1 Overview of Hyperglycemia and Diabetes

Diabetes mellitus is a heterogeneous metabolic disorder characterized by the presence of hyperglycemia due to impairment of insulin secretion, defective insulin action or both. The chronic hyperglycemia of diabetes is associated with relatively specific longterm microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD) (Punthakee et.al, 2018).

According to World Health Organization, diabetes can be classified into two major classes: type 1 diabetes (T1DM) and type 2 diabetes (T2DM). Type 1 diabetes results from the body's failure to produce insulin, and presently requires the person to inject insulin. The majority of type 1 diabetes is of the immune-mediated nature, where *β*-cells loss is a T-cell mediated autoimmune attack. T2DM is mainly initiated by insulin resistance, the condition in which cells fail to use insulin properly, often followed by gradual failure of *β*-cells to secrete enough insulin, resulting in even more severe diabetic conditions (Kumar et.al, 2012).

There are many metabolic pathways that cause insulin resistance inperipheral tissues. They provoke inflammation and stress-induced kinases such as IkB kinase-β (IKKβ) and JUN N-terminal kinase (JNK). These kinases are known to efficiently participate in pathogenesis of diabetes. IKKβ may potentiate the activation of nuclear factor-κB (NF-κB), which in turn induces pro-inflammatory cytokines (TNF-α and IL-1β) in liver and adipose tissues. These cytokines result in insulin resistance in peripheral tissues (Arkan et al, 2005). However, JNK potentiates activating transcription factor-2 (ATF2) and ELK1.

TNF-α and IL-1β, which are produced by the activation of NF-κB are also known to stimulate both NF-κB and JNK in response to feed-forward mechanism through the involvement of their particular receptors (Donath and Shoelson, 2011). Other then NF-κB and JNK pathways, FFAs and advanced glycation end-products may promote insulin resistance and overt T2DM by the activation of toll like receptors (TLRs) and receptors for advanced glycation end-products (RAGE) (Shi et al., 2006). These extracellular stimuli bind these cell surface receptors by activating intracellular pathways that unite on both JNK and NF-κB. Activation of these pathways takes place in liver and adipose tissues and upregulates the production of TNF-α, IL-1β,and IL-6 (Sabio et al., 2008). Since, these NF-κB and JNK pathways are activated in many tissues and play crucial role in tissue inflammation, blocking the activity of these pathways may stop the prevalence of inflammation.

2.2 The Insulin Signaling Pathway

Insulin is an anabolic hormone that acts on various target tissues, including the liver, skeletal muscle, and fat tissue. The activity of enzymes that govern metabolic responses, such as glycogen synthesis, glycogenolysis, gluconeogenesis, and lipogenesis, is rigorously controlled via intracellular signaling mechanisms downstream of the insulin receptor. Additionally, insulin promotes the uptake of circulating glucose into its target tissues, such as skeletal muscle and fat tissue, and thereby reduces the blood glucose level (Saltiel and Kahn, 2001).

The insulin receptor belongs to the family of receptors with tyrosine kinase (Tyr) intrinsic activity. Autophosphorylated residues are then recognized by different adaptor proteins, which include members of the family of the insulin receptor substrate (IRS), out of which IRS-1 and IRS-2 are the two main substrates and most common

intermediaries in insulin signal propagation initial stage. IRS acts as an adaptor molecule that organizes the formation of molecular complexes and triggers intracellular signaling cascades (Jensen and De Meyts, 2009). Most insulin actions are carried out by activation of two main signaling pathways: the phosphatidylinositol-3-kinase (PI3K) /Akt pathway, also known as protein kinase B (PKB), responsible for most its metabolic actions, and the mitogen-activated protein kinases/Ras pathway (MAPK/Ras), which regulates gene expression and insulin-associated mitogenic effects (White, 2003).

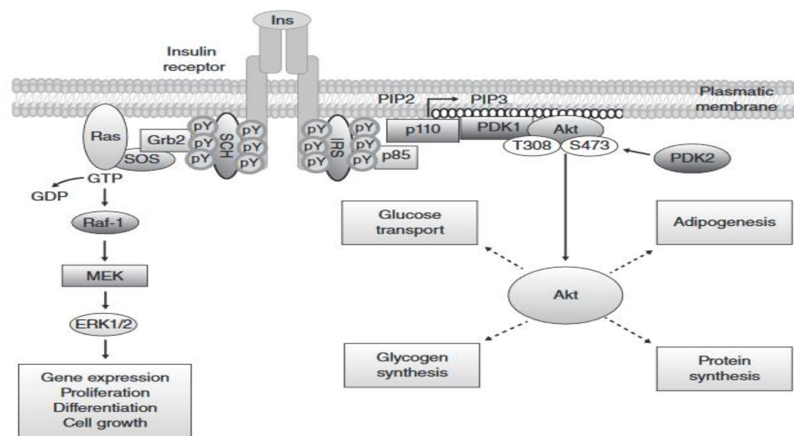

Figure 2.1. The insulin signaling pathway. When interacts with its receptor. Adapting proteins are recruited and phosphorylated, including : IRS, principal mediator of insulin metabolic actions, and SHC, which mediates cell proliferation and growth actions. Main IRS-mediated pathways include the PI3K/Akt pathway, which plays a central role in activation and regulation of several metabolic processes, including glucose transport stimulation, glycogen and protein synthesis and adipogenesis. In the case of SHC, it is associated with MAP kinases pathway activation to regulate its proliferative and growth functions (Taniguchi et.al, 2006).

In the case of the PI3K/Akt pathway, the Akt kinase plays a central role in insulin signaling, since its activation leads to phosphorylation of an important number of substrates with key functions in a wide variety of biological processes, including enzymes, transcription factors, cell cycle regulating proteins and apoptosis and survival proteins. To date, three Akt isoforms have been identified (Akt 1, 2 and 3), out of which Akt2 appears to play an important role in insulin metabolic actions, including muscle and adipose tissue glucose uptake through GLUT-4 translocation from intracellular compartments to the cell membrane, to increase glucose uptake. Additionally, Akt participates in the synthesis of glycogen through GSK-3 β inhibition, synthesis of proteins via mammalian target of rapamycin/ribosomal protein S6 kinase, of 70 kDa (kilo-daltons), and synthesis of lipids (Manning and Cantley, 2007) .

On the other hand, insulin is known to be a potent growth factor; its growth-promoting effects are mediated by MAP/Ras pathway activation. Activation of this pathway involves Tyr phosphorylation of IRS proteins and/or SH2 domain-containing protein (SHC), both of which, in turn, interact with growth factor receptor-binding protein 2 (Grb2), which recruits Sons of Sevenless (SOS) guanine nucleotide exchange factor to the plasmatic membrane for small G protein Ras activation, catalyzing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in Ras, which enables its activation. Ras-GTP operates as a molecular “switch”, stimulating the MAPK cascade through Raf, MEK and ERK1/2 sequential activation. Once active, ERK1/2 translocate to the nucleus and catalyze the phosphorylation of transcription factors that regulate gene expression and promote cell growth, proliferation and differentiation (Fig. 2.1) (Taniguchi et.al, 2006).

Different homeostatic regulatory mechanisms have been identified at the receptor level, at IRS and in proteins located downstream of both, including PI3K, Akt or GLUT-4 (Fig. 2.2). PTP-1B over expression in the pancreatic β cell line INS-1 decreased both receptor and IRS-1 insulin-stimulated Tyr phosphorylation, Akt phosphorylation and glucose stimulated insulin secretion (Lu et.al, 2016). Another molecular mechanism associated with insulin receptor regulation is the phosphorylation of the β-subunit on Ser/Thr residues. There is evidence indicating that this phosphorylation affects receptor kinase activity in response to insulin binding, an alteration that has been observed in states of resistance and obesity both in rodents and in humans. The main receptor phosphorylation-associated kinase is protein kinase C (PKC), which phosphorylates it in different intracellular regions of β-subunit (Youngren, 2007). However, it has also been reported that other Ser/Thr kinases phosphorylate the insulin receptor and decrease its activity, such as protein kinase A (PKA), c-Jun amino-terminal kinase (JNK) and p38-kDa mitogen-activated protein kinase. Among Ser/Thr possible phosphorylation sites, several are found close to autophosphorylation sites or within the catalytic domain, which might affect receptor conformation or access to Tyr residues (Youngren, 2007).

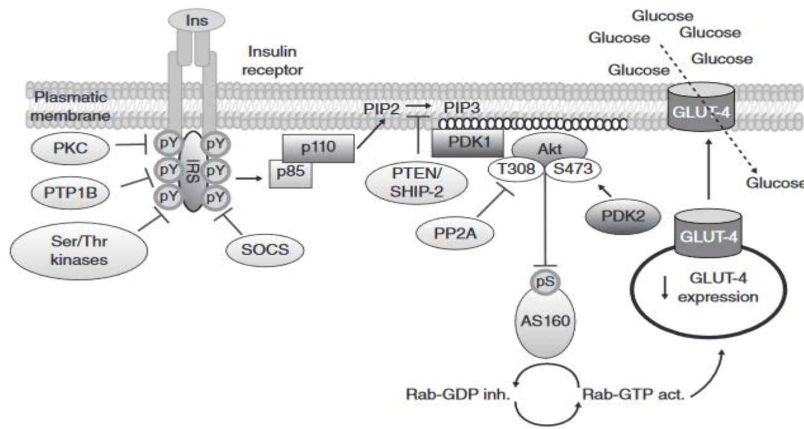

Figure 2.2. Insulin actions regulation. Insulin actions are highly regulated in order to promote adequate functioning of its metabolic, growth-pro-moting and cell proliferation actions. At the receptor level, several regulatory mechanisms have been described, including endocytosis and recycling; dephosphorylation of Tyr key residues that participate in receptor activation and association with adapting proteins, by PTP-1B action, and receptor phosphorylation on Ser/Thr residues by PKC and other Ser/Thr kinases, which affects insulin receptor enzymatic activity. These mechanisms alter receptor activity by disarranging protein complexes formation and regulating their number and cell location. There are other receptor-downstream insulin signaling regulation check points: at the level of IRS proteins, by Ser/Thr residues phosphorylation and by SOCS action; at the Akt level, by phosphatase PP2A action and, at the level of PIP3 synthesis, by PTEN and SHIP-2 lipid phosphatase action, which specifically antagonize PI3K/Akt signalling. Grey arrows and lines indicate negative regulation pathways (Lu et.al, 2016).

2.3 Insulin Resistance

Insulin resistance is a state in which cells do not respond to insulin appropriately, so glucose in the blood are not absorbed by cells. To compensate, the pancreas secrete more insulin to induce glucose uptake

by cells, resulting in hyperinsulinemia (high blood insulin) that occurs in the early stage of the development of type 2 diabetes. Thus, gradually, the over work of β-cells causes their failure in secreting enough insulin, leading to insufficient insulin in the later stage of type 2 diabetic patients.

Development of insulin resistance is mainly associated with low grade tissue specific inflammatory responses induced by various pro-inflammatory and/or oxidative stress mediators notably pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), numerious chemokines and adipocytokines, epigenetic factors, glucolipotoxicity, various transcriptional and metabolic pathways. Chronic exposure of pro-inflammatory mediators stimulates the activation of cytokine signaling proteins which ultimately block the activation of insulin signaling receptors in β-cells of pancreatic islets (Feve and Bastard, 2009).

LPS is the major component of the outer membrane of the Gram negative bacteria. This endotoxin is composed of three modules: a highly variable O-antigen constituted of repeating oligosaccharide units, a core oligosaccharide and lipid A. Lipid A component is responsible for much of LPS toxicity. Toll-like receptors (TLR) of the innate immune system recognize lipid A and then trigger immune and inflammatory responses (Raetz and Whitfield, 2002).

Integrity breakdown and increased intestinal permeability favor LPS translocation from the intestinal lumen to the bloodstream, causing metabolic endotoxemia (Musso et.al, 2011). LPS has a short half-life, so LPS-binding protein (LBP) has been used as a metabolic endotoxemia marker. LBP is an acute-phase protein synthesized in the liver. The

binding of LBP – LPS complex to cluster of differentiation 14 (CD14), which is mainly expressed by macrophages and neutrophils, mediates signal transduction, including nuclear factor kappa B (NF- κ B) activation via TLR4, leading to the activation of innate and adaptive inflammatory responses. Considering that LBP represents the innate immune response triggered by LPS, assessing LBP concentrations is an indirect way to evaluate active LPS. Consequently, LBP is a good marker of metabolic endotoxemia (Liu et.al, 2014). Animal and human studies indicate LPS as an antigen that activates the immune system, playing an important role in the pathogenesis of metabolic chronic diseases related to subclinical inflammation, such as obesity, IR, T2DM, and dyslipidemia (Frazier et.al, 2011).

Hagiwara et.al (2011) determined the effect of insulin therapy on cardiac function in a rat model of systemic inflammation with treated lipopolysaccharides with or without insulin. Cytokine levels and cardiac function were significantly reduced in diabetic rats compared to non-diabetic rats. Moreover, insulin treatment was associated with higher cytokine levels and decreased cardiac function. In systemic inflammatory conditions, diabetes increases various proinflammatory mediators and inhibits cardiac function; insulin treatment exacerbates these effects.

Chronic elevation of circulating intestinal-generated lipopolysaccharide (LPS) (i.e., metabolic endotoxemia) could play a role in the pathogenesis of insulin resistance. LPS increased JNK phosphorylation and MCP-1 and IL-6 gene expression. This inflammatory response led to reduced insulin-stimulated IRS-1, Akt and AS160 phosphorylation and impaired glucose transport. Both pharmacologic blockade of TLR4 with TAK-242, and TLR4 gene silencing, suppressed the inflammatory response and insulin resistance

caused by LPS in human muscle cells. Taken together, these findings suggest that elevations in plasma LPS concentration found in T2DM subjects could play a role in the pathogenesis of insulin resistance (Liang et.al, 2013).

2.4 Treatment for Insulin Resistance

Drugs that reduce insulin resistance is metformin and thiazolidinediones. Metformin is the only biguanide in the UK. It increases insulin action at some unknown intracellular locus, and has no direct action on the pancreatic β-cells. In type 2 diabetes, the main actionof metformin is to potentiate the action of insulin, thus decreasing hepatic glucose production by reducing both gluconeogenesis and glycogenolysis. In addition metformin improves peripheral glucose utilisation in muscle. Metformin is particularly useful for obese type 2 diabetes patients as it does not cause weight gain, but rather a little weight loss. The United Kingdom Prospective Diabetes Study (UKPDS) showed that, compared with conventional treatment with diet, metformin reduced the risk of diabetes-related deaths by 42 % and reduced myocardial infarction (MI) by 39 % over a 10-year follow-up period. These benefits were not seenin overweight type 2 diabetes subjects given a sulphonylurea or insulin therapy. This vascular-protective effect of metformin has now established it as the drug of choice in type 2 diabetes patients with a BMI >25 where diet and lifestyle measures fail to achieve glycaemic control (Nesto et.al, 2004).

Thiazolidinediones: pioglitazone (Actos) and rosiglitazone (Avandia), these drugs are also known as TZDs or glitazones. They act at the level of the genome,modifying the transcription of a number of genes that regulate insulin action and lipid metabolism (Nesto et.al, 2004). The thiazolidinediones (rosiglitazone and pioglitazone) mechanism of action

involves binding to and activating the peroxisome proliferator-activated receptor-Υ (PPAR-Υ). PPAR-Υ expression is highest in adipocytes, intestinal cells, and macrophages but low in most other tissues including skeletal muscle (Stumvoll and Haring 2002).

The thiazolidinedione (TZD) family of drugs is widely used for treatment of type 2 diabetes. PPARγ ligands and TZD (e.g., rosiglitazone and troglitazone) have been proposed to exert anti-inflammatory effects because they may inhibit phorbol myristyl acetate induced secretion of proinflammatory cytokines (such as tumor necrosis factor-α (TNF-α) and interleukin 6 (Woster and Comb, 2007) by monocytes and block lipopolysaccharide (LPS) induced expressions of the inducible nitric oxide synthase (iNOS) and/or cyclooxygenase-2 (COX-2) (Giri et.al, 2004). Thus, these agents have a potential application in inflammation treatment.

Thiazolidinediones, also known as PPAR γ agonists, (rosiglitazone, pioglitazone) improve insulin sensitivity in target organs. Use of these drugs has been controversial, with the first in class troglitazone withdrawn because of liver toxicity. Rosiglitazone is now used infrequently because of adverse cardiovascular outcomes (Mahaffey et al, 2013). These drugs are associated with durable control (Kahn et al, 2011) and improve HbA1c concentration by up to about 1%.

2.5 Bacterial EPS

Polysaccharides are extremely complex and not encoded in the genome; therefore, until recent decades, they have gradually been determined to have various biological functions, such as antioxidant, immunomodulation, antitumour, radioprotection, antidiabetes, hepatoprotection and antimicrobial (Chen et al., 2013).

Exopolysaccharide (EPS) is one type of metabolite in many microorganisms. They are usually biocompatible, edible, and nontoxic to humans and the environment (Shih, 2010). Recently, there has been an increased interest in exploiting the EPS for their biological activities including antitumor, immunostimulatory, cholesterol-lowering activity, and antioxidant activities (Chen et.al, 2006).

Exopolysaccharides (EPS), produced by both prokaryotes (Gram-positive and Gram-negative bacteria) and eukaryotes (fungi, some algea, and phytoplankton), Various exopolysaccharides produced by bacteria have novel and unique physical characteristics. Microbial exopolysaccharides (EPS) are heterogeneous polymers that are formed of wide range of homo- or hetero-carbohydrates as well as organic and inorganic substituents, the monosaccharides are linked together through glycosidic bonds (Zong et.al., 2012).

Bacteria release EPS in the environment in the form of capsules or slime to help these microorganisms cope with adverse environmental conditions as desiccation prevention and adhesions by forming biofilms. (Wijesekara et al., 2011). Several factors and parameters influence the production of EPS among these are the composition of the medium, especially carbon and nitrogen sources, pH, temperature, and incubation time. The highest EPS yield (292 mg/ ml), from *Bacillus thuringienisis* S13 was observed on using glucose as carbon source (58.5 mg/ml), peptone (49.5 mg/ml) at pH 7.0 this EPS have anti proliferative activity on A549 lung cancer cells (Parthiban et al., 2014).

Among these properties EPS shows efficacy as anti cancer, antioxidant and show immune stimulation activities evidences that

made EPS gained increasing attention as source of potential new drugs for Cancer, one of the top ten leading causes of mortality worldwide, as recent treatment strategies shows limitations because severe side effects and multidrug resistance occurred in the clinical application. For example it has been found that MD-b1-derived polysaccharides show significant therapeutic activities against gastric tumors from the endophytic bacteria *Bacillus amyloliquefaciens* (Chen et al., 2013).

Exopolysaccharide from *Lachnum* YM40 (LEP-2a) and its derivatives were able to relieve the cardiovascular disease in the diabetic mice, especially the 200 mg/kg dose LEP-2a. Histopathological observation revealed that myocardial structure disorder and confluent necrosis of cardiac muscle fibers were relieved in the diabetic mice after the treatments of LEP-2a and its derivatives, which indicated that LEP-2a have more significant cardioprotective effect, supporting them as an important role on treating cardiovascular complication in diabetes mellitus (Xu et.al, 2017).

The other study was designed to investigate the anti-hyperglycaemia, hypolipidemia and renoprotective effects of two extracellular polysaccharides (EPS) from *Pleurotus eryngii* SI-04 (EPS1 and EPS2) in mice with streptozotocin (STZ)-induced diabetic nephropathy (DN), a common microvascular complication of diabetes mellitus (DM). The glomerular proliferation and tubular necrosis were considerably recovered by the administration of EPS1 and EPS2, indicating that intervention with EPS1 and EPS2 can protect the kidneys of diabetic mice and inhibit the progression of DN (Zhang et.al, 2018).

Huang et al. (2015) isolated EPS from *Enterobacter cloacae* Z0206 and in the present experiment showed that the severely impaired glucose tolerance confirmed the insulin resistant state of the KKAy mice. Improved oral glucose tolerance, reduced serum insulin levels as well as decreased serum triglycerides (TG), cholesterol (TC) and low density lipoprotein cholesterol (LDL-c) were observed after treatment with EPS. The results suggested that EPS had the hypoglycemic effect. Limited papers have reported EPS with anti-diabetic function. EPS of *B. amyloliquefaciens* is the first EPS of probiotic which is found to lower blood glucose. EPS from *B. Amyloliquefaciens* is very unique because it have hypoglycemic effect. It molecular weight is 23,29 x 10^4 g/mol with monosaccharide ratio (%) are 96,9 mannose and 3,1 glucose (Han et.al, 2015).

CHAPTER III. MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell Line

IEC 18 cells from rat ileum epithelium were obtained from Bioresource Collection and Research Center (BCRC, 60230, Hsinchu Taiwan).

3.1.2 Media

3.1.2.1 Dulbecco's Modified Eagle Medium (DMEM)

DMEM containing 5% Fetal Bovine Serum (FBS, Gibco, Mexico) was used as growth medium for IEC-18 cells. One liter of the medium was prepared by dissolving one pack of pre-mixed DMEM powder (high glucose, GIBCO, Grand Island, USA) into 700-800 ml distilled water. After the addition of 1.5 g of sodium bicarbonate ($NaHCO_3$) and adjustment to pH 7.4, the volume was adjusted to 950 ml by distilled water. 50 ml of FBS (Fetal Bovine Serum, Gibco, Mexico) was added to the medium for serum containing media. The media was filtered through 0.22 µm filter membrane (Milipore, Salt Lake City, USA) and stored in sterile bottles at 4°C.

3.1.2.2 Modified Eagle Medium (MEM)

One liter of the medium was prepared by dissolving one pack of pre-mixed MEM powder (M0268-10X1L, Sigma, Spruce Street, St Louis, USA) into 900 ml double distilled water. After the addition 2.2 g of sodium bicarbonate ($NaHCO_3$) and adjustment to pH 7.4, the volume was adjusted to 1000 ml by distilled water. The media was filtered through 0.22 µm filter membrane and stored in sterile bottles at 4°C.

3.1.3 *Bacillus amylolliquefaciens* exopolysaccharides (EPS)

EPS were isolated from *Bacillus amyloliquefaciens* amy-1. Culture *Bacillus amyloliquefaciens* amy-1, centrifuge 6000 x g at 4^0C for 30 min, and collect the supernatant. After that, Filtrate the supernatant through a whatman N0.44 filter paper, followed by filtration through a 0.45 μm top filter, and place the supernatant into sterile Nalgene centrifuge bottles. Incubate at 100^oC for 20 30 min to denature proteins. Allow the sample to cool down in room temperature. Centrifuge at 6000 x g at 4^0C for 30 min.

EPS is insoluble in ethanol. Thus, add 95% ethanol to the sample (95% ethanol : supernatant = 2:1, v/v) ([ethanol] will become 75%) to precipitate EPS, incubate at 4^oC, overnight. The next day, prepare sterile distilled water and sterile centrifuge tubes/bottles, and weigh the tubes/bottle. Centrifuge the sample by 6000 x g, 4^oC, 10 min. Remove the supernatant. Add sterile water to completely dissolve EPS. Place the sample into centrifuge tubes/bottles. Precipitate EPS again using 95% ethanol : EPS solution = 2:1. Incubate at 4^oC, overnight. The next day, centrifuge the sample by 6000 x g, 4^oC, 10 min. Remove the supernatant. Place the pellet into -80^oC for 6 hours, followed by freeze drying. Furthermore, calculate the weight of dried EPS. Dissolve EPS in appropriate amount of sterile water before use, and filtrate by 0.22 μm filter.

Quantitation of EPS use phenol-sulfuric acid method to determine the concentration of carbohydrates in the preparation of EPS. Dilute EPS 100X and 1000X, withdraw 1 mL to glass tubes for analysis. Prepare 80% phenol, with 95% ethanol as the solvent. Then, mix 1 mL diluted EPS + 2.5 µl 80% phenol + 2.5 mL 95% sulfuric acid in a glass tube. Meanwhile, prepare glucose solutions (20, 40, 60, 80, 100 mg/L, 1 mL

for each concentration) to perform the same reaction for deriving the standard curve. Mix well and incubate in room temperature for 10 min, followed by incubation in a water bath of room temperature to cool down the tubes. Withdraw 200µl to a 96-well plate to check OD at 490 nm. Use the data from glucose solutions to derive a standard curve. Finally, calculate the carbohydrate content in the EPS solution based on the standard curve.

3.1.4 Reagent for Cell Treatment

A. LPS (Lipopolysacharides)

Lipopolysaccharides was purchased from Sigma-Aldrich and dissolved in ddH20 at concentration 100 µg/ml as stock solution. The solution was aliquoted and stored at 20^0C.

B. Insulin

Bovine insulin powder was purchased from Sigma-Aldrich and dissolved in PBS at concentration 10 mg/ml as stock solution. The solution was aliquoted and stored at 20^0C.

C. Rosiglitazone (RZD)

RZD (Sigma-Aldrich) was dissolved in DMSO at a concentration 25 mM as a stock solution. The solution was aliquoted and stored at 4^0C.

3.1.5 Reagent for Western Blotting Analysis

A. Cell Lysis Buffer

Composition	Volume
PMSF (phenylmethylsulfonyl fluoride)	10 µl
Pepstatin	1 µl
Leupeptin	1 µl
Aprotinin	1 µl
Phosphate Inhibitor	100 µl
5x Lysis buffer (Promega, USA)	200 µl
ddH20	687 µl
Total	1000 µl

B. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Composition	12% of Separating Gel	3% of Upper Gel
30% acrylamide solution	3600 µl	400 µl
2M Tris-HCl (pH 8.8)	1688 µl	-
0.5 Tris-HCl (pH 6.8)	-	1000 µl
10 % SDS	108 µl	48 µl
0.5 EDTA (pH 7.5)	36 µl	16 µl
10% APS	90 µl	40 µl
H20	3469 µl	2492 µl
TEMED	9 µl	4 µl
Total Volume	9000 µl	4000 µl

C. 10X TGS Buffer (SDS-PAGE Running Buffer)

Composition	
Tris-base	30.28 g
Glycine	150.14 g
SDS (Sodium dodecyl sulfate)	10 g

Those materials were dissolved into 1L double-distilled water and stored at 4°C. The 10X TGS buffer was diluted by 10folds (1X TGS buffer) prior to be used as a running buffer.

D. Transfer Buffer (Western Blotting Running Buffer)

Composition	
Tris-base	2.42 g
Glycine	11.26 g
NaCl	0.5845 g
Methanol	200 ml
SDS (Sodium dodecyl sulfate)	0.5 g

Those materials were dissolved into 1L double-distilled water and stored at 4°C.

E. 10X PBS-Tween 20 Buffer (Western Blotting Washing Soluion)

Composition	
KCl	2 g
Na2HPO4	14.4 g
NaCl	80 g
KH2PO4	2.4 g
Tween-20	20ml

Those materials were dissolved into 1L double-distilled water, adjusted to pH 7.4 by 0.1 M HCl 0r 0.1 NaOH, and stored at 4 ^{0}C. Prior to be used as a washing solution, the 10X PBS-Tween 20 buffer was diluted 10 folds (1X PBS-Tween 20), and stored at 4°C.

F. Substrate for horseradish peroxidase-conjugated primary and secondary antibodies

- SignalBoost™Immunoreaction enhancer kit

I. Molecular-Weight Markers

- Prestained standard protein marker 10 – 170 kDa (Thermo Scientific)

J. Antibody

Primary antibodies for specific protein	Provider (catalog number)
Akt	Cell Signaling (4691)
pAkt	Cell Signaling (4058)
Secondary antibodies	
Goat anti-rabbit IgG antibody	St.Cruz (2060)

3.1.6 Other Chemical and Reagents

- APS (Ammonium persulfate, 31117, Sigma-Aldrich)

- TEMED (Tetramethylethylenediamine, 0591C348, AMRESCO, Solon, OH, USA)

- Acrylamide (UN2074, Millipore, Billerica, MA, USA)

- Tris-base (12791,USB, Cleveland, OH, USA)

- Methanol (M5XG2H, Burdick & Hackson, Ulsan, Korea)

- Glycine (33226, Vivantis, Chino, California, USA)

- Bradford Assay Reagent (Bio-Rad, Hercules, Caliornia, USA)

- Glucose Kit (GOF FS, 1 2550 99 10 023Dyasis, Holzheim, Germany)

3.2 Instruments

3.2.1 Instruments for Cell Culturing

- Laminar Flow (Hipoint, Jih Her Tyan Scientific, Taiwan)

- CO2 Incubator (Model MCO-15AC, Sanyo, Nagoya, Japan)

- Microscope (Olympus IX51, Olympus Corporation, Tokyo, Japan)

- Liquid nitrogen storage barrels (LNC-35R, AirLiquid, Taiwan)

3.2.2 Instruments for Western Blotting and Protein Analysis

- Low-temperature high-speed centrifuge (Biofuge Fresco, Germany)

- Spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan)

- Dry bath (MDO-2, Fisher Scientific, Pittsburgh, USA)

- Protein electrophoresis apparatus (Bio-Rad, Herculas, CA, USA)

- Stirrer plate (HP 3000, Jeio Tech, Korea)

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- UVP Biospectrum Imaging System Vision WorksLS 6.0 (Level Biotechnology Inc)

- Shaker (Kansin Instruments, Taiwan)

3.2.3 Instruments for Glucose Uptake Assay

- ELISA reader (Spectra max Plus 384, Molecular Devices LLC, USA)

- Orbital shaker (Kansin Instruments, Taiwan)

3.2.4 Instruments for Western Blotting

- UVP analysis

3.3 Experimental Procedures

3.3.1 Cell Culture

IEC-18 cells were sub-cultured when the confluence reached 90%-100%. The old medium was removed and the cells were washed twice with sterile PBS. One ml of TrypRC (Genedirect) was added into the dish of 10-cm diameter and incubated for 5 min at 37°C. The suspension was centrifuge at 500 x g for 5 min. The supernatant was removed, and the cell pellet was resuspended in an appropriate volume of fresh DMEM containing 5% FBS also containing 0.1 units/ml insulin. The cell suspension was then divided into 2 or 3 dishes of 10-cm diameter and incubated at 37°C in incubator supplemented with 5% CO_2 for 3 days to reach 90%-100% confluence.

3.3.2 Western Blot Analysis

3.3.2.1 Cell Preparation

Cell were seeded in 3.5-cm dishes incubated at 37°C CO_2 5%. After reached 90% confluence, cells were incubated for 24 h in serum-free DMEM and insulin-free. Subsequently, cells were incubated in containing 100 ng/ml LPS to induce insulin resistance. The old medium was removed and cells were washed with PBS twice, followed

by incubation in serum-free DMEM containing 100 nM of insulin, 200 µg/ml of EPS, or 50 µM of RZD.

3.3.2.2 Cell Lysis for Total Protein Extraction

Cell culture was washed twice with PBS. For a 3,5-cm dish, 100 µl of cell lysis buffer (see section 3.1.5 A) was added to the dish, and cells was scrapped off the plate on ice. The resulting cell lysate was kept on ice prior to centrifugation at 3000 rpm in 4°C for 10 min. The supernatant containing the protein crude extract was transferred into a new sterile tube and used directly for Western blotting.

3.3.2.3 Protein Quantification

Protein concentration was measured using Bradford assay reagent. Bovine serum albumin was used to make a standard curve of protein concentration. Various concentration of BSA (0, 1.5, 2, 4, 6, 8, and 10 µg/ml) were prepared by diluting 0.1 mg/ml BSA in distilled water to the final volume of 800 µl. Simultaneously, 2 µl of protein crude extract was diluted into 798 µl of distilled water. Either BSA or protein sample were then added with 200 µl of Bradford assay reagent, mixed well, and incubated at room temperature for 10 min. The optical density (O.D.) of protein samples and the standards were detected at the wavelength of 595 nm. Both sample and the standards were performed in duplicate. The O.D. of BSA standards were plotted to make a standard curve using Excel program and the equation of the curve was obtained using linear regression. The concentration of the sample was calculated according to the standard curve.

3.3.2.4 Electrophoresis of Proteins by SDS-PAGE

Sample was heated at 100°C for 5 min prior to loading into an SDS-PAGE gel that already formed to the formulation described in 3.1.5 B. Fresh 1X TGS buffer (see section 3.1.5 C) was added into the inner

tank as the running buffer. Electrophoresis was set firstly at a voltage of 30 mV for 40 min to allow the protein sample pass throught the stacking gel, followed by a voltage of 100 mV for 1 h 30 min to allow the separation of proteins in the separating gel.

3.3.2.5 Blotting of Proteins onto PVDF Membrane

The PVDF (Polyvinylidene fluoride) membrane (Millipore, USA) was immersed in 100% methanol for 5 minutes, then in transfer buffer for a few minutes. The transfer sandwich was set up following an order of fiber pad, filter paper, SDS-PAGE gel, PVDV membrane, filter paper, and fiber pad. The sandwich was put into the transfer tank containing the transfer buffer (see section 3.1.5 D). Electrophoresis was set at a voltage of 40 mV for 1 hour. After electrophoresis, the PVDF membrane was immersed with shaking for 1 hour in a blocking solution.

3.3.2.6 Antibody Detection

The PVDF membrane was cut according the molecular weights of target protein. The strip was incubated with the specific primary antibody diluted in the SignalBoost immunoreaction enhancer kit solution at an appropriate ratio for overnight at 4ºC with shaking. Then, the antibody solution was removed and the strip was washed 3 times with PBS-Tween 20. The secondary antibody specific for the primary antibody was diluted into SignalBoost immunoreaction enhancer kit solution in an appropriate ratio, and incubated for 1-2 hours at room temperature with gently shaking. The strip was washed 3 times with PBS-Tween at room temperature.

3.3.2.7 Visualization of Protein

For visualization of proteins on PVDF membrane, the PVDF membrane was immersed in 600 µl of Supersignal West Femto Maximum Sensitivity Substrate containing equal volume of solution A and B or

alternatively immersed in the luminance solution. Protein bands were visualzed by scanning using UVP Biospectrum Imaging System Vision WorksLS 6.0, and band intensities analyzed by the supplied program.

3.3.3 Glucose Uptake Assay

To determine glucose uptake of IEC-18 cells, cells were seeded in 96-well dishes in a density of $1x10^5$ cell/well. Cells were incubated overnight in DMEM containing 5% FBS. After overnight incubation at 37^0C, cells were incubated in serum-free MEM containing 100 ng/ml LPS for 16 h to induce insulin resistance. The medium was removed and 50 µl of serum-free MEM containing 100 nM of insulin, or insulin and the indicated concentration of EPS, or insulin and 50 µM RZD was added, cells were incubated for 5 h. 5 µl of the supernatant was aliquoted and mixed with 200 µl of Glucose Kit in a 96-well plate and incubated at room temperature for 10 min. Absorbance at 500 nm was then determined using a microplate reader. Meanwhile, the cells were subjected to WST-8 assays (the next paragraph) to analyze the relative cell number between wells. The result of glucose uptake are normalized by the cell number in each well (WST-8 assay)

3.3.4 WST-8 Assay

The cell Counting Kit 8 (WST-8) is a convenient and robust way of measuring cell viability. The kit uses a water-soluble tetrazolium salt to quantify the number of live cells by producing anorange formazan dye upon bio-reduction in the presence of an electron carrier. After did a glucose uptake assay, all the medium was removed and was replaced with MEM serum free mixed with WST-8 reagent with ratio 10:1 (the final volume of the WST-8 reagent). 50 µl of WST-8 reagent was added in each well and blank wells (medium without cells). Cells incubated at 37^oC

for 3 hours. After incubation, absorbance 450 nm and 690 nm was then determined using a microplate reader.

3.3.5 Cytotoxicity Assay

To determine toxicity of EPS and LPS by WST-8 assay, cells were seeded in 96 well dishes in a density of $1x10^5$ cell/well. Cells were incubated in MEM free serum containing a various concentration of EPS and LPS for 24 hours. After 24 hours, the medium was removed and 50 µl of WST-8 mix with MEM free serum was added, then cells was incubated at 37°C for 3 hours. After incubation, absorbance 450 nm and 690 nm was then determined using a microplate reader.

3.3.6 Statistic Analysis

Glucose uptake assay and cytotoxicity is statistically analyzed by one-way ANOVA, followed by *Scheffe's post hoc* test. Significance was considered when $P < 0.05$ and $F > 35546$.

CHAPTER IV. RESULTS

4.1 The Induction of Insulin Resistance by LPS

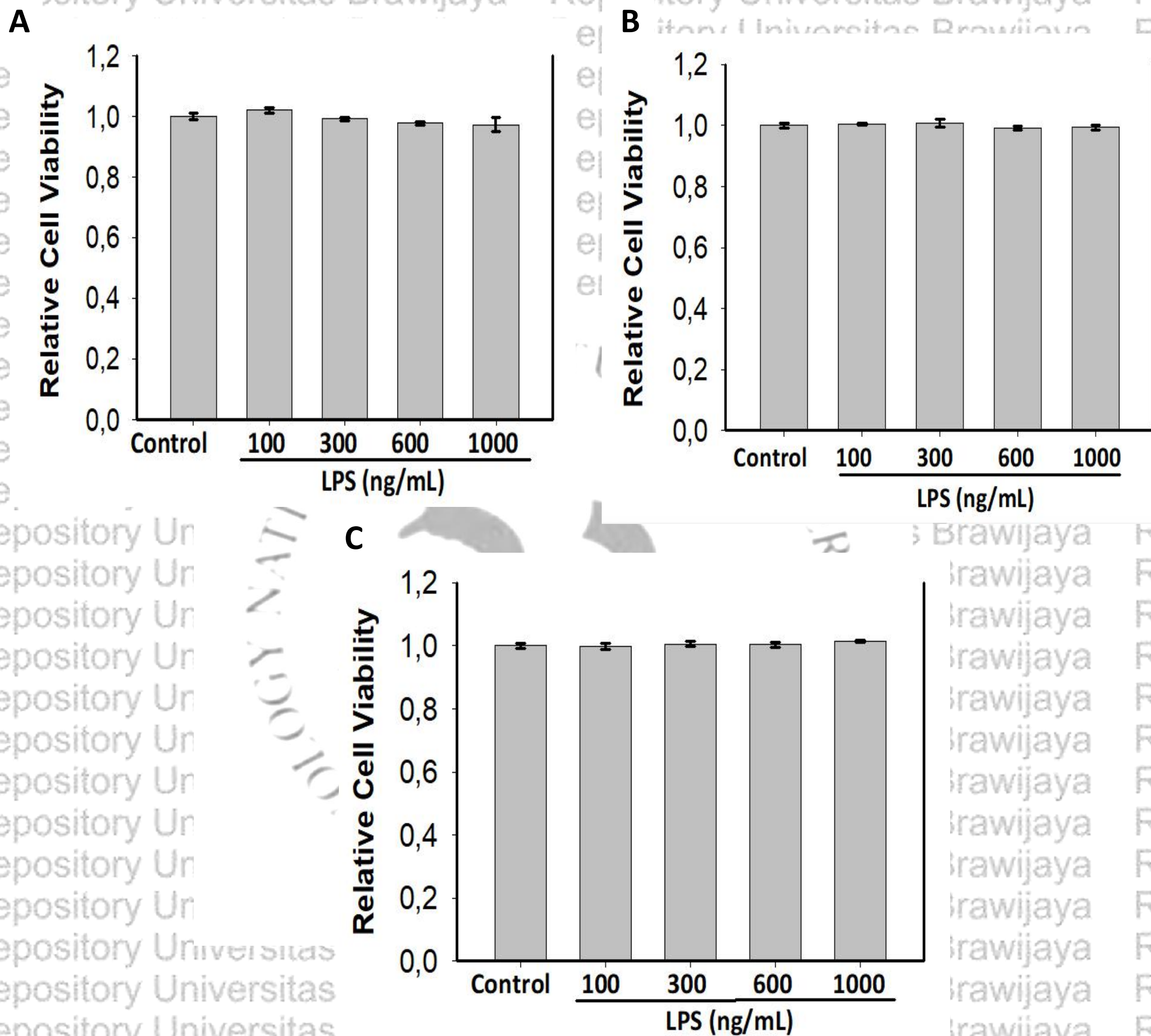


Figure 4.1. The citotoxicity of LPS on IEC-18 cells. Cells were treated with 100, 300, 600, or 1000 ng/ml LPS, or water (the solvent; control) for 24 hours. Cell viability relative to the control is calculated. A, B and C are three independent experiments, each in triplicate. Data represent mean ± SD.

The development of diabetes is closely related to inflammation, and insulin resistance can be induced by inflammatory factors such as LPS. Thus, it was tested whether LPS could induce insulin resistance in IEC-18 cells. The toxicity of LPS to IEC-18 cells was analysed first. As shown in Figure 4.1, when IEC-18 cells were treated with 100 to 1000 ng/ml LPS, there was no obvious inhibition on cell growth, suggesting that 100 to 1000 ng/ml LPS is not toxic to IEC-18 cells.

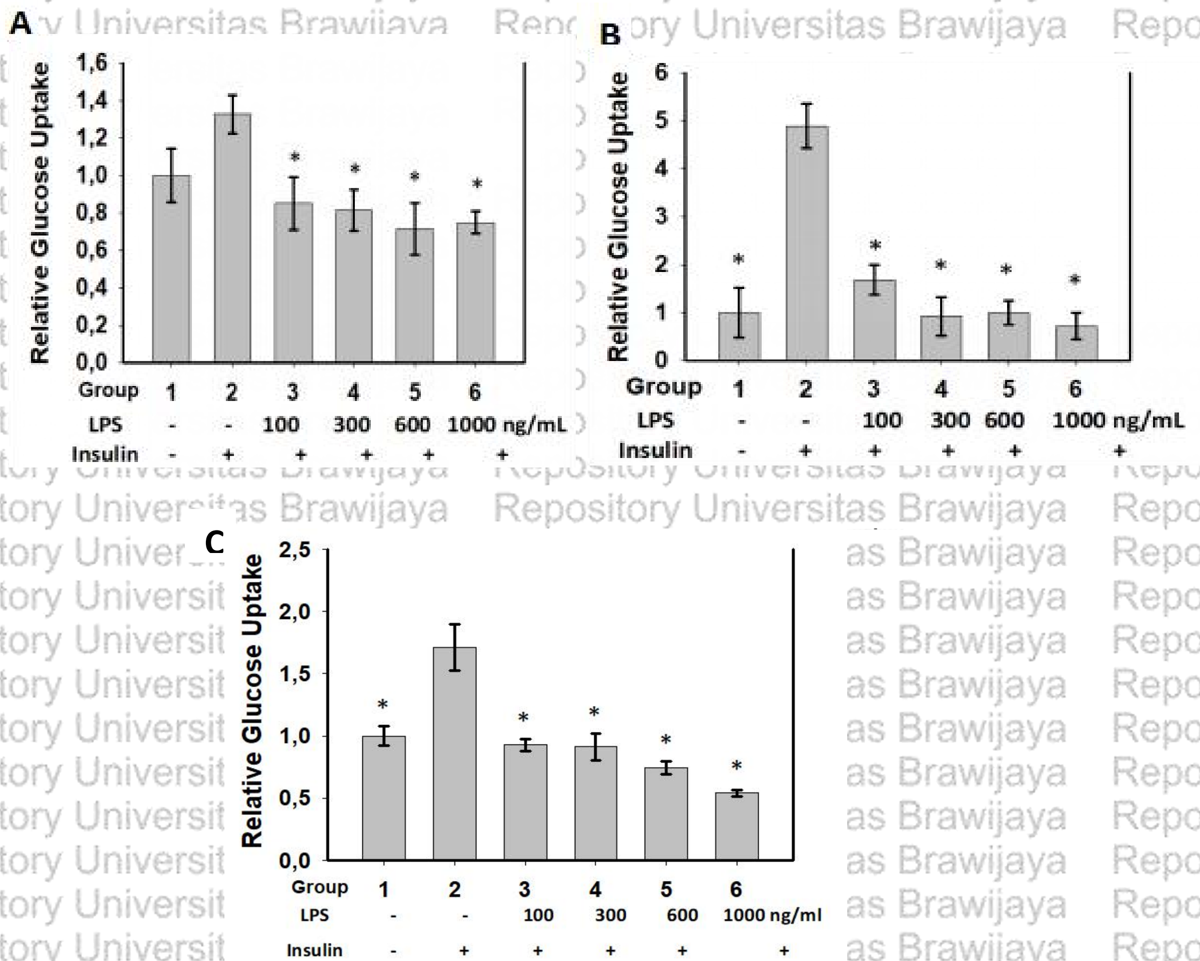


Figure 4.2. Induction of insulin resistance by LPS. Cells were treated with 100, 300, 600, or 1000 ng/ml of LPS for 16 h, followed by stimulation with 100 nM insulin for 5 h. Relative glucose uptake was measured. A, B and C are three independent experiments, each in triplicate. Data represent mean ± SE. *P<0,05 versus Group 2.

Therefore, IEC-18 cells were treated with 100, 300, 600 and 1000 ng/ml LPS for 16 h to induce insulin resistance and insulin-induced glucose uptake of the treated cells was assayed. As shown in Figure 4.2, insulin obviously promoted the glucose uptake of cells (Group 2 vs Group 1), whereas 100 – 1000 ng/ml LPS pre-treatment all effectively suppressed the insulin-induced glucose uptake (Groups 3 - 6).

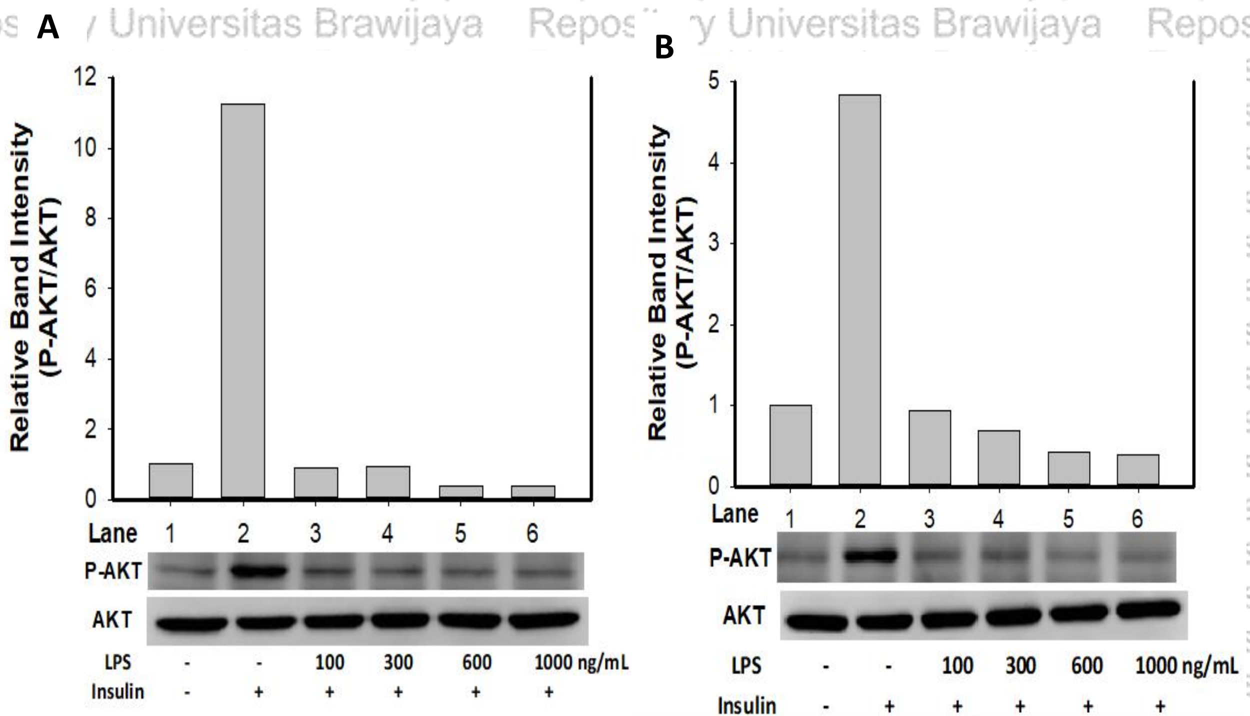


Figure 4.3. LPS inhibited insulin-induced activation of Akt. IEC-18 cells were treated with 100, 300, 600, 1000 ng/ml LPS for 16 h, followed by treating with 100 nM insulin for 1 h. A and B are two independent experiments. The relative band intensity versus Lane 1 in each assay was shown in the graph.

LPS-treated cells were also subjected to western blotting to assay the insulin-induced activation of Akt. As shown Figure 4.3, insulin obviously increased the phosphorylation of Akt (Lane 2), yet 100 - 1000 ng/mL LPS pre-treatment apparently inhibited the effect of insulin on Akt (Lanes 3-6). Overall, Figures 4.2 and 4.3 demonstrated that pre-treatment

by 100 – 1000 ng/mL LPS effectively resulted in insulin resistance in IEC-18 cells. Thus, 100 ng/mL LPS was used to induce insulin resistance in IEC-18 cells in the following assays.

4.2 The Effect of EPS on Insulin-Resistant IEC-18 Cells

The toxicity of EPS on IEC-18 cells was analysed first. As shown in Figure 4.4, when IEC-18 cells were treated with 50 300 µg/ml EPS for 24 hours, there was no obvious inhibition on cell growth, suggesting that 50 300 µg/ml EPS is not harmful to IEC-18 cells.

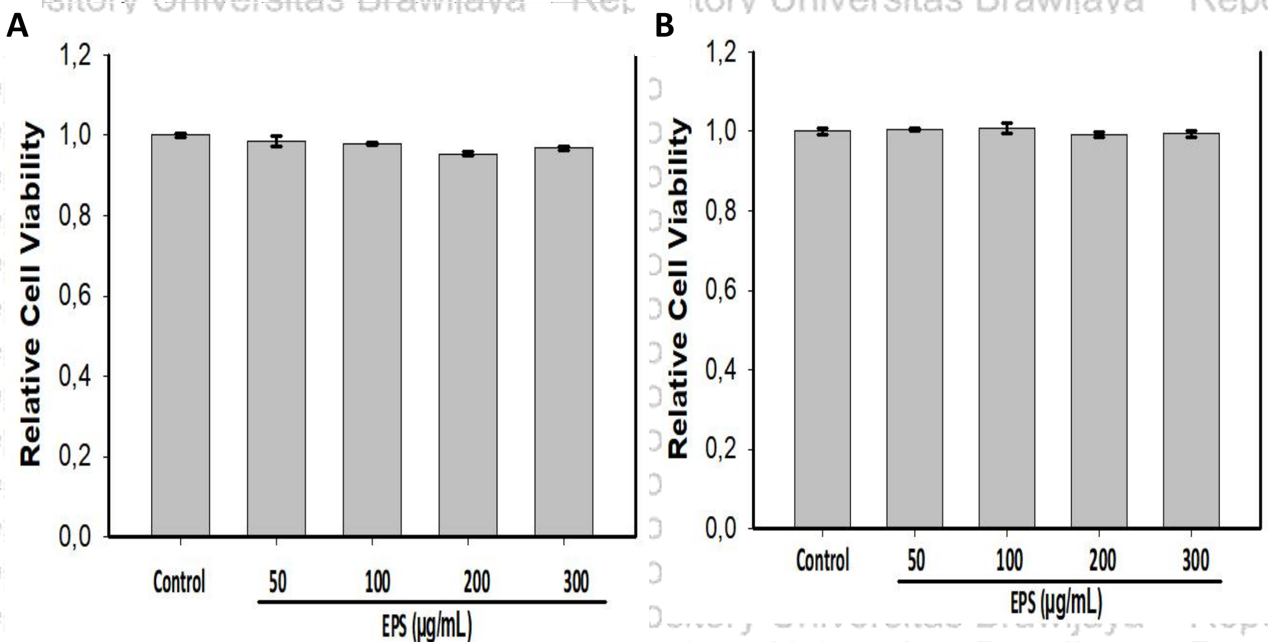


Figure 4.4. The citotoxicity of EPS on IEC-18 cells. Cells were treated with 50, 100, 200, or 300 µg/ml EPS, or water (the solvent; control) for 24 hours. Cell viability relative to the control is calculated. A and B are two independent experiments, each in triplicate. Data represent mean ± SD.

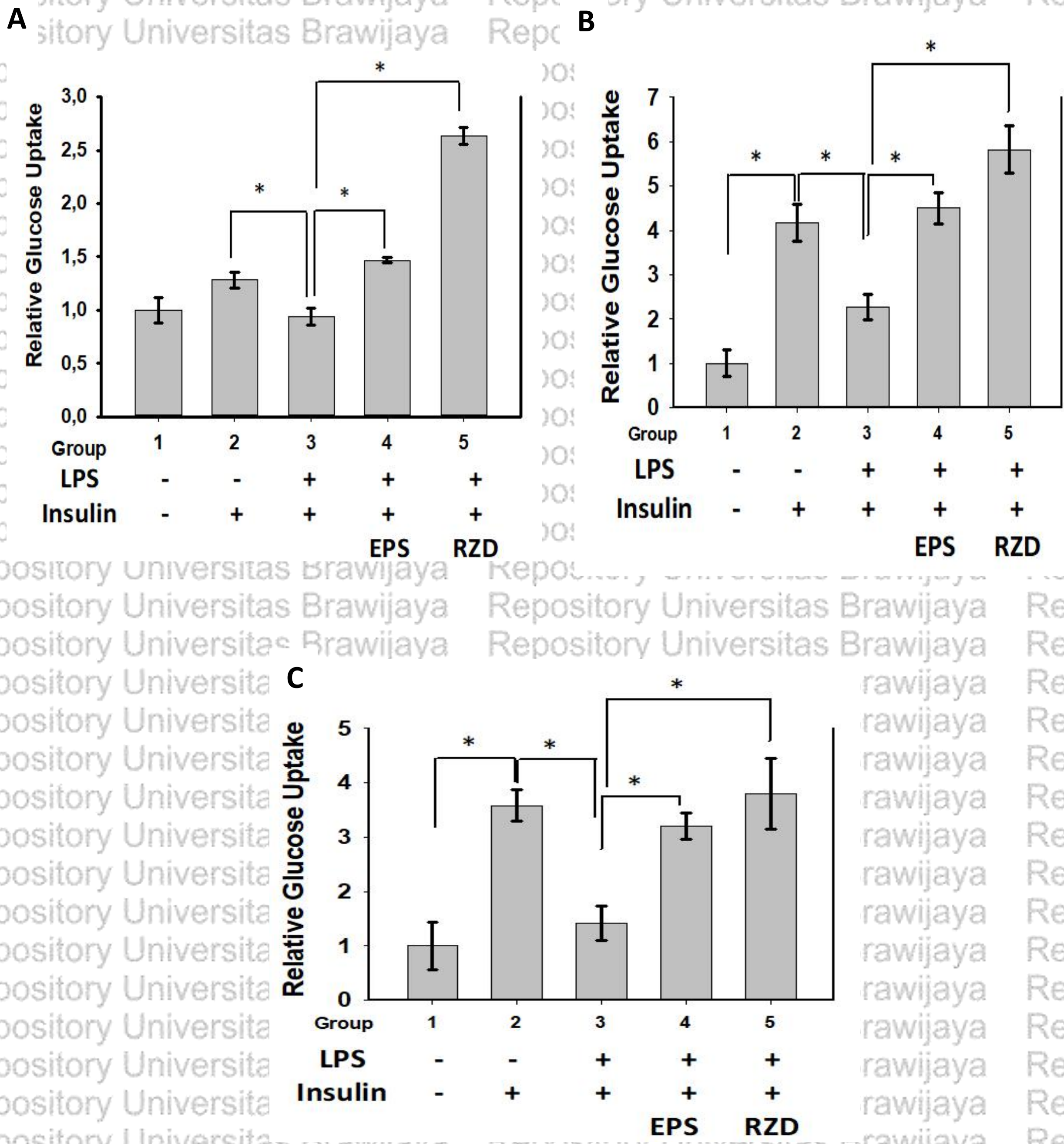
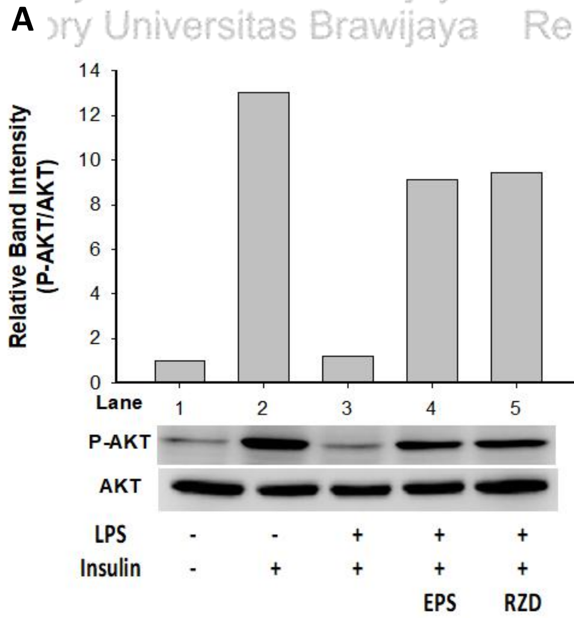
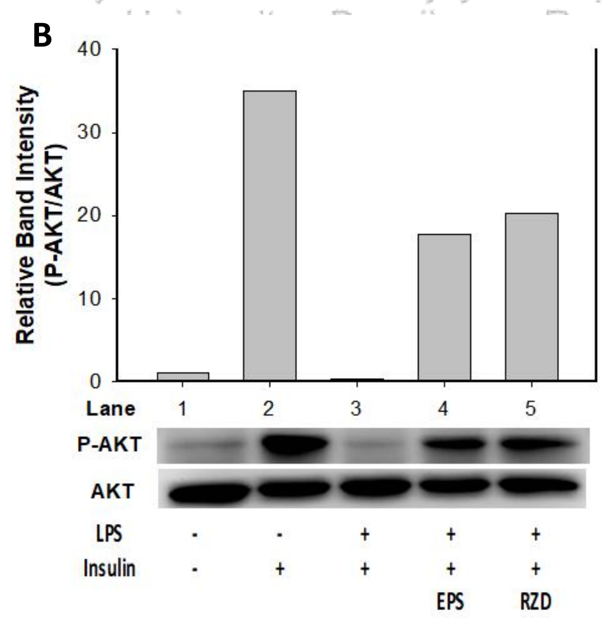


Figure 4.5. The effect of EPS on the glucose uptake of insulin-resistant IEC-18 cells. IEC-18 cells were treated with 100 ng/ml LPS for 16 hours, followed by treating with 100 nM insulin, 200 µg/ml EPS, or 50 µM rosiglitazone (RZD) for 5 hours. A, B, and C are the results of three independent experiments, each in triplicate. Data represent mean ± SE. *$P < 0.05$ between the indicated groups.

Subsequently, IEC-18 cells were treated with 100 ng/mL LPS to induce insulin resistance, followed by treatment with 100 nM insulin and 200 µg/ml EPS, or 50 µM rosiglitazone (RZD, as a positive control). As shown in Figure 4.5, insulin obviously promoted the glucose uptake of cells (Group 2), and LPS effectively suppressed the effect of insulin (Group 3), suggesting the generation of insulin resistance. EPS (Group 4) and rosiglitazone (Group 5) obviously elevated the level of glucose uptake of cells. These results supported that EPS could recover the glucose uptake of insulin-resistant IEC-18 cells.

Therefore, whether EPS could activate Akt was examined next. Figure 4.6 showed that insulin obviously increased the phosphorylation of Akt in IEC-18 cells (Lane 2), but LPS inhibited the effect of insulin (Lane 3), confirming the development of insulin resistance in these cells. When EPS (Lane 4) or RZD (Lane 5) was added with insulin in LPS-treated cells, they both obviously promoted the phosphorylation of Akt as compared to insulin added alone (Lane 3). The data indicated that EPS was able to recover the activation of Akt, an effector in the insulin-signaling pathway.





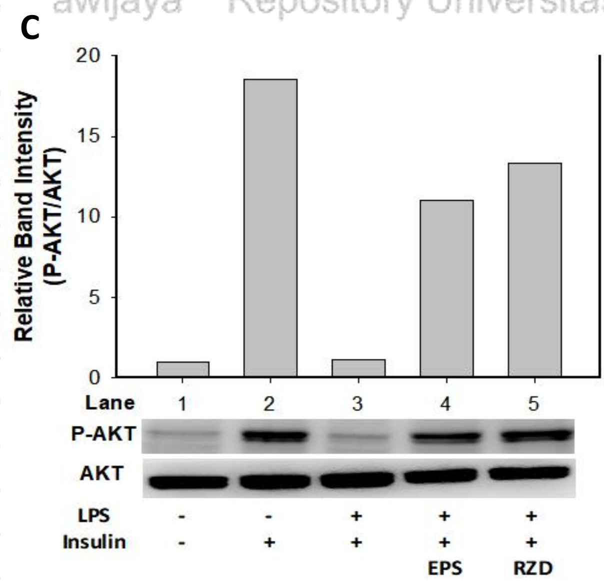
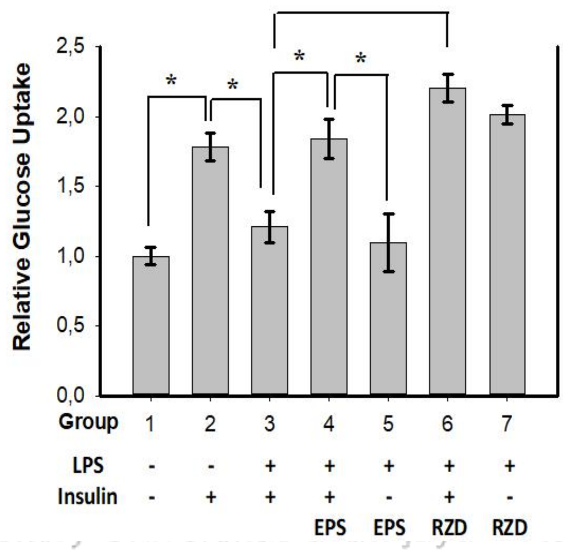
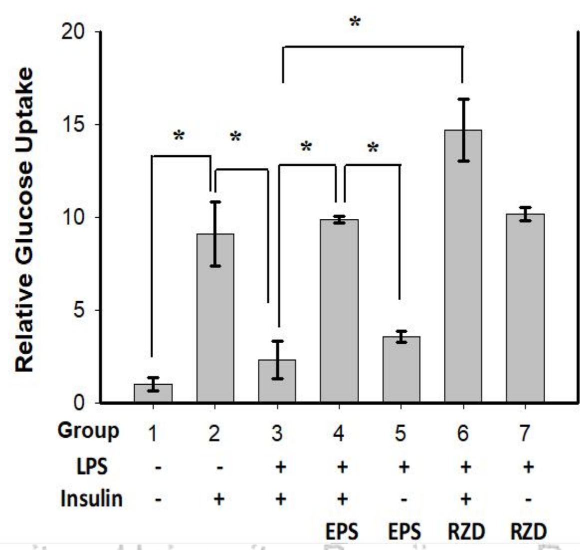


Figure 4.6. EPS activated Akt in insulin-resistant IEC-18 cells. IEC-18 cells were treated with 100 ng/ml LPS for 16 hours, followed by treating with 100 nM insulin, 200 μg/ml EPS, or 50 μM rosiglitazone (RZD) for 1 hours. A, B, and C are three independtly experiments. The relative band intensity versus Lane 1 in each assay was shown in the graph.





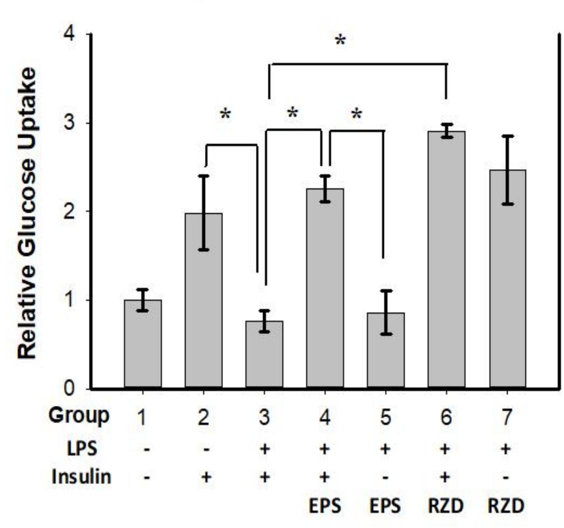
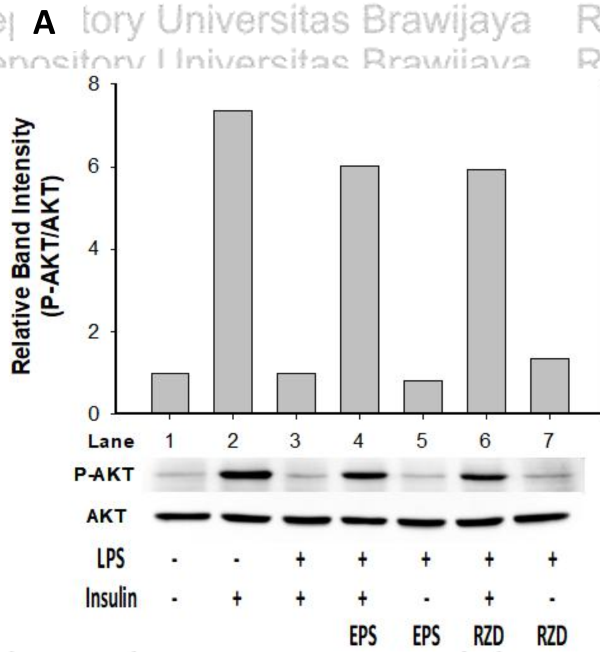
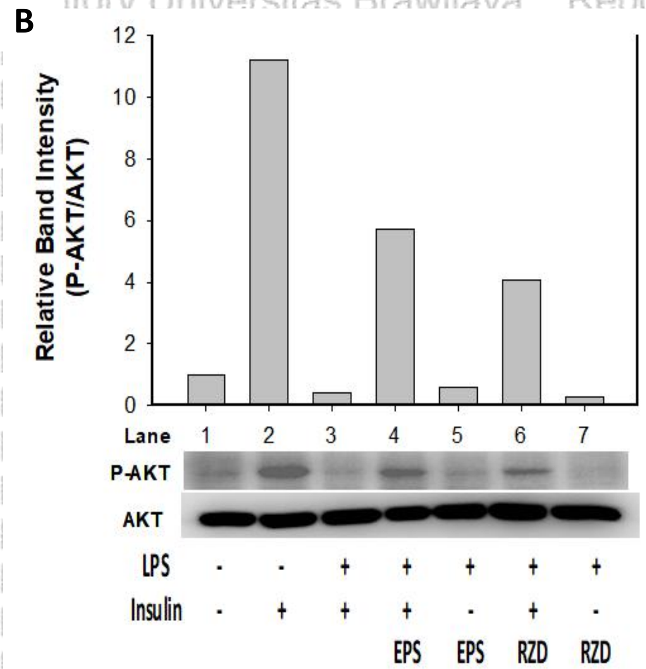


Figure 4 7 EPS worked as an insulin sensitizer in insulin-resistant IEC-18 cells. IEC-18 cells were treated with 100 ng/ml LPS for 16 hours, followed by treating with 100 nM insulin alone (Group 3), 200 μg/ml EPS in the presence (Group 4) or absence (Group 5) of insulin, or 50 μM RZD with (Group 6) or without (Group 7) insulin. A, B, and C are three independent experiments, each in triplicate. Data represent mean ± SE. **p* < 0,05 between the indicated groups.

To further explore the mechanism of EPS, IEC-18 cells were treated with LPS to induce insulin resistance, and the cells were then treated with EPS in the presence or absence of insulin. As shown in Figure 4.7, EPS and insulin together could recover the glucose uptake inhibited by LPS (Group 4). However, EPS alone did not show this function (Group 5). The data indicated that EPS acted as an insulin sensitizer. EPS alone was not able to promote the glucose uptake of insulin-resistant cells, but it helped insulin to recover the level of glucose consumption by the cells. In contrast, RZD as a positive control could promote the glucose uptake in insulin-resistant with or without insulin (Group 6 and Group 7).





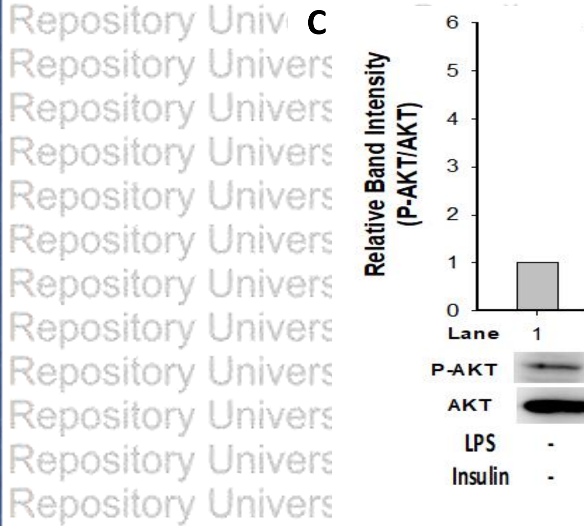


Figure 4.8. Effect of EPS on Akt in insulin-resistant IEC-18 cells. IEC-18 cells were treated with 100 ng/ml LPS for 16 hours, followed by treating with 100 nM insulin (Lane 3) , 200 µg/ml EPS in the presence (Lane 4) or absence (Lane 5) of insulin, or 50 µM RZD (Lane 6 and Lane 7) for 1 hour. A, B and C are three independently experiments. The relative band intensity versus Lane 1 was presented in the histogram after normalization by the amount of total Akt.

Similarly, Figure 4.8 revealed that insulin plus EPS could enhance Akt phosphorylation in LPS-treated cells (Lane 4), but EPS alone could

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not (Lane 5). Interestingly, RZD also needed the presence of insulin to recover the phosphorylation of Akt (Lane 6), it did not work when added alone (Lane 7). Overall, Figures 4.7 and 4.8 demonstrated that EPS worked as an insulin sensitizer in that it recovered insulin stimulated glucose uptake and Akt activation in insulin-resistant cells. EPS itself did not promote glucose consumption of insulin-resistant cells.

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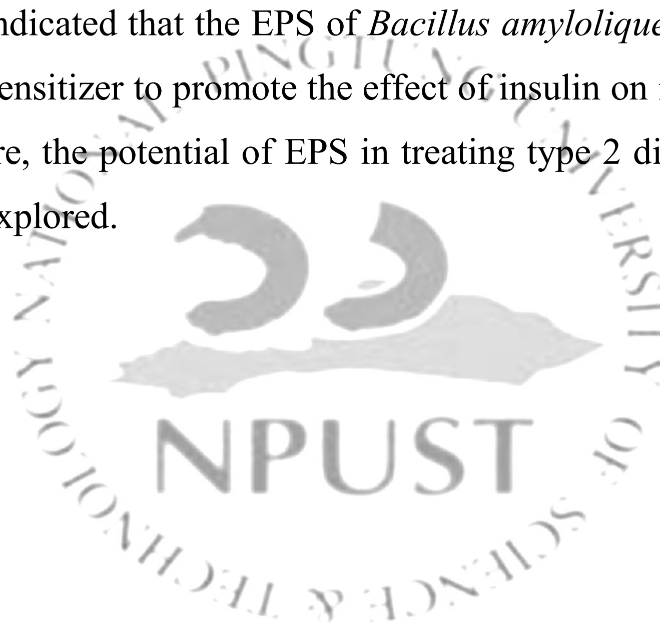
CHAPTER VI. CONCLUSION

The results of this study showed that EPS obviously enhanced the glucose consumption of intestinal epithelial cells and activated Akt in insulin-resistant cells. EPS works as an insulin sensitizer in that its recovers the activation of Akt only in the presence of insulin. Furthermore, EPS also could increase glucose uptake in LPS-stimulated insulin resistance under insulin stimulation, as insulin sensitizer. Together, these results indicated that the EPS of *Bacillus amyloliquefaciens* worked as an insulin sensitizer to promote the effect of insulin on insulin-resistant cells. Therefore, the potential of EPS in treating type 2 diabetes deserves to be further explored.



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