Regulation of glucose metabolism by TNF-α signalling and MIG1 gene switching

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Statement

As an author of the dissertation presented, I state that the dissertation represents my original work and does not include material already published, submitted for publication or presented as a PhD thesis by other authors, unless mentioned or cited in accordance with proper rules.

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Abstract

In this study, two important aspects of glucose metabolism were investigated, such as (1) effect of TNF-α elimination on insulin signaling and metabolic processes; (2) role of MIG1 gene in metabolic switching of fermentation /oxidation. The activation of the insulin signaling pathway regulates glucose and lipid metabolism, protein synthesis, gene expression, cell survival and differentiation. It seems that saturated fatty acids, especially palmitate inhibit insulin signaling pathway through increased expression of inflammatory cytokine TNF-α. So, the inhibition of TNF-α activity could be a potential therapeutic target in insulin resistance induced by fatty acids. On the other side, documented evidences from animal models with knockdowned TNF-α gene under high-fat diet, exhibited an increase in insulin sensitivity and lower blood glucose. Thus, down-regulation of TNF-α causes interfering with fatty acid signaling pathway and can eliminate the negative impact of fatty acids on the insulin signaling pathway. However the mechanism of decreasing of fatty acid induced insulin resistance by TNF-α down-regulation is still unknown. This study was conducted in order to answer this question. Initially TNF-α expression was reduced through shRNA technique in liver cells, HepG2 cell line, under *in vivo* condition. The shRNA was introduced into cells through transfection. Furthermore, the both modified HepG2 and normal control cells were treated with suitable concentrations of palmitate. The rate of insulin resistance in cells was measured through either western blot technique or radioactive glucose uptake test. To evaluate the intra cellular insulin signalling pathway under palmitate and insulin treatment conditions, two signalling proteins (IRS-1 and Aks) are capable candidates. We determined the phosphorylation of proteins IRS and Aks in TNF-α knockdown and control hepatic (HepG2) cells under the presence and absence of palmitate. According to our data, palmitate can deregulate the insulin signaling pathway and causes diabetic insulin resistance by increased expression of TNF-α in hepatocytes. These data support the evidence that TNF-α downregulation contributes to the improvement of insulin

sensitivity in hepatic cells, even in the presence of palmitate. Taking these findings together, the inhibition of TNF-α signaling has a potential possibility for treatment target. The results obtained may be used in management of metabolic and cardiovascular diseases, lipid-induced insulin resistance, and type 2 diabetes.

Another issue of our study was investigation of the role of MIG1 gene in metabolic switching of fermentation/oxidation. The glucose repression effect can direct metabolism of yeast to preferably anaerobic conditions. This leads to higher ethanol production and less efficient production of recombinant products. The general glucose repression system is constituted by MIG1, TUP1 and SSN6 factors. The role of MIG1 is known in glucose repression but the evaluation of effects on aerobic/anaerobic metabolism by deletion of MIG1 and constructing an optimal strain brand remains unclear and an objective to be explored. To find the impact of MIG1 in induction of glucose-repression, the Mig1 disruptant strain (ΔMIG1) of yeast *Saccharomyces cerevisiae* was produced for comparing with its congenic wild-type strain (2805). The analysis approached for changes in the rate of glucose consumption, biomass yield, cell protein contents, ethanol and intermediate metabolites production. This study showed the amount of ethanol measured by HPLC method in mutant and wild strain cultivations which determined less production of Δ*MIG1*. Enhancement of biomass production in Δ*MIG1* strains can lead to higher amount of intracellular protein content and carbohydrates. In the current research, results of strain Δ*MIG1* 2805 could confirm similar achievements rather than wild types. As to peripheral functions, efforts have already been exerted in production of partially derepressed galactose, maltose and sucrose metabolisms. For central functions, industrial interest could promote attempts in the metabolic engineering of strains that exhibit higher specific growth rates for baker's yeast or recombinant protein production, or of distiller's yeast strains that give higher ethanol yields. Results suggest that ΔMIG1 compared to the wild-type strain can significantly present less effects of glucose repression. The constructed strain has more efficient growth in aerobic

cultivations and it can be a potential host for biotechnological recombinant yields and industrial interests.

Key words: Hepatic Cell, Insulin Signaling Pathway, TNF-α downregulation, shRNA transfection,Western Blot Analysis, Mig1 gene, *Saccharomyces cerevisiae*, Glucose Repression, PCR analysis, DNA cloning.

აბსტრაქტი

წარმოდგენილ ნაშრომში შესწავლილია გლუკოზის მეტაბოლიზმის ორი მნიშვნელოვანი ასპექტი, როგორიცაა (1) TNF-α-ს ელიმინაციის ეფექტი ინსულინის სასიგნალო სისტემაზე და მეტაბოლურ პროცესებზე; (2) MIG1 გენის როლი ფერმენტაცია/ჟანგვის მეტაბოლურ შეცვლაში. ინსულინის სასიგნალო გზის გააქტიურება არეგულირებს გლუკოზისა და ლიპიდების მეტაბოლიზმს, ცილების სინთეზს, გენების ექსპრესიას, უჯრედების გადარჩენას და დიფერენციაციას. როგორც ჩანს, გაჯერებული ცხიმოვანი მჟავები, განსაკუთრებით კი პალმიტატი, ახდენს ინსულინის სასიგნალო გზის ინჰიბირებას ანთებითი ციტოკინი TNF-α-ს გაძლიერებული ექსპრესიით. ამრიგად, TNF-α- ას აქტივობის ინჰიბირება შეიძლება იყოს პოტენციური თერაპიული სამიზნე ცხიმმჟავებით გამოწვეულ ინსულინ-რეზისტენტობისთვის. მეორეს მხრივ, გათიშული TNF-α გენის მქონე ცხოველურ მოდელებზე მიღებული მონაცემებით, ცხიმიანი დიეტის დროს, გამოვლინდა ინსულინის მიმართ მგრძნობელობის ზრდა და სისხლში გლუკოზის დაწევა. ამრიგად, TNF-α- ის დაღმავალი რეგულაცია აბრკოლებს ცხიმოვანი მჟავების სასიგნალო გზას და შეუძლია აღმოფხვრას ცხიმოვანი მჟავების უარყოფითი გავლენა ინსულინის სასიგნალო სისტემაზე. ამასთან, ჯერ კიდევ უცნობია ცხიმოვანი მჟავებით გამოწვეული ინსულინ-რეზისტენტობის შემცირების მექანიზმი TNF-α- ის დაღმავალი რეგულაციის დროს. წარმოდგენილი კვლევა ჩატარდა ამ კითხვაზე პასუხის გასაცემად. თავდაპირველად, TNF-α-ს ექსპრესია შემცირდა მოკლე რნმ-ების (shRNA) ტექნიკით ღვიძლის უჯრედებში, HepG2 უჯრედულ ხაზში, *in vivo* პირობებში. shRNA შეიტანეს უჯრედებში ტრანსფექციის გზით. შემდგომ, მოდიფიცირებული HepG2 და ნორმალური საკონტროლო უჯრედები დამუშავდა შესაბამისი კონცენტრაციების პალმიტატით. უჯრედებში ინსულინის რეზისტენტობის დონე განსაზღვრულ იქნა ვესტერნ-ბლოთინგის ტექნიკით ან რადიოაქტიური გლუკოზის შემცველი ტესტით. პალმიტატითა და ინსულინით

დამუშავების პირობებში ინტრა უჯრედული ინსულინის სასიგნალო სისტემის შესაფასებლად კარგი კანდიდატებია ორი სასიგნალო ცილა (IRS-1 და Aks). ჩვენ განვსაზღვრეთ IRS და Aks ცილების ფოსფორილირება TNF-α-ს გათიშული გენით მოდიფიცირებულ და საკონტროლო ღვიძლის (HepG2) უჯრედებში, პალმიტატის არსებობის და არარსებობის პირობებში. ჩვენი მონაცემებით, პალმიტატს შეუძლია დაარღვიოს ინსულინის სასიგნალო სისტემის რეგულაცია და გამოიწვიოს დიაბეტური ინსულინ-რეზისტენტობა ჰეპატოციტებში TNF-α- ს გაზრდილი ექსპრესიით. ეს მონაცემები ადასტურებს, რომ TNF-α-ს დაღმავალი რეგულაცია ხელს უწყობს ღვიძლის უჯრედებში ინსულინის მიმართ მგრძნობელობის გაუმჯობესებას, თუნდაც პალმიტატის თანაობისას. ეს აღმოჩენები მიუთითებს, რომ TNF-α-ს სასიგნალო სისტემის ინჰიბირებით პოტენციურად შესაძლებელია სამიზნის მკურნალობა. მიღებული შედეგები შეიძლება გამოყენებული იქნეს პრაქტიკაში სხვადასხვა დაავადებების მართვისას, როგორიცაა მეტაბოლური და გულსისხლძარღვთა დაავადებები, ლიპიდებით გამოწვეული ინსულინ-რეზისტენტობა და მე-2 ტიპის დიაბეტი.

ჩვენი კვლევის შემდეგი საკითხი შეეხება MIG1 გენის როლის გამოკვლევას ფერმენტაცია/ჟანგვის მეტაბოლურ შეცვლაში. გლუკოზის რეპრესიის ეფექტს შეუძლია საფუარის მეტაბოლიზმი წარმართოს უპირატესად ანაერობულ პირობებში. ეს იწვევს ეთანოლის წარმოების გაზრდას და რეკომბინანტული პროდუქტების ნაკლებად ეფექტურ წარმოებას. ზოგადად, გლუკოზის რეპრესიის სისტემა შედგება MIG1, TUP1 და SSN6 ფაქტორებისგან. გლუკოზის რეპრესიაში MIG1– ის როლი ცნობილია, მაგრამ ჯერჯერობით გაურკვეველია და შესასწავლია MIG1– ის დელეციისა და კონსტრუირებული შტამების გავლენა აერობულ / ანაერობულ მეტაბოლიზმზე. იმისათვის, რომ გაგვერკვია MIG1– ის გავლენა გლუკოზის რეპრესიის ინდუქციაში, შეიქმნა საფუარის *Saccharomyces cerevisiae* შტამი (ΔMIG1), რომელსაც ჰქონდა დაზიანებული MIG1–ის გენი. მისი შედარება მოხდა მონათესავე ველური ტიპის შტამთან (2805). ჩატარებული ანალიზით შემოწმდა ცვლილებები

გლუკოზის მოხმარების სიჩქარეში, ბიომასის წარმოებაში, უჯრედების ცილის შემცველობაში, ეთანოლისა და შუალედური მეტაბოლიტების წარმოებაში. კვლევის დროს გაიზომა ეთანოლის ოდენობა HPLC მეთოდით მუტანტურ და ველური შტამის კულტურებში, დადგინდა ΔMIG1– ის მიერ ეთანოლის ნაკლები წარმოება. ΔMIG1 შტამებში ბიომასის წარმოების გაძლიერებამ შეიძლება გამოიწვიოს უჯრედშიდა ცილის შემცველობისა და ნახშირწყლები რაოდენობის გაზრდა. ჩვენს კვლევაში, ΔMIG1 2805-ის შტამის შედეგებმა უფრო მეტად დაადასტურა მსგავსი მონაცემები, ვიდრე ველური ტიპის შტამების შედეგებმა. რაც შეეხება პერიფერულ ფუნქციებს, უკვე განხორციელდა ძალისხმევა გალაქტოზას, მალტოზისა და საქაროზის ნაწილობრივ დაქვეითებული მეტაბოლიზმის მისაღებად. ცენტრალურ ფუნქციებთან მიმართებაში, ინდუსტრიულმა ინტერესმა შეიძლება ხელი შეუწყოს მეტაბოლურ ინჟინერიას ისეთი შტამებისთვის , რომლებსაც ექნებათ სპეციფიკური უფრო მაღალი ზრდის ტემპი მცხობელის საფუარის ან რეკომბინატული ცილების წარმოებისთვის, ან ლუდის საფუარის შტამების მიმართ, რომლითაც შესაძლებელია ეთანოლის გაზრდილი წარმოება. შედეგების საფუძველზე ივარაუდება, რომ ველური ტიპის შტამთან შედარებით ΔMIG1 შტამი მნიშვნელოვნად ნაკლებ ეფექტს ახდენს გლუკოზის რეპრესიაზე. კონსტრუირებული შტამი ეფექტურად იზრდება აერობული კულტივირებისას და ის შეიძლება იყოს პოტენციური მასპინძელი ბიოტექნოლოგიური რეკომბინანტული წარმოებისას და სამრეწველო ინტერესებისთვის.

საკვანძო სიტყვა: ღვიძლის უჯრედი, ინსულინის სასიგნალო სისტემა, TNF-α-ს დაღმავალი რეგულაცია, მ-რნმ-ით ტრანსფექცია, ვესტერნ-ბლოთინგი, Mig1 გენი, *Saccharomyces cerevisiae*, გლუკოზის რეპრესია, პჯრ-ანალიზი, დნმ-ის კლონირება

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Abbreviations

body mass index (BMI)

double-stranded RNA (dsRNA)

Dulbecco's Modified Eagle's Medium (DMEM

extracellular signal regulated kinase (ERK)

fasting plasma glucose (FPG)

family of transcription factors (FOXO)

factor-κβ (Iκβ).

fetal bovine serum (FBS

glycogen synthase kinase 3 (GSK3)

glucose transporter 4 (GLUT4)

glucose 6-phosphatase (G-6-Pase)

green fluorescent protein (GFP)

insulin receptor (IR),

interleukin-6 (IL-6)

initiating factor (IF)

inhibitor of nuclear factor-κβ kinase (IKK)

Jun N-terminal kinase (JNK)

mitogen-activated protein kinase (MAPK)

MAP kinase kinases (MAPKKs)

microRNAs (miRNAs)

nuclear factor-κβ (NF-κβ)

oral glucose tolerance test (OGTT)

peroxisome proliferator-activated receptor-γ co-activator (PGC)-1α

phosphatidylinositol 3-kinase (PI3K)

phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2)

phosphoinositide-dependent kinase (PDK).

protein kinase C (PKC)

protein kinase C (PKC),

RNA-induced silencing complex (RISC)

short interfering RNA (siRNA)

small interfering RNA (siRNA)

Son of sevenless (SOS)

Src-homology-2 (SH2)

suppressor of cytokine signaling 3 (SOCS3)

TNF receptor-associated death domain (TRADD).

TNF receptor-associated factor 2 (TRAF2)

tumor necrosis factor-α (TNF-α)

1. INTRODUCTION

1.1. DEFINITION

Diabetes mellitus, or simply diabetes, is a group of diseases characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. It is a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage (retinopathy, nephropathy and neuropathy). It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life (www.who.int/diabetes).

Several pathogenetic processes are involved in the development of diabetes. These include processes, which destroy the beta cells of the pancreas with consequent insulin deficiency,and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (Report of a WHO Consultation, 1999). Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. Often symptoms are not severe, or may be absent.

1.2. HISTORY

For 2,000 years diabetes has been recognized as a devastating and deadly disease. In the first century A.D. a Greek physician, Aretaeus, described the destructive nature of the affliction, which he named "diabetes" from the Greek word for "siphon" (www.diabeteshealth.com). Physicians in ancient times, like Aretaeus, recognized the symptoms of diabetes but were

powerless to treat it effectively. In the 17thcentury a London physician, Dr. Thomas Willis, determined whether his patients had diabetes or not by sampling their urine. If it had a sweet taste he would diagnose them with diabetes mellitus-"honeyed" diabetes. This method of monitoring blood sugars went largely unchanged until the 20th century.

Before the discovery of the insulin little could be done for patients suffering from diabetes. Low calorie diets prolonged their lives but left them weak and near starvation. But in 1921, doctors in Canada treated patients dying of diabetes with insulin and managed to drop high blood sugars to normal levels. Since then, medical breakthroughs have continued to prolong and ease the life of people with diabetes.

In the ’50s, it was discovered that there were two types of diabetes: "insulin sensitive" (type I) and "insulin insensitive" (type II). Two thousand years have passed since Aretaeus spoke of diabetes as "the mysterious sickness". It has been a long and arduous process of discovery, as generations of physicians and scientists have added their collective knowledge to finding a cure. It was from this wealth of knowledge that the discovery of insulin emerged in a small laboratory in Canada. Since then, medical innovations have continued to make life easier for people with diabetes. In the 21st century, diabetes researchers continue to pave the road toward a cure. Today, it is unclear what shape the road will take; perhaps another dramatic discovery like insulin waits around the corner, or possibly researchers will have to be content with the slow grind of progress (Satley, 2008).

1.3. PATHOPHYSIOLOGY

An understanding of the pathophysiology of diabetes rests upon knowledge of the basics of carbohydrate metabolism and insulin action. Following the consumption of food, carbohydrates are broken down into glucose molecules in the gut. Glucose is absorbed into the bloodstream elevating blood glucose levels. This rise in glycemia stimulates the secretion

of insulin from the beta cells of the pancreas. Insulin is needed by most cells to allow glucose entry. Insulin binds to specific cellular receptors and facilitates entry of glucose into the cell, which uses the glucose for energy. The increased insulin secretion from the pancreas and the subsequent cellular utilization of glucose results in lowering of blood glucose levels. Lower glucose levels then result in decreased insulin secretion.

If insulin production and secretion are altered by disease, blood glucose dynamics will also change. If insulin production is decreased, glucose entry into cells will be inhibited, resulting in hyperglycaemia. The same effect will be seen if insulin is secreted from the pancreas but is not used properly by target cells. If insulin secretion is increased, blood glucose levels may become very low (hypoglycemia) as large amounts of glucose enter tissue cells and little remains in the bloodstream. Multiple hormones may affect glycemia. Insulin is the only hormone that lowers blood glucose levels. The counter-regulatory hormones such as glucagon, catecholamines, growth hormone, thyroid hormone, and glucocorticoids all act to increase blood glucose levels, in addition to their other effects (Meley et al, 2006).

1.4. DIAGNOSIS

The diagnosis of diabetes mellitus is easily established when a patient presents the classic symptoms of hyperglycaemia and has a random blood glucose value of 200 mg/dL (11.1 mmol/L) or higher, and confirmed on another occasion. The following tests are used for the basic diagnosis:

A fasting plasma glucose (FPG) test measures blood glucose in a person who has not eaten anything for at least 8 hours. This test is used to detect diabetes and prediabetes. An oral glucose tolerance test (OGTT) measures blood glucose after a person fasts at least 8 hours and 2 hours after the person drinks a glucose-containing beverage. This test can be used to diagnose diabetes and prediabetes. The FPG test is the preferred test for diagnosing diabetes because of its convenience and low cost. However, it may miss some diabetes or prediabetes

that can be found with the OGTT. The FPG test is most reliable when done in the morning. Research has shown that the OGTT is more sensitive than the FPG test for diagnosing prediabetes, but it is less convenient to administer. A random plasma glucose test, also called a casual plasma glucose test, measures blood glucose without regard to when the person being tested last ate. This test, along with an assessment of symptoms, is used to diagnose diabetes but not prediabetes. Test results indicating that a person has diabetes should be confirmed with a second test on a different day (Twillman, 2002). The current WHO diagnostic criteria for diabetes should be maintained – fasting plasma glucose ≥ 7.0mmol/l (126mg/dl) or 2–h plasma glucose ≥ 11.1mmol/l (200mg/dl).

1.5. TYPES OF DIABETES MELLITUS

The first widely accepted classification was published by the WHO in 1980 (Second Report, 1980). Two major classes of diabetes mellitus were proposed: IDDM (Type I) and NIDDM (Type II). Other types as well as gestational diabetes were also included. The modified form of 1985 (Diabetes Mellitus: Report of a WHO Study Group, 1985) was widely accepted and is used internationally.It was recommended that the terms "insulin-dependent diabetes mellitus" and "non-insulin-dependent diabetes mellitus" should no longer be used, because patients were classified according to treatment rather than pathogenesis. The terms Type I and Type II were introduced to describe the cases which are primarily due to pancreatic islet beta-cell destruction the former and the common major form of diabetes resulting from defects in insulin secretion the latter (Goodpaster, 2010).

1.5.1. DIABETES TYPE I

Type I accounts for only about 5—10% of all cases of diabetes; however, its incidence continues to increase worldwide and it has serious short-term and long-term implications. Type I indicates the process of beta-cell destruction in the pancreas that may ultimately lead to diabetes mellitus in which “insulin is required for survival” to prevent the development of ketoacidosis, coma and death (Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Report of a WHO Consultation, 1999). Management of Type I diabetes is best undertaken in the context of a multidisciplinary health team and requires continuing attention to many aspects, including insulin administration, blood glucose monitoring, meal planning, and screening for diabetes-related complications. These complications consist of microvascular and macrovascular disease, which account for the major morbidity and mortality associated with Type I diabetes (Daneman, 2006).

1.5.2. DIABETES TYPE II

Type II is the most common form of diabetes. Millions of people around the world have been diagnosed with Type II diabetes, and many more remain undiagnosed. People with diabetes are at a greater risk of developing cardiovascular diseases such as heart attack and stroke if the disease is left undiagnosed or poorly controlled. They also have elevated risks for sight loss, foot and leg amputation due to damage to the nerves and blood vessels, and renal failure requiring dialysis or transplantation (Pasinetti, 2011).

Before people develop Type II diabetes, they almost always have "prediabetes" – bloodglucose levels that are higher than normal but not yet high enough to be diagnosed as diabetes. Recent research has shown that some long-term damage to the body, especially the heart and circulatory system, may already be occurring during prediabetes(DePaula, 2008).

The increased concentration of blood glucose **(Table 1)** seen in type 2 diabetic patients is caused by a combination of insulin resistance in tissues such as skeletal muscle,

adipose tissue and liver, and a reduced function of the insulin producing β-cells in the pancreas.

Type 2 diabetes develops gradually and blood glucose levels begin to rise when insulin production becomes insufficient to overcome peripheral insulin resistance. In turn, high blood glucose levels can lead to both microvascular and macrovascular complications. Microvascular complications such as retinopathy, nephropathy and neuropathy are common in diabetes, and ultimately lead to loss of vision, foot ulcers/amputation and kidney failure. Furthermore, macrovascular complications, including coronary artery disease, myocardial infarction and stroke, are main causes of death among diabetic patients. Indeed, cardiovascular morbidity is two to four times greater in patients with diabetes compared to non-diabetic individuals (Adlerberth et al., 1998; Zimmet et al., 2001). Clearly, type 2 diabetes is not only a health burden for the affected individual, but also has an impact on society as a whole due to high costs for medical care.

	Fasting plasma glucose	2h plasma glucose
T2D	≥7.0 mmol/l	≥11.1 mmol/l
IGT	≥6.1<7.0 mmol/l	≥7.8<11.1 mmol/l

Table 1. Diagnostic criteria for type 2 diabetes (T2D) and impaired glucose tolerance (IGT) according to WHO. Type 2 diabetes can be diagnosed by measuring glucose levels in the plasma after an 8-14 h fast or by assessing the glucose levels two hours after a 75 g oral glucose load.

1.6. PREVALENCE

The number of people with diabetes is increasing rapidly worldwide and the disease is considered to have reached epidemic-like proportions (**Figure 1**). The World Health Organization (WHO) estimates that more than 180 million people around the world have diabetes, which corresponds to 2.8% of the world population. This number is likely to more than double by 2030 (International Diabetes Federation, 2006; Wild et al., 2004; World Health Organization, 2009). The diabetes "epidemic" relates primarily to type 2 diabetes since it accounts for 90% of all cases of diabetes and the increase in the incidence of type 2 diabetes encompasses both developed and developing nations. In the United States 7.7% of the adult population was diagnosed with diabetes in 2006. Remarkably, when the number of patients with undiagnosed diabetes was added to these numbers the prevalence of diabetes was as high as 12.9%. In addition, another 30% of the population had elevated blood glucose levels and can thus be considered to be in a prediabetic state (Cowie et al., 2009). This is a large increase compared to 10-15 years earlier, when 5.1% of the population had diabetes (Cowie et al., 2009; Gregg et al., 2004), and 60 years earlier as little as 0.37% of the population was diagnosed with diabetes (Kenny SJ, 1995).

The largest increase in the prevalence of diabetes is expected in China and India (King et al., 1998; Zimmet et al., 2001). In fact, the "epidemic" is just beginning in the two most populated countries in the world. An increased lifespan, genetic vulnerability and urbanization, coupled with a sedentary lifestyle and more energy-rich food, are factors thought to contribute to the rapid increase in diabetes in this part of the world (Diamond, 2003; King et al., 1998; Zimmet et al., 2001).

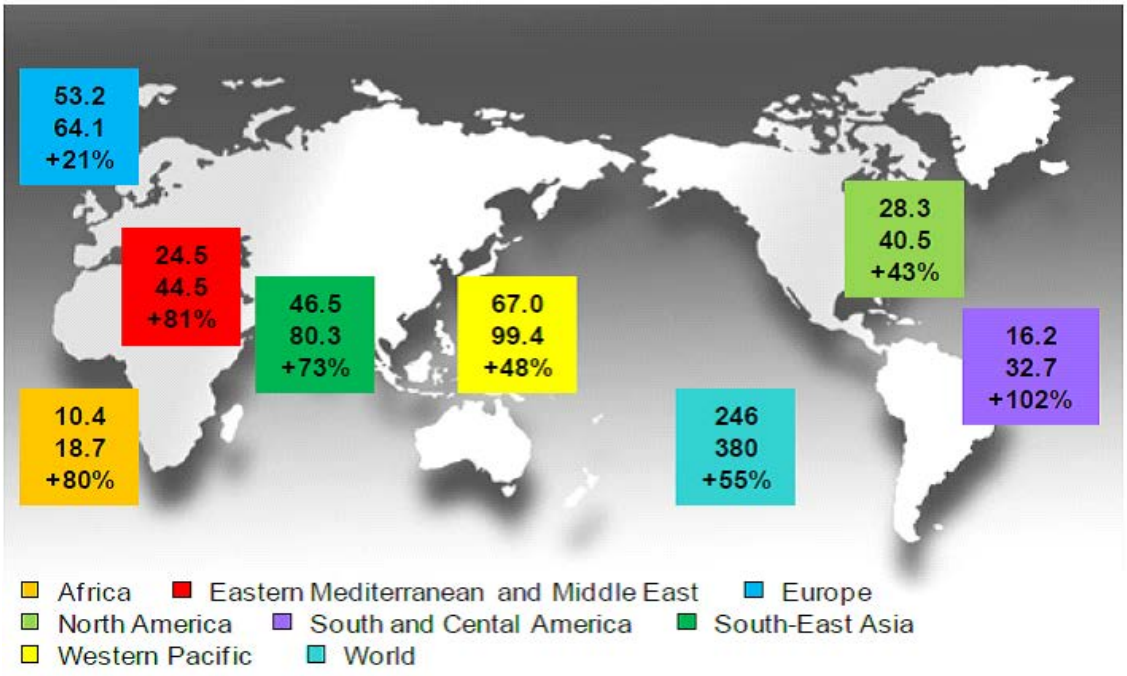


Figure 1. Estimation of diabetes prevalence in the adult population worldwide. Data is presented as million people with diabetes estimated for the years 2007 and 2025, with the increase expressed in percent.

The primary cause of type 2 diabetes is unknown, and might differ between individuals (Martin et al., 1992; Yki-Jarvinen, 1994). Most likely, a combination of both genetic and environmental factors is involved in the etiology of type 2 diabetes. The impact of genetic factors is illustrated by the fact that the concordance rate for type 2 diabetes in monozygotic twins is 50% (Poulsen et al., 1999). Evidence supporting the influence of environmental factors is found in two populations of Pima Indians living in Arizona, the United States, and Sonora, Mexico. Despite sharing a similar genetic background the prevalence of type 2 diabetes differs 5-fold between these two groups. The westernized lifestyle of the Pima Indians in Arizona is thought to be the underlying factor behind the difference (Schulz et al., 2006).

1.7. GLUCOSE METABOLISM; INTEGRATION OF THE WHOLE BODY

Type 2 diabetes is a complex disease in the sense that defects can be found in all major organs involved in metabolic control of glucose homeostasis. Metabolism has to be considered in the context of the whole body, since "crosstalk" exists between different metabolic tissues (**Figure 2**).

In healthy humans, blood glucose levels are constantly held within a narrow range of approximately 5 mmol/l (Kopf et al., 1973). This is critical to ensure an adequate energy supply to organs relying of glucose as their energy source (for example the brain) and for protecting the blood vessels from damage due to dangerously high levels of glucose. Insulin is the primary hormone responsible for the strict maintenance of a balanced blood glucose level. Insulin is produced by the pancreatic β-cells in response to increased blood glucose levels after a meal and consequently stimulates glucose uptake in peripheral tissues such as skeletal muscle and adipose tissue while inhibiting hepatic glucose production. Insulin mediates its biological effects via interaction with the cell surface insulin receptor, resulting in activation of intracellular signaling pathways. To define the contribution of each tissue in the development of insulin resistance and altered glucose homeostasis tissue-specific ablation of the insulin receptor in mice has been used. Deletion of the insulin receptor in the liver (Michael et al., 2000) causes hyperglycemia and hyperinsulinemia. These changes are due to an inability of insulin to suppress hepatic glucose production, as well as decreased insulin clearance. Likewise, tissue-specific ablation of the insulin receptor in pancreatic β-cells causes glucose intolerance (Kulkarni et al., 1999) with a loss of glucose-stimulated insulin secretion. On the contrary, mice with muscle-specific insulin receptor deletion have normal blood glucose and insulin levels, and respond appropriately to a glucose tolerance test (Bruning et al., 1998).

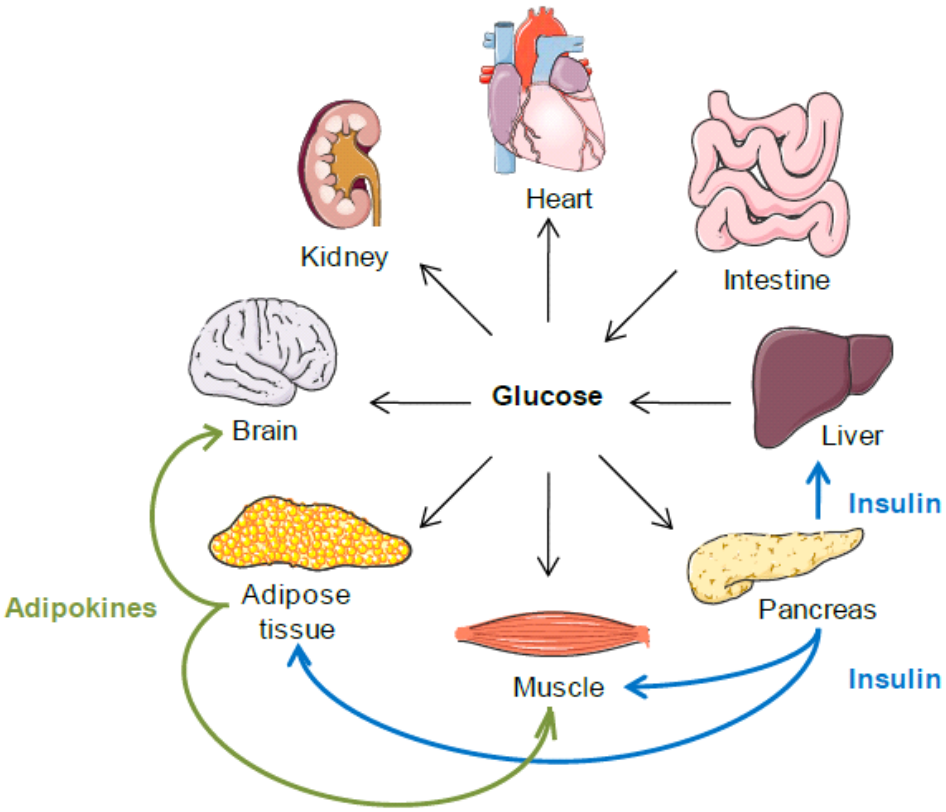


Figure 2. Crosstalk between different tissues in the regulation of glucose metabolism. Glucose enters the blood stream via uptake by the intestine after a meal or by release from the liver. Glucose is then used as an energy source and/or stored as glycogen in different tissues. Hormones and other signaling molecules are secreted from several tissues, and regulate whole body metabolism.

The decreased glucose uptake in the skeletal muscle of these animals is compensated by increased glucose uptake in adipose tissue, with a subsequent increase in body fat content and circulating free fatty acids and triglycerides (Kim et al., 2000). Thus, even though skeletal muscle-specific insulin-receptor knock-out mice do not develop glucose intolerance, impairments often seen in the diabetic patient are observed. Surprisingly, deletion of the insulin receptor in adipose tissue leads to a slight improvement in glucose homeostasis

(Bluher et al., 2002). This is probably due to changes in levels of secreted factors from the adipose tissue, which affect other organs involved in metabolic control in a positive way. Finally, genetic ablation of the insulin receptor in the hypothalamus increases food intake and body fat content (Obici et al., 2002). These mice have normal blood glucose and insulin levels, but have increased hepatic glucose production.

The pancreas, which secretes insulin and glucagon, is not the only tissue releasing bioactive mediators that influence whole body metabolism. The "crosstalk" between the tissues involved in maintaining nutrient homeostasis is further achieved via the release of numerous adipokines and cytokines by several tissues, in particular adipose tissue. Adiponectin is the most abundant protein secreted by adipose tissue and it is negatively correlated to obesity and type 2 diabetes in humans (Weyer et al., 2001). Adiponectin improves whole body insulin sensitivity by increasing glucose uptake and lipid oxidation in skeletal muscle (Fruebis et al., 2001; Yamauchi et al., 1996), while reducing hepatic glucose production (Berg et al., 2001).

The level of the adipose-derived hormone leptin is directly related to the amount of adipose tissue. Leptin controls food intake and energy expenditure by acting in the central nervous system (Munzberg and Myers, 2005; Zhang et al., 1994). In peripheral tissues leptin increases fatty acid oxidation (Muoio et al., 1997) and glucose metabolism (Kamohara et al., 1997). Leptin resistance has been observed in obesity, where high levels of this hormone fail to promote weight loss (Munzberg and Myers, 2005). Other bioactive mediators derived from the adipose tissue that modulate metabolism include resistin (Steppan et al., 2001), retinol binding protein 4 (Yang et al., 2005) and possibly visfatin (Fukuhara et al., 2005; Sun et al., 2009).

Several pro-inflammatory cytokines are released from the adipose tissue with increased levels in obesity, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). IL-6 has been implicated in insulin resistance (Bastard et al., 2002), but high concentrations of IL-6 are also secreted from contracting skeletal muscle, thereby increasing

peripheral insulin sensitivity (Steensberg et al., 2002), making the role of IL-6 in whole body metabolism unclear (Glund and Krook, 2008). In conclusion, balanced whole body metabolism requires coordinated regulation and involves an integration of multiple organs. Thus, it is not surprising that defects are noted in multiple tissues in type 2 diabetic patients.

1.8. INSULIN SIGNALING IN GLUCOSE TRANSPORT

In skeletal muscle and liver activation of the insulin signaling pathway regulates glucose and lipid metabolism, protein synthesis, gene expression, cell survival and differentiation (**Figure 3**).

Insulin receptor Insulin signaling is initiated by insulin binding to the insulin receptor (IR), expressed at the cell surface of all insulin-sensitive tissues. The IR belongs to the tyrosine kinase receptor family and consists of four subunits; two extra-cellular insulin-binding α-subunits linked to two transmembrane β-subunits which possess intrinsic tyrosine kinase activity. Upon insulin stimulation, conformational changes trigger autophosphorylation of the β-subunits. This will activate the receptor and lead to tyrosine phosphorylation of a large number of substrate molecules setting off the signaling cascade (Baron et al., 1992; Rosen, 1987; White, 1997).

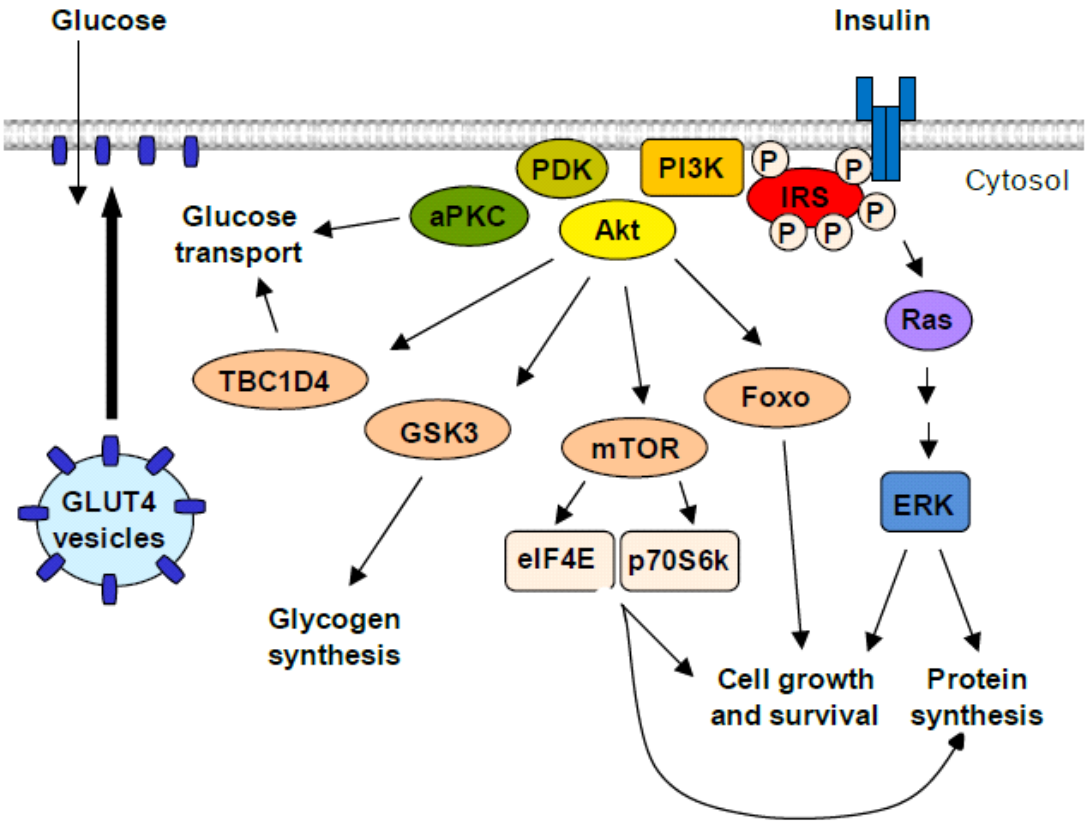


Figure 3. The insulin signaling cascade in skeletal muscle.

Insulin receptor substrates There are at least 12 substrates of the insulin receptor, including four different isoforms of insulin-receptor substrates (IRSs) (Sun XJ Nature 1991, 1648180, Sun XJ Nature 1995, 7675087, Lavan BE JBC 1997, 9111055, Lavan BE JBC 1997, 9261155), as well as Shc isoforms, Gab-1, p62, Cbl and APS (Baumann et al., 2000; Cai et al., 2003; White, 2003). IRS proteins have multiple tyrosine phosphorylation sites, and once phosphorylated they act as regulatory docking proteins that direct the insulin signaling cascade towards either metabolic or mitogenic events by binding to numerous proteins containing Src-homology-2 (SH2) domains.

Phosphatidylinositol 3-kinase Among the downstream proteins of IRSs phosphatidylinositol 3-kinase (PI3K) has been shown to be essential for the action of insulin

on glucose and lipid metabolism. Chemical inhibition of PI3K blocks insulin-stimulated glucose uptake (Yeh et al., 1995). PI3K consist of a regulatory (p85) and a catalytic (p110) subunit, with several isoforms of each subunit (Taniguchi et al., 2006). When the regulatory subunit associates with IRS the catalytic subunit is brought close to the plasma membrane. PI3K is then able to phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2) in the plasma membrane, leading to the production of the lipid second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn can bind to other signaling molecules and alter their activity or cellular localization. A key molecule downstream of PI3K is phosphoinositide-dependent kinase (PDK). PDK is consequently involved in the regulation of several kinases, including the serine/threonine kinase Akt and atypical protein kinase C (PKC) (Chou et al., 1998).

Akt (also known as protein kinase B) Three different isoforms of Akt have been identified (Brodbeck et al., 1999; Cheng et al., 1992; Jones et al., 1991), which relay the insulin signal via phosphorylation of multiple downstream targets to pathways triggering glucose metabolism, protein synthesis and cell survival.Once recruited to the plasma membrane Akt needs to be phosphorylated at Ser473 and Thr308 for full activation of its kinase activity (Alessi et al., 1996). PDK1 is the kinase responsible for the Thr308 phosphorylation (Alessi et al., 1997), whereas the identity of the Ser473 kinase (PDK2) has been a subject of debate. There is increasing evidence that mammalian target of rapamycin (mTOR) is a primary regulator of the Ser473 site (Sarbassov et al., 2005).

Akt activation promotes glucose metabolism by inducing glycogen synthesis through phosphorylation and inhibition of the enzyme glycogen synthase kinase 3 (GSK3) (Cross et al., 1995), which normally acts as a block on the pathways governing glucose incorporation into glycogen. In addition, phosphorylation of the Akt substrate of 160 kDa (AS160/TBC1D4) stimulates glucose uptake (Kane et al., 2002; Sano et al., 2003). Recently another Akt substrate with a similar size to TBCID4 has been identified and it is termed TBC1D1 (Roach et al., 2007). Insulin controls glucose uptake in skeletal muscle by regulating the movement

of intracellular vesicles containing glucose transporter 4 (GLUT4) (Birnbaum, 1989; Fukumoto et al., 1989; James et al., 1989) from an intracellular localization to the cell membrane (Hirshman et al., 1990; Karlsson et al., 2009). TBC1D1 and 4 have a GTPase-activating domain for Rabs, which are small G-proteins important for membrane trafficking and/or docking shown to be important for the GLUT4 translocation (Miinea et al., 2005).

Akt regulates cell growth and survival through inhibition of several pro-apoptotic agents including Bad and forkhead box O family of transcription factors (FOXO). Protein synthesis is regulated via the mTOR pathway, where mTOR controls the translation machinery by phosphorylation and activation of the ribosomal initiating factor eIF-4E, and p70S6K activated ribosomal biosynthesis (Manning and Cantley, 2003).

Mitogen-activated protein kinases Insulin exerts its mitogenic control via activation of the mitogen-activated protein kinase (MAPK) pathway. This occurs through IRS activation of Grb2, recruitment of Son of sevenless (SOS) exchange protein to the plasma membrane and subsequent activation of Ras. Ras then activates a serine kinase cascade where MAP kinase kinase kinases (MAPKKKs) stimulate MAP kinase kinases (MAPKKs), which ultimately stimulate the three classes of MAPKs including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38. Chemical inhibition of ERK reveals this kinase to be essential for insulin-stimulated cell growth without effecting glucose metabolism (Lazar et al., 1995).

1.9. CRITICAL NODES IN THE INSULIN SIGNALING CASCADE

The intracellular insulin signaling pathway in skeletal muscle is a complex cascade of phosphorylation events, which regulates glucose and fatty acid transport and metabolism, protein synthesis and gene expression. The existence of multiple isoforms for some of the signaling molecules adds further complexity to the signaling cascade.

The concept of "critical nodes" in the insulin-signaling pathway was introduced by Taniguchi *et al* (Taniguchi et al., 2006). A critical node is defined as a group of related proteins in a signaling network, where two or more of these related proteins have unique biological roles and therefore act as a junction where the signaling can take different tracks. A node is highly regulated, both positively and negatively, and is a site for "cross talk" with other signaling pathways. In the insulin signaling pathway, at least three different critical nodes have been described; the insulin receptor and its substrates (IRS), PI3K and Akt (**Figure 4**) (Taniguchi et al., 2006).

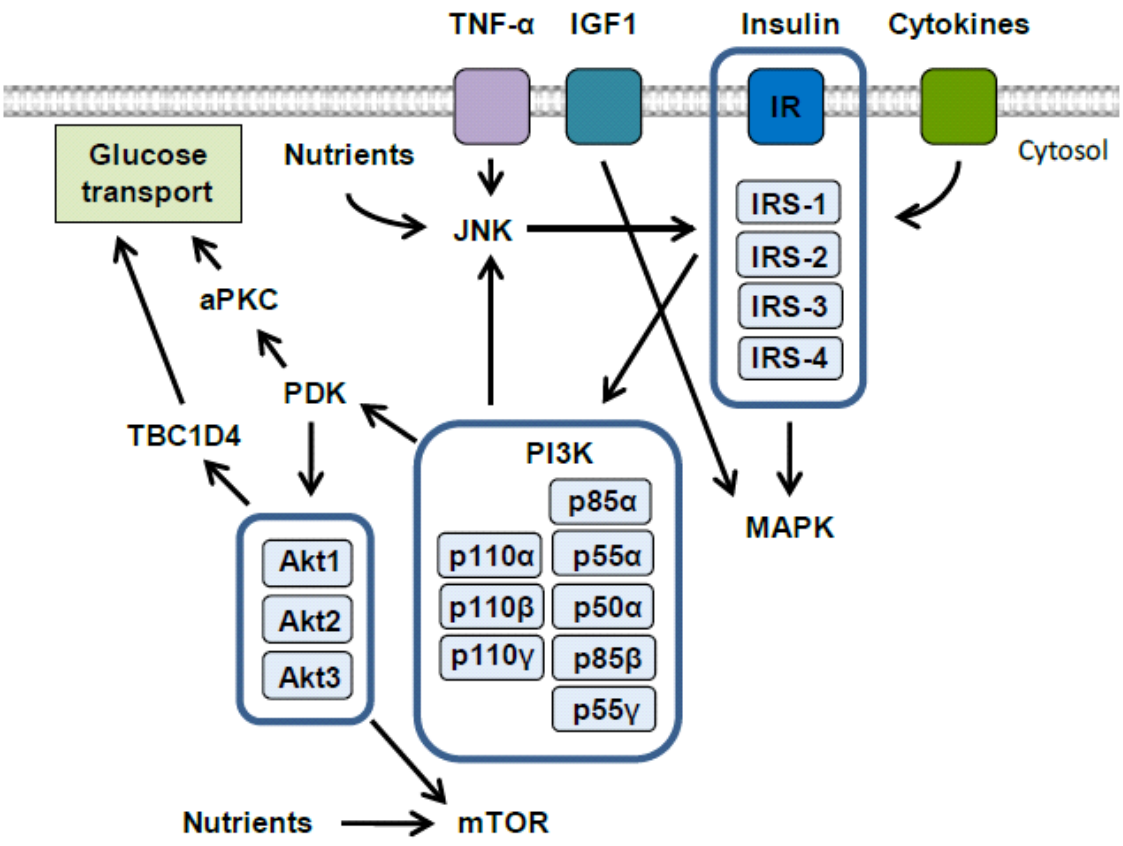


Figure 4. Critical nodes in the insulin signaling cascade. At each critical node (framed in blue), several related proteins have unique biological roles and therefore the node acts as a junction where the insulin signal can take different tracks. Modified from Taniguchi *et al*, (Taniguchi et al., 2006).

Studies performed on genetically modified animal models have given insights into the specialized roles of the different IRS and Akt isoforms in the insulin signaling pathway. Four structurally related IRS isoforms are expressed in mammals; IRS1-4, where IRS-1 (White et al., 1985) and IRS-2 (Araki et al., 1994) are the most widely expressed isoforms (Wang et al., 2015; Zhang et al., 2015).

Mice with a genetic ablation of IRS-1 have a marked reduction in intrauterine growth and remain small throughout life. Even though deletion of IRS-1 does not cause diabetes, the animals develop impaired glucose tolerance (Araki et al., 1994; Tamemoto et al., 1994). The importance of IRS-1 in peripheral glucose metabolism is highlighted by the fact that skeletal muscle and adipose tissue isolated from IRS-1-deficient mice have reduced insulin-stimulated glucose uptake (Araki et al., 1994; Tamemoto et al., 1994; Yamauchi et al., 1996). The phenotype of IRS-2 deficient mice differs from the phenotype of IRS-1-deficient mice. Disruption of IRS-2 only leads to mild growth retardation, but the mice develop overt diabetes (Araki et al., 1994; Tamemoto et al., 1994).

In addition to peripheral insulin resistance deletion of IRS-2 causes progressive pancreatic β-cell failure, making the mice unable to compensate for the increased insulin demand (Withers et al., 1998). However, insulin-stimulated glucose uptake is intact in muscle isolated from animals with genetic ablation of IRS-2 (Higaki et al., 1999).

The IRS-3 and IRS-4 isoforms seem to be less important for the regulation of metabolism in skeletal muscle. IRS-3 is mainly expressed in liver and lung in rodents (Sciacchitano and Taylor, 1997), but expression is undetectable in humans (Bjornholm et al., 2002). Furthermore, IRS-3 depletion does not alter growth or glucose homeostasis in mice (Liu et

al., 1999). Likewise, IRS-4 knockout mice only show mild defects in glucose homeostasis (Fantin et al., 2000).

Three mammalian Akt isoforms exist, which are encoded by distinct genes. Despite close sequence homology between the three isoforms, knockout studies have revealed distinct roles for the Akt proteins. The Akt1 isoform is ubiquitously expressed and Akt1 deficiency leads to increased neonatal mortality and severe growth retardation (Cho et al., 2001b) due to a defect in placental development (Yang et al., 2003). However, glucose homeostasis is unaltered in these mice (Chen et al., 2001; Cho et al., 2001).

Akt2 deficiency leads to peripheral insulin resistance, as well as mild growth retardation and age-dependent loss of adipose tissue. Insulin-stimulated glucose transport is impaired in skeletal muscle from mice lacking Akt2 (Cho et al., 2001a; Garofalo et al., 2003). Furthermore, Akt2 is the most abundant isoform in insulin-responsive tissue (Altomare et al., 1998; Yang et al., 2003). Some compensatory effects between the Akt1 and Akt2 isoforms might exist, since mice with deletion of both isoforms die directly after birth, show dwarfism, have impaired adipogenesis and impaired development of skin, skeletal muscle and bone (Peng et al., 2003). Akt3 is expressed predominantly in brain and testis, and genetic ablation of Akt3 leads to reduction in brain size without any detected changes in glucose metabolism (Easton et al., 2005; Tschopp et al., 2005).

1.10. HEPATIC GLUCOSE PRODUCTION

The liver plays a central role in both glucose and lipid metabolism. Hepatic insulin resistance is thought to be the main factor in the development of fasting hyperglycemia (Biddinger et al., 2006). Type 2 diabetes is associated with an increased rate of basal HGP despite 2- to 4-fold elevations in insulin concentrations suggesting hepatic resistance to insulin (Bays et al.,

2004). In order to understand the contribution of liver insulin resistance to insulin resistance, liver insulin receptor knock out mice were generated. These mice developed hyperglycemia and compensatory hyperinsulinemia due to the inability to respond to insulin and suppress HGP. Increased HGP results from elevated glycogenolysis and gluconeogenesis. Hepatic gluconeogenesis alone contributes 50-60% of HGP and is thought to be primarily responsible for increases in fasting glucose levels in individuals with type 2 diabetes (Bays et al., 2004). Gluconeogenesis is mainly regulated by the regulatory enzymes- phosphoenolpyruvate carboxy kinase (PEPCK), glucose 6-phosphatase (G-6-Pase), and fructose 1,6 bis-phosphatase. The rate of gluconeogenesis is regulated by hormones such as insulin which suppresses HGP, glucagon and glucocorticoids which stimulate gluconeogenesis, and adipokines adiponectin and leptin which suppress HGP (Bays et al., 2004). Sustained hyperglycemia, a characteristic of type 2 diabetes, enhances gluconeogenesis by enhancing PEPCK and G-6-Pase gene expression (Shao et al.,2005). In addition to chronic hyperglycemia, high circulating concentrations of FFA stimulate HGP by increasing activities of PEPCK and G-6-Pase. Studies have shown that infusion of FFA in normal subjects and obese insulin resistant individuals enhances HGP by stimulating gluconeogenesis (Bevilacqua et al., 1987) and is attributed to impairments in the insulin signaling pathway.

1.11. INSULIN RESISTANCE

The diminished ability of a given concentration of insulin to exert its usual biological effects is referred to as insulin resistance (Bays et al., 2004). Insulin resistance syndrome is a composite of abnormalities, including obesity, glucose intolerance, dyslipidemia and hypertension that eventually lead to type 2 diabetes (Biddinger et al., 2006). Liver, skeletal muscle and adipose tissue are the major sites for insulin resistance (Bays et al., 2004). Insulin resistance is characterized by increased glucose production by the liver, decreased glucose

uptake and utilization by skeletal muscle, and increased lipolysis in the adipose tissue (Bays et al., 2004). Insulin resistance syndrome is increasing at an alarming rate and affects 27% of adults in the United States. Moreover, approximately 50% of severely obese children have the insulin resistance syndrome (Biddinger et al., 2006).

1.11.1. Molecular mechanism of insulin resistance

Insulin resistance can be attributed to multiple mechanisms such as decreased synthesis, increased degradation of IR and signaling molecules, inhibitory serine phosphorylation of IRS proteins, alteration in the ratios of signaling molecules, and interaction of IR with inhibitory proteins (**Figure 5**). For instance, recent studies have demonstrated that an increase in SREBP-1c can result in a decrease in IRS-2 transcription (Ide et al., 2004). Insulin receptor and IRS proteins can undergo phosphorylation at the serine residue by protein kinase C (PKC), extracellular signal regulated kinase (ERK), JNK and IKKβ which decreases its activity. Insulin resistance can also be produced by interaction of inhibitory proteins with molecules in the insulin signaling pathway. For example, inflammatory cytokines induce suppressors of cytokine signaling proteins (SOCS) which bind to the IR and block its signaling (Biddinger et al., 2006; Wang et al., 2015; Zhang et al., 2015).

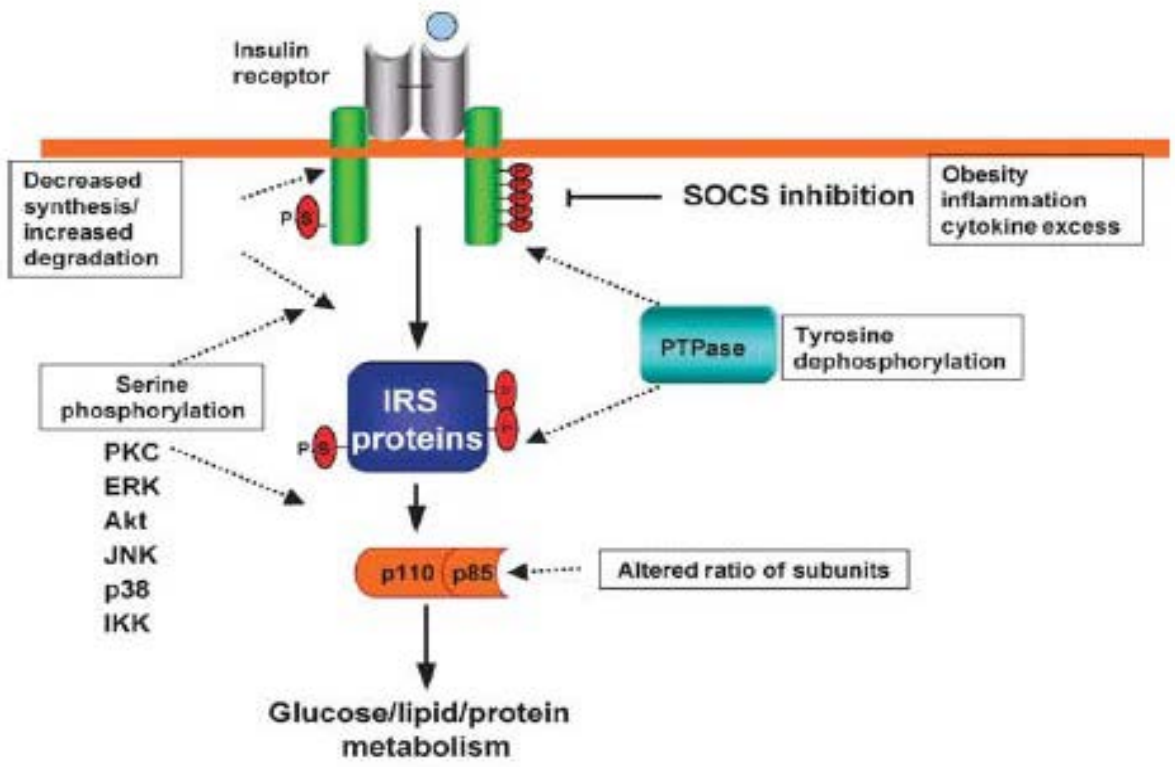


Figure 5. Molecular mechanisms of insulin resistance

The molecular mechanisms of insulin resistance have been demonstrated extensively. in rodents and humans over the past two decades. Using 13C magnetic resonance spectroscopy (MRS) to measure intracellular levels of glycogen synthesis in the muscle of individuals with type 2 diabetes (Rothman et al., 1992) demonstrated that insulin stimulated muscle glycogen synthesis was decreased by over 50% in patients with type 2 diabetes compared to normal individuals under hyperinsulinemic (80μU/ml)-hyperglycemic (10mmol/l) clamp conditions. Furthermore, using 13C and 31P MRS studies to measure intracellular levels of glucose, glucose-6-phosphate and glycogen. demonstrated that glucose transport was the rate limiting step in insulin stimulated glycogen synthesis in muscle (Cline et al., 1992). In addition, studies with offspring of subjects with type 2 diabetes exhibited similar abnormalities. Results demonstrated that fasting plasma fatty acids were a good predictor for insulin

resistance in the healthy cohort with type 2 diabetic parents. Subsequent studies using 1H MRS to detect intramyocellular lipid showed that lipid content in the muscle was a better predictor for insulin resistance than plasma fatty acids in both adults and children (Krssak et al., 1992).

The mechanisms by which FFA induce insulin resistance in both humans and rodents have been elucidated. In the skeletal muscle, accumulation of intramyocellular fatty acyl CoAs and DAG from plasma or impaired β-oxidation activate serine/threonine kinases such as PKC (PKC-θ in rodents and PKC-δ in humans). Activated PKC-θ/δ phosphorylates IRS-1 on the serine residue instead of tyrosine residue which seems to negatively regulate IRS signaling. Similar to FFA, cytokines also induce similar defects in insulin signaling observed in insulin resistant states. Phosphorylation of serine residues in IRS-1 decreases insulin stimulated Akt2 activity, thereby decreasing GLUT-4 translocation and glucose uptake by the skeletal muscle.

In the liver, increased levels of DAG resulting from elevations in plasma FFA or suppressed β-oxidation activate PKC-ε leading to reduced IRS-2 tyrosine phosphorylation (Samuel et al., 2004). This decreases activation of glycogen synthase and glycogen synthesis, and increases glucose output due to impaired FOXO inhibition. In addition to these data, when certain Ser/Thr kinases (e.g., JNK, IKK-β, PKC-θ) were either knocked down or inhibited using pharmacological inhibitors, high fat diet induced insulin resistance was prevented in rodent models. Furthermore, recent studies in mice with specific Ser to Ala mutations in muscle IRS-1 (IRS-1 Ser- Ala302, Ser- Ala307 and Ser-Ala612) revealed protection against high-fat diet-induce resistance (Morino et al., 2006). Similarly, when mitochondrial glycerol-3phosphate acyl transferase, a key enzyme in de novo lipogenesis is knocked down, high fat induced synthesis of DAG is inhibited resulting in decreased PKC- θ activation and fat-induced liver insulin resistance.

Collectively, these data suggest that phosphorylation of key Ser residues on IRS plays an important role in the development of muscle and liver insulin resistance and have

important implications for the development of novel treatment agents to prevent insulin resistance and NAFLD.

1.11.2. Mitochondrial dysfunction in insulin resistance

Mitochondrial dysfunction, whether associated with β-cell dysfunction or impaired oxidative and phosphorylative capacity of the skeletal muscle, plays an important role in the pathogenesis of type 2 diabetes (Petersen et al., 2003) demonstrated a 40% decrease in muscle oxidative and phosphorylation capacity along with increased intramyocellular and intra-hepatic lipid content in healthy lean elderly subjects with severe insulin resistance compared to body mass index (BMI) and activity matched young control subjects. These data suggest that ageing may predispose individuals to loss of mitochondrial function which results in insulin resistance. Similar results in loss of mitochondrial function was observed in young lean insulin resistant offspring of parents with type 2 diabetes. In addition to an 80% increase in intramyocellular lipid content in these subjects, mitochondrial density was decreased by 38% determined by electron microscopy suggesting that mitochondrial dysfunction may be a predisposing factor for the development of insulin resistance. Key factors that have been identified as regulators of mitochondrial density in the skeletal muscle include peroxisome proliferator-activated receptor-γ co-activator (PGC)-1α and AMPK.

The cellular switch, AMPK, is activated during exercise and ischemia when there is a decrease in the ATP/AMP ratio. AMPK mediates its effects on increasing mitochondrial biogenesis via increase in PGC-1α (Morino et al., 2006). It thus seems that the phenotype of insulin resistance depends on the components and tissues in which insulin signaling is altered and therapy should be directed at the specific pathway and tissue affected.

1.12. DEFECTS IN INSULIN SIGNALING

Defects in skeletal muscle metabolism appear to be an early event in the development of insulin resistance since decreased insulin-stimulated glucose uptake is found in healthy first-degree relatives of type 2 diabetic patients (Eriksson et al., 1989; Vaag et al., 1992). In view of the fact that GLUT4 protein levels are normal in skeletal muscle from type 2 diabetic patients (Eriksson et al., 1992; Kahn et al., 1992), while the GLUT4 translocation and glucose uptake are impaired (Dohm et al., 1988; Zierath et al., 1996) a large number of studies have focused on identifying defects in the insulin signaling cascade.

Impaired expression and tyrosine phosphorylation of the IR have been observed in skeletal muscle from morbidly obese type 2 diabetic subjects (Goodyear et al., 1995b). In contrast, insulin binding, protein expression and tyrosine phosphorylation of IR is normal in skeletal muscle from moderately overweight type 2 diabetic subjects (Arner et al., 1987; Krook et al., 2000). Multiple reports have described changes in phosphorylation patterns of IRS in insulin resistance. Decreased insulin-stimulated IRS-1 tyrosine phosphorylation and consequently activation of PI3K has been observed in skeletal muscle biopsies from patients with type 2 diabetes (Bjornholm et al., 1997; Kim et al., 1999; Krook et al., 2000, Cusi, 2000) and cultured myotubes from type 2 diabetic patients (Bouzakri et al., 2003; Nikoulina et al., 2001).

In addition to tyrosine phosphorylation IRS can be phosphorylated at multiple serine and threonine residues, which will either inhibit or enhance insulin signaling (Gual et al., 2005). Using a serine phosphatase inhibitor, increased serine phosphorylation was shown to interfere with the insulin receptor-mediated tyrosine phosphorylation of IRS-1 and its docking with PI3K. Multiple systemic factors associated with insulin resistance, for example free fatty acids and TNF-α, increase IRS-1 serine phosphorylation and thus inhibit its function (Gual et al., 2005, Yu et al., 2002). Impaired insulin-dependent activation of Akt has

been reported in some (Krook et al., 1998), but not all (Kim et al., 1999) studies. The differences in Akt response between different groups of diabetic patients might be due to the existence of different Akt isoforms and later studies have reported isoform-specific defects. In skeletal muscle from obese subjects signaling defects were found at the level of Akt2 and Akt3, but not Akt1 (Brozinick et al., 2003). Moreover, decreased Ser473 phosphorylation on Akt2 and decreased Thr308 phosphorylation on Akt1 has been observed in cultured myotubes (Cozzone et al., 2008). Finally, insulin-stimulated phosphorylation of the Akt substrate TBC1D4 is impaired in skeletal muscle from type 2 diabetic subjects (Karlsson et al., 2005).

1.13. DOWNSTREAM SIGNALING OF TNF-α

The negative role of TNF-α in insulin resistance has been supported by the fact that removal of TNF-α function improves insulin sensitivity and glucose homeostasis in obese mice. In addition, an acute TNF-α infusion in healthy humans leads to insulin resistance through impaired insulin signaling and decreased glucose uptake (Plomgaard et al., 2005). The binding of TNF-α to the cell surface receptor TNF-R1 leads to the activation of two major transcription factors; c-Jun and nuclear factor-κβ (NF-κβ), which subsequently results in activation of genes involved in the inflammatory process, stress responses and cell growth and development. TNF-α binding causes conformational changes in the receptor, which recruits the adaptor protein TNF receptor-associated death domain (TRADD). TRADD then acts as a platform for binding of other proteins, including receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2), leading to the activation of JNK and NF-κβ pathways (Chen and Goeddel, 2002; Zhang et al., 2015).

The activity of NF-κβ is dependent on cellular localization and under basal conditions it is controlled by inhibitor of nuclear factor-κβ (Iκβ). Iκβ masks the nuclear localization

signal and thus prevents NF-κβ from entering the nucleus. Upon TNF-α stimulation the serine kinase complex inhibitor of nuclear factor-κβ kinase (IKK) is activated to phosphorylate the Iκβ complex and mark it for ubiquitination and proteasomal degradation. This releases NF-κβ and permits translocation to the nucleus, where genes involved in the inflammatory process can be activated (Ghosh and Karin, 2002). The IKK complex consists of three subunits, IKKα and IKKβ, which have regulatory functions, and IKKγ, which acts as a scaffolding protein. IKKβ is the most important subunit for NF-κβ activation.

In addition to the involvement of TNF-α signaling in the inflammatory process, activation of the signaling molecules JNK and IKKβ have been implicated in insulin resistance (**Figure 6**) (Aguirre et al., 2000) . Clues to the importance of the IKKβ/NFκβ axis in diabetes date back to 1876 when high doses of sodium salicylate (4-10 g/day) were observed to reduce glucosuria in diabetic patients. The effect of salicylate on blood glucose concentrations in diabetic patients was later verified (Hundal et al., 2002) and high does of salicylates were shown to reverse hyperglycemia and hyperinsulinemia in obese animal models (Yuan et al., 2001). The molecular target of salicylate was discovered to be IKKβ. Heterozygote IKKβ +/- mice have decreased blood glucose and insulin levels, and are partly protected from diet- or lipid-induced insulin resistance (Kim et al., 2001). The inflammatory cytokines like TNF-α, IL-6, and IL1β can impair the insulin signaling pathway leading to insulin-resistant metabolic conditions (Wang et al., 2015).

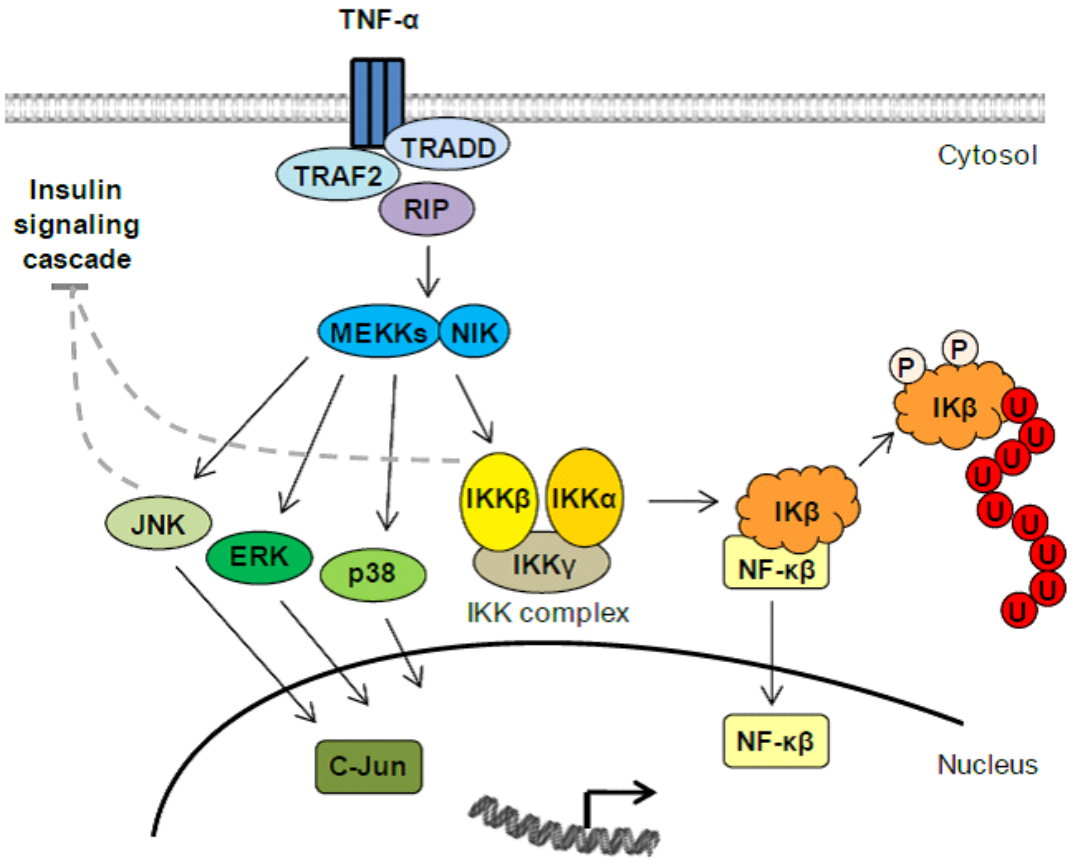


Figure 6. TNF-α signaling. Initiation of TNF-α signaling leads to activation of two major pathways; JNK and NF-κβ . Both pathways have been implicated in the negative regulation of insulin signaling. TNF-α mediated activation of the IKK complex leads to pho sphorylation of IKβ, which marks it for ubiquitination and proteasomal degradation. This sequence of events permits NF-κβ to enter the nucleus and regulate gene expression.

1.13.1. TNF-α mediated effect on IRS phosphorylation

Increased IRS-1 serine phosphorylation is associated with negative effects on the insulin signaling pathway and has been described in connection with TNF-α-induced insulin

resistance (Hotamisligil et al., 1996). This led us to investigate the effect of TNF-α treatment and IKKβ silencing on IRS-1 serine phosphorylation in human cells.

TNF-α treatment increased Ser312 (corresponding to Ser307 in rodents) phosphorylation of IRS-1 as previously described (Aguirre et al., 2000). Ser312 phosphorylation is thought to induce a conformational change of the IRS-1 molecule leading to reduced affinity for the binding between IR and IRS-1 and thus interfere with the propagation of the insulin signaling (Aguirre et al., 2002). JNK has been shown to be involved in the IRS-1 Ser312 phosphorylation (Aguirre et al., 2000).

1.13.2. TNF-α, insulin resistancy and dibetes type II

There is increasing evidence that suggests that both obesity and type 2 diabetes are inflammatory states. One such adipose derived inflammatory cytokine is TNF-α, that plays an important role in insulin resistance. **(Figure 7)** Elevated TNF-α levels have been reported in obesity and other insulin resistant states (Hotamisligil et al., 1993). In addition to TNF-α, the cytokine interleukin (IL-6) is secreted by adipocytes and is implicated in muscle insulin resistance and β-cell apoptosis (Shimabukuro et al., 1998). When TNF-α is infused into rodents, it is associated with development of insulin resistance, increased lipolysis, activation of inflammatory mitogen activated protein kinase (MAPK) isoforms including c-Jun N-terminal kinase 1 (JNK1) that mediates serine phosphorylation of insulin receptor substrate 1 (IRS-1), activation of nuclear factor κB (NF-κB), induction of suppressor of cytokine signaling 3 (SOCS3), and production of ROS.

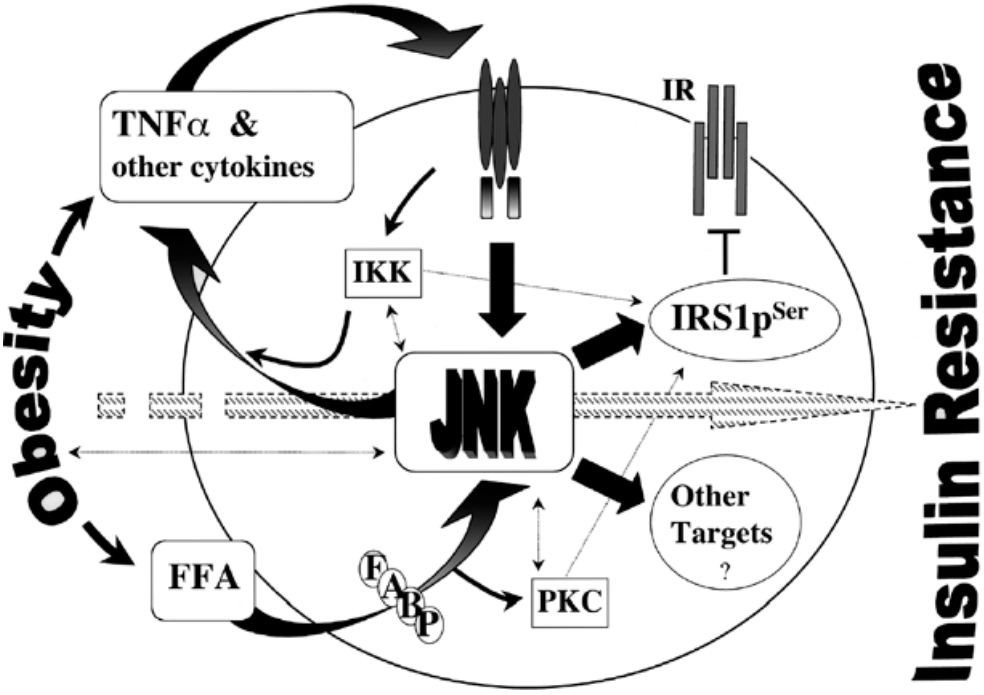


Figure 7. Inflammatory mechanism of obesity and insulin resistancy in diabetes

1.14. RNA INTERFERENCE

Over the last decade RNA interference has emerged as a powerful tool in target identification and dissection of signaling pathways. The phenomenon of RNA interference, where double-stranded RNA (dsRNA) triggers sequence-specific gene silencing, was first described in the nematode *Caenorhabditis elegans* in 1998 (Fire et al., 1998). For their discovery, Andrew Fire and Craig Mello received the Nobel Prize in Medicine.

In nature several processes can generate dsRNA. RNA interference exists *in vivo* as a protection system against viral infection (Covey and Al-Kaff, 2000) and to secure the stability of the genome by keeping mobile elements (transposons) silent. Furthermore, endogenous small RNA molecules, called microRNAs (miRNAs), play a role in developmental regulation and are likely involved in several diseases including metabolic disturbances (Esau et al., 2006). Thus, RNA interference is not only a powerful research tool,

but might be used in functional genomics or as therapeutic agents/targets in the future (Dorsett and Tuschl, 2004; Jeyaseelan et al., 2007).

1.14.1. Mechanism of RNA interference

Regulated gene expression is fundamental for all living cells. According to the central dogma of molecular biology the genetic information in double-stranded DNA is transcribed into single-stranded messenger RNA (mRNA) in the cell nucleus and subsequently translated into protein in the cytoplasm. In RNA interference mRNA is destroyed before it can serve as a template for the translation into protein. Before Fire and Mello discovered the mechanism whereby RNA interference occurs gene silencing had been described in plants. With the purpose of deepening the color of petunias a chimeric gene important for color synthesis was introduced into the flower. Surprisingly, the transgene did not cause a deeper color, but instead the color disappeared, indicating that not only was the transgene inactive, it also blocked the endogenous expression. Sense or antisense single-stranded RNA was later discovered to have little effect on mRNA levels, but small amount of dsRNA could efficiently and specifically silence homologous mRNA (Fire et al., 1998).

Long dsRNA is cut into 21-23 nucleotide dsRNA fragments called short interfering RNA (siRNA) by a ribonuclease III-like nuclease known as Dicer. Thereafter, a large complex called RNA-induced silencing complex (RISC) uses the siRNA fragments as template whereby the complex can be guided to and recognize homologous mRNAs, which will be cleaved and subsequently destroyed (**Figure 8**). The RISC complex contains at least one member of the argonaute protein family, which acts as an endonuclease and cuts the homogenous mRNA (Hammond et al., 2000).

Shortly after the mechanism of gene silencing by RNA interference was revealed techniques were developed to use RNA interference as a research tool to investigate the role of a specific

protein. In mammalian cells, the introduction of long dsRNAs leads to non-specific stress reactions, but the problem was solved by the introduction of short (21 nucleotides) siRNA (Caplen et al., 2001). Since then siRNA has been used extensively in the dissection of signaling pathways (Al-Khalili et al., 2003a; Huang et al., 2008; Puri et al., 2007) and to identify new targets involved in the regulation of metabolism (Powelka et al., 2006).

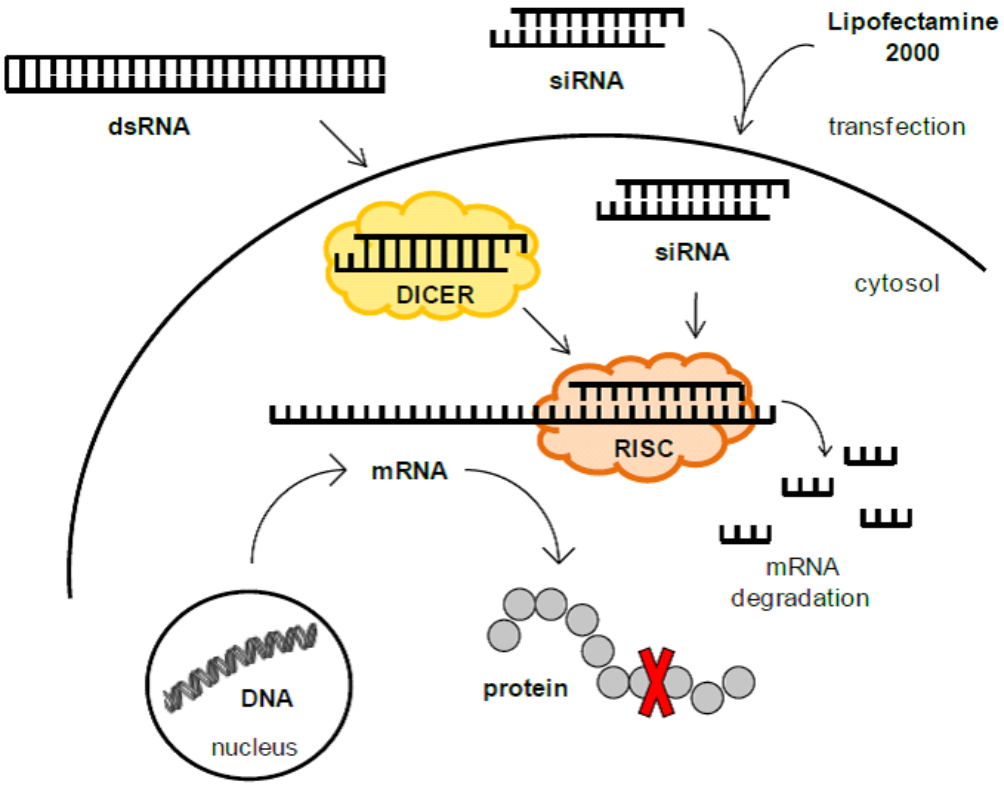


Fig 8. Mechanism for siRNA transfection and mRNA degradation. After entry into the cell, long dsRNA sequences are cut into smaller pieces by Dicer. The RISC complex then uses the produced siRNAs to cleave homologus mRNA, and thereby interferes with the translation of mRNA into a protein.

1.15. Key role of MIG1 gene in metabolic switching

1.15.1. MIG1 gene, Glucose Repression and Metabolic Processes

The presence of glucose in industrial cultivation media has a he metabolism of other sugars. Glucose repression reduces the transcription rate of repressible genes, and is the most investigated mechanism of glucose control. So, it is of importance to develop strains in which glucose repression is relieved. Therefore, all sugars should be utilized, preferably simultaneously, to achieve optimal economic yield. Invertase (EC 3.2.1.26), the sucrose-hydrolyzing enzyme, is expressed under control of glucose repression. In S. cerevisiae, six SUC genes (SUC1 to SUC5 and SUC7) are responsible to encode Invertase of which SUC2 is the most common one 2. Invertase is encoded by SUC2 including two forms, secreted and cytoplasmic. The secreted form of invertase is glycosylated and located in periplasmic space, digesting extracellular sucrose to glucose and fructose, both of which can be transported into the cell 6 (Galello et al., 2017). The cytoplasmic form is not glycosylated, and acts physiologically to cleave sucrose, transported across the plasma membrane. SUC2 gene repression following glucose uptake constitutes a regulatory cascade through a large number of regulatory elements. Mig1p, a zinc finger DNA-binding protein, is the main involved component that mediates glucose induced repression of sugars (Xu et al., 2018). Inactivation of MIG1 gene derepresses the expression of invertase encoded by SUC2. This protein resembles the mammalian Egr and Wilms' tumor proteins, the CreA repressor in Aspergillus nidulans and the Migl repressor in Kluyveromyces lactis. The MIG1 promoter has been reported to be autoregulated. Mig1p prevents transcription of SUC2 through the mechanism that mediates the binding of the general repressor Ssn6p-Tup1p complex to the regulatory part of SUC2. Snf1 kinase regulates glucose derepression of genes required for utilization of alternative carbon sources. The main mechanism is through glucose dependent

dephosphorylation of transcriptional repressor protein Mig1 (Shashkova et al., 2017). One important strategy to reveal the mechanism of Mig1-mediated repression is to investigate the physiological consequences of MIG1 deletion/disruption and/or MIG1 over expression. No absolute evidence could well explain the physiological changes by Mig1-redundant protein in a ΔMIGl mutant, i.e. a protein that is similar to Miglp and can partly be a substitute. A second repressor, Mig2p, which is 71% identical to Miglp, as well as a related protein, YerO28, have been identified. In microarray approached combinatorial control, MIG1 and MIG2 repress a largely overlapping set of genes but MIG3 does not seem to overlap in function with MIG1 and MIG2. Instead, MIG3 down regulates the SIR2 gene responsible for gene silencing and the control of aging. There are two approaches to silence MIG1 gene of which one is usually accomplished by disruption and substitution of the target gene with a selecting marker introduced by homologous recombination (Shashkova et al., 2017, Xu et al., 2018). The second alternative approach, which has been applied and researched in plant, is antisense gene expression. Disruption and replacement of MIG1 can derepress promoters of genes which are repressed by glucose. Prototrophic MIG1 disruptant (Δ MIG1) as well as its congenic wild-type strain (2805) were analyzed for expected physiological changes in peripheral metabolism (batch cultivations on sugar mixtures) and central metabolism. During the cultivations, an attempt was made to monitor the parameters of glucose consumption, biomass production, intracellular protein content and patterns of metabolite production to understand how the effect of MIG1 removal can shift to oxidative pathway of metabolism. The strain can be optimized as a host for further processing of recombinant production with advantages of being eukaryotic.

2. MATERIALS AND METHODOLOGY

2.1. CELL CULTURE

An assured cell culture is the basis of success in the process of gene transfer. Different cells require special conditions such as serum, culture media and complementaries. According to the source of cells, they grow attached or suspended. Adherent cells needs to a anchorage environment and usually grow in the form of monolayers in cell culture dish. This connection is essential for cell proliferation and, many after reaching the stage of Confluent (in which cells cover all levels of culture dish) would lose the power of self-replication and die. Most of the isolated cells from tissues are attached type. The suspended types not required anchorage to grow. Hematopoietic cells derived from the blood, spleen and bone marrow are of this type (Sato et al., 2000). In terms of proliferation capacity, cells divided into three groups: Primary, Finite and Continuous. The primary type is of animal tissue cells which are then separated by enzymatic, chemical or mechanical methods and grown in culture. These cells attached to the culture dish and are morphologically like tissue. These cells proliferated in a limited number then enter to a stable phase and finally die. in overall, working with these cells is far more difficult than cell lines.

These cells are physiologically similar to in vivo cells environment. The finite cells, are actually the Primary cells with limited power of proliferation. However, these cells will ultimately die. The continuous cell type or cell lines, have unlimited replication potential and this character of immortality makes the possibility to be easily work on it. Even though it should also be considered to these cells because of changes occurring in their genotypic and phenotypic markers are not of in vivo conditions (Sato et al., 2000).

2.2. Hepatic cell line, HepG2

Hepatocellular carcinoma cells (HepG2 cells) possess the same bioactivity as normal hepatic cells, which are valuable for investigating liver-associated functions and stable during many passages. The HepG2 cell line was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% v/v penicillin/streptomycin antibiotic solution at 37 C under 5% CO2 atmosphere. The medium was changed every other day. For mRNA or protein analysis, HepG2 cells were cultured for 24 h in DMEM supplemented with 1% FBS in the presence or absence of PA (25 mM). For the analysis of insulin signaling, treated HepG2 cells were incubated with or without 100 nM of insulin for 20 min before harvest (Alipourfard et al., 2019a).

2.3. Matreials and equipments reqiured for cell culture

- 6 wells plate
- A variety of filtered pipettes
- 37 ° C incubator
- Falcon (plastic pipe) 15 and 50 ml
- 70% alcohol
- Rack for tubes
- DMEM culture medium
- FBS (Fetal Bovine Serum)
- Inverted microscope
- culture flask

- PBS solution 1X
- EDTA-Trypsin solution
- Antibiotics streptomycin (100 μl / mL) and penicillin (100 unit / mL)

2.3.1. The preparation of FCS (Golestani et al., 2006)

- Fetal bovine serum (Gibco), a bottle of 500 ml
- capped sterile large tubes, 10 pieces

Because of the recommended temperature for storing FCS, 20- ° C, so initially put the container FCS from freezing outside and it was given the opportunity to be processed. To deactivate the complement then, for 30 minutes in water bath at 56 ° C was used. Because the FCS should not be freezed frequently, so it is best that after a first melt to be divided into smaller volumes. Commonly each purchased bottle containing 100 ml of FCS, so after deactivation under the hood and with the help of a sterile pipette, was divided in ten sterile falcon tubes. The tubes were stored at -20 ° C.

2.3.2. The preparation of buffer PBS 1X [Qiagen Transfection Kits]

dissolving of 137 mM NaCl, 7.2 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4 in 800 ml of sterile distilled water and after adjusting pH in 4/7 should reach to one liter capacity by adding sterile distilled water. to sterile the buffer, transfer 100 ml of it to a autoclavable jar cast and autoclaved for 15 minutes. PBS sterile solution is stored at 4 ° C.

2.3.3 . The Preparation of Trypsyn- solution EDTA (Hodges et al., 1973)

25.0 g trypsin and 2.0 g EDTA dissolved in 70 ml of PBS 1X buffer lacking calcium and magnesium ions and then with PBS 1X solution the final volume is brought to 100 ml. After

sterilizing it with a syringe filter (2.0 micrometers) kept at -20. Small amounts of this solution can be stored for 1-2 weeks at 8-2 ° C.

2.3.4. The preparation of cell culture medium [Dulbecco & Freeman method]

- ingredients:
- DMEM powder
- Net deionized water
- L-Glutamine Powder
- Sodium bicarbonate powder (Cell culture grade)
- penicillin / streptomycin solution
- FCS

To prepare 500 ml of medium DMEM, 5 grams of DMEM powder, 85.1 grams of sodium bicarbonate and 2.0-3.0 gr of L-Glutamine was dissolved in 450 ml pure water, and after djusting the pH to 2.7 by hydrochloric acid, the final volume was reached to 500 ml. Then the medium was filtered through 2.0 micrometer sterile syringe filter under laminar hood and sterile conditions. This incomplete DMEM stored at 4° C. To maintain complete DMEM were added v/v 10% FCS and v/v1% solution of penicillin / streptomycin it.

2.4. PREPARATION of HepG2 CELL CULTURE

2.4.1. Preparation of HepG2 cells

Firstly DMEM culture without FCS and antibiotics (1%) are placed in a hot water bath at 37 ° C. afterwards, FCS (10%) and penicillin / streptomycin (1%) solution added in the sterile condition to provide the complete culture. A vial of HepG2 cells frozen in liquid nitrogen is

placed in a hot water bath. After melting, it will be added to 4 ml of culture medium in a Falcon and mixed gently few times with a pipette, then centrifuged at 1200 rpm speed for 5 minutes. Following the remove of supernatant cell precipitates homogenized slowly with 1 ml complete culture by Pasteur pipette. Homogenized product with 5 ml culture transferred to a flask 25 mL incubated overnight in 5% CO2 and at 37 ° C. The next day, the flask medium is replaced with fresh DMEM culture. the cells should observe daily in terms of contamination and growth under the microscope. After 3-2 days reaching to 80-90 % confluency cells are ready for trypsynation.

2.4.2. Trypsynation of the Cells

In first, the medium and floating cells were removed from flasks and cells attached to the bottom of the flask were washed twice with PBS 1X. Then, 1 ml PBS 1X and 1ml Trypsyn-EDTA was added to the monolayer cells in flask. Then for 1-2 minutes flask placed in incubator at 37 ° C. A few hits the wall of the flask, would liberate the attached cells. 4 ml complete DMEM culture was added to the flask to inactivate trypsyn by serum available in culture. mixing with Pasteur pipette several times separate the cells from each other. The cells are transferred to a Falcon to be centrifuged for 5 minutes 1200rpm. after discharging the supernatant, 1 ml culture medium added to precipitated cells slowly with Pasteur pipette and homogenized. The number of cells are counted using a Neubauer slide using Trypan blue for cell counting solution.The colored cells considered as dead and non-colored cells as living cells. After the count, the desired number of cells for subsequent experiments were transfered to the container. For example, in this study for transfection experiments, 50 cells were added to each well of the 6 well plate.

2.5. Decreased expression of genes through siRNA

siRNA as a powerful tool to disrupt gene expression discovered accidentally within experiences need to enter the RNA to cells. It was observed that the insertion of a specific double-stranded RNA molecules into cells leads to inhibition of expression of the genes containing the sequences in the molecule of double-stranded RNA. So, entering a specific RNA molecules can interfere with the expression of a particular gene (Hutvagner and Zamore., 2002). RNA interference mechanism is largely known. When a double-stranded RNA molecule enters into a cell, an enzyme called Dicer will break it to fragments of approximately 21 nucleotides. Each piece of siRNA compose of 19 base pairs and two unpaired base at the end of '5 either. After separation, single-stranded RNA molecules that are either called small interfering RNA (siRNA) binds to an enzymatic complex called RNA induced silencing complex (RISC). The Single-stranded piece of RNA that is bound to the enzyme acts as a guide and allows RISC to destroy sequences which is complementary with siRNA (Hutvagner and Zamore., 2002). siRNA can be chemically synthesized for a particular gene and transfected into eukaryotic cells, leading to reduced expression of the gene specifically. Unfortunately, this type of inhibition of gene expression is temporary.

Application of shRNA based on plasmid or virus expression has made possible to reduce expression in long-term (Haghani et al., 2015). In this method, a fragment including desired target siRNA, a sequence corresponding antisense and middle strand sequence would be cloned inside is a plasmid. After transcription, the sequence between compose the formation of a hairpin structure, which allows pairing sequence provides sense and antisense. This RNA duplex then broken by Dicer into 21 neucleotide pieces and eventually leads to decreased expression of the target gene through the RISC complex (Bantounas et al., 2004). In order to knock down TNF-α gene, shRNA-mediated technique was performed (Haghani et al., 2015) by using shRNA lentiviral particles (Santa Cruz Biotechnology Inc., Heidelberg, Germany), which were designed to suppress the production of TNF-α in liver cells (Alipourfard et al., 2019a).

2.2.4. Temporary plasmid DNA transfection into Hep-G2 cell line by using calcium phosphate method (Alipourfard et al., 2019a).

Transfection, transfer of foreign molecules such as DNA or RNA into eukaryotic cells emerged as a powerful tool for the study and control of gene expression respectively. Inserted genes may be investigated in biochemical characteristics, mutation analysis, gene regulation effect on cell growth. The Select of appropriate technology for gene transfer to cells strongly affect the results of this process. A favorite technique should be a convenient way of speed, and have ease, efficiency, reliability and minimal cytotoxicity. Transfection methods are generally classified into three groups: (Prokop et al., 2007)

1) chemical (calcium phosphate, DEAE- dextran, cationic liposomes and non-liposomic lipids)

2) Physical (electroporation and microinjection)

3) viral (virus vectors)

In this study, the calcium phosphate chemical method was used. The procedure was performed according to the following steps.

2.6.1. Required solutions:

A) HEBS 20X

18.8 g NaCl, 95.5 HEPES and 0.106 g NaHPO4 in 40 ml of pure distilled water, and the final volume to be placed 50 ml solution placed. Then, this solution filtered and stored in -20^{o}C.

B) 2 mM CaCl2, 1.61 mM Tris-HCL

2.22 g CaCl2, 1.61 ml solution of 1 M Tris-HCl (7.5= pH) and 0.65 ml of 1 M NaOH were dissolved in 8 ml of pure distilled water, and pH adjusted to 7.5 then final volume reached to 10 ml. This solution is then sterilized with filter and kept at a temperature -20^{o}C.

C) HEBS 2X

1 ml solution X HEBS and 0.1- 0.15 ml solution of 1 M NaOH with pure distilled water reached to a final volume of 10 milliliters. PH should be 7.1- 7.2. This solution is then sterilized with filter and maintained at -20°C.

2.6.2. Method

1. Day first : 3 × 10 cells were cultured in each well of a six-well plate, in DMEM (1% antibiotics and FCS 10%).
2. Day second : when 60-70% of the plate floor was covered by cells, replaced the culture medium with fresh one and the cells were incubated for 2 hours in a CO2 incubator.
3. Then DNA solution was prepared by the following method:
32.5 microliter of solution 2 mmol CaCl - 1.61 mM Tris-HCl added to 10 micrograms of plasmid and the final volume reached to 525 ml with distilled water.
4. 525 microliters of solution HEBS 2X was added at the bottom of a Falcon. Then within vortex of the Falcon, DNA solution was added drop by drop (at this point is solution becomes milky).
5. 30 minutes incubation at room temperature was carried out.
6. Then 1 ml of falcon contain added to 2 ml culture in each wells and mixed by cross like shaking to distribute DNA calcium-phosphate precipitant.
7. 16 hours after transfection medium in each well removed and the cells were washed twice with DMEM without FCS and antibiotics till calcium phosphate is almost eliminated.
8. 2 ml complete DMEM added to each well placed environments.
9. after 48 hours cells transfected with GFP plasmid were observed by inverted fluorescence microscope and transfected cells with plasmid smashed with the lysis buffer and their protein contents extracted for Western blot test.

2.7. WESTERN BLOT (Bakhtiyari et al., 2010; Alipourfard et al., 2019a).

To confirm the down-regulation of TNF-α protein level, Western blot method was used. The base of this method is linkage between antibodies and their specific proteins.in this trial antibodies specific for TNF-α were used. In addition, to eliminates errors due to unequal loaded amounts of protein samples, the structural protein beta actin was used as housekeeping gene. Using software TotalLab density of different bands of protein measured and the density of TNF-α band of each sample was divided to the density of beta actin on the same sample to cause loading related error to be removed.

2.7.1. Required solutions

1. Storage Akrylamide solution 30%: 29.2 gr Akrylamide, 0.8 gr bis Akrylamide in final volume of 100 ml.
2. Down gel buffer: 18.2 gr Tris-base, 0.4 gr SDS in final volume of 100 ml, PH=8.8.
3. Up gel buffer: 6.1 gr Tris-base, 0.4 gr SDS in final volume of 100 ml, PH=6.8.
4. Tank buffer: 3 gr Tris-base, 14.4 gr Glycine, 1 gr SDS in final volume of 1000 ml, PH=8.3.
5. Sample loading buffer 5X: 10 ml up gel buffer, 5ml Glycerol, 1 gr SDS, 0.5 ml bromo-phenol blue solution (0.1% in ethanol), 1 ml 2-Mercaptoethanol in final volume of 20ml.
6. Ammonium persulphate 10%: 0.1 gr Ammonium persulphate solved in 1 ml distilled water.
7. Protease inhibitor cocktail: 1 tablet solved in 1 ml distilled water.
8. Lysis buffer: 2.5 gr sodium deoxycholate (DOC), 2.5 ml NP-40, 2.5 ml EDTA 0.5 M, 95 mgr EGTA in final volume 250 ml, PH=7.4. when using added 10% v/v protease inhibitor and PMSF with 2 mM concentration.

9. Washing buffer or TBS-T: 1.21 gr Tris-base, 8.77 gr NaCl, 5 ml Tween-20 in final volume 1000 ml, PH=7.5.

10. Blocking buffer: 5% skimmed milk in TBS-T buffer.

11. Transfer buffer: 6 gr Tris-base, 28.8 gr Glycine, 300 ml Methanol in final volume of 2000 ml, PH=8.3.

2.7.2. Protein extraction

1. cells after trypsination, washed 2 times with cold PBS buffer and was centrifuged for 5 minutes each time.

2. Then for each 1 million cells was added 200 microliter lysis buffer included 10% v / v protease inhibitor and 2mM PMSF to the vial containing the cells.

3. The cells were homogenized with lysis by insulin syringe 5-7times.

4. The mixture for 30 minutes on ice with gentle Shaking were incubated.

5. The above vials was centrifuged in 13000 rpm for 10 minutes and the temperature of 4 ^{o}C.

6. supernatant containing protein extracts were isolated, and its concentration was determined using Bradford method. Protein extracts were then kept in -70^{o}C.

2.7.3. SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was performed according to the following steps:

1. First electrophoresis system components including glassy plates, spacer and comb, thoroughly washed , and were cleaned with cotton soaked in alcohol.

2. By Use the spacer, the gel form was and placed on its location respectively.

3. Resolving gel 8% prepared with appropriate volumes of the following solutions: *3.25 ml storage 30% AKrylamide solution, 3 ml sample buffer, 6.2 ml distilled water, 0.05 ml Ammonium persulphate 10%, 0.05 ml TEMED, Total Vol: 12.1 ml.*

4. The solution prepared in the previous step was delivered to 2.5 cm distance before the end of the mold and immediately 1 ml of distilled water was slowly added.
5. When the gel formed, distilled water was removed.
6. Stacking gel 4% was prepared as follow storage solutions and was poured in the form of gel. The comb was put in place.

0.65 ml storage 30% Akrylamide solution, 1.25 ml stacking gel buffer, 3.05 ml distilled water, 0.05 ml Ammonium persulphate, 0.015 TEMED, Total Vol: 5 ml.

7. When stacking gel formed, comb was removed and the wells washed with the electrode buffer then prepared mold was placed in the electrophoresis tank.

8. Samples were mixed at a ratio of 1: 4 with sample buffer and then placing it in a boiling water bath for 5 minutes, later loaded in wells. At the same time in a well standard molecular weight was shed.

9. After throwing electrophoresis buffer tank, flow was restored to 100 volts for one hour.

10. With the arrival of color reagents to the end of the gel, the electrical flow stopped and gel separated in order to transfer of proteins from gel to the PVDF sheets (purchased from Sigma company).

2.7.4. Transfer of proteins from gel to PVDF sheets

Since the antibodies cannot bind to the proteins on the gel, the proteins should transfer to PVDF sheets. So the following steps for the transfer was performed:
1. After SDS-PAGE, PVDF paper was cut to the right size with the gel placed for 10 minutes in the cold transfer buffer. some filter papers fitted with gel size and Western blot sponges were placed in transfer buffer.
2. After wetting all the above components, western sandwich was made from the bottom to the up containing of: sponge, filter paper, gel, PVDF sheet, filter paper and sponge.

3. The mentioned sandwich placed in tank of somehow to locate the gel at cathode (-) side and the PVDF of anode (+) side. Then the tank filled out with transfer buffer to cover thoroughly the form and has been connected to the power supply. The transfer voltage of 100 V for 1 hour in 4 °C was done.

2.7.5. Blotting stage

After discontinuation of electrical flow blotting was performed according to the following steps:

1. To prevent non-specific connection of antibodies to PVDF sheet blank parts should be blocked. For this purpose PVDF transfer out of the tank and incubated overnight in blocking buffer in 4 °C.
2. The next day PVDF twice, each time for 5 minutes with gentle shaking rinsed by washing buffer. In each wash buffer was replaced with fresh buffer.
3. The appropriate dilution of primary antibodies (usually 1/1000) prepared in washing buffer containing BSA 1% and PVDF sheet was incubated in it for 1 hour with gentle shaking.
4. PVDF paper once for 15 minutes and three times for 5 minutes rinsed with gentle shaking by washing buffer.
5. In next stage, appropriate dilution of secondary antibodies conjugated with HRP (usually 1/10000) was prepared in washing buffer containing BSA 1% and PVDF sheet was incubated for 1 hour with gentle shaking.
6. Again, washing like step 4.
7. to reveal the bands of PVDF, was incubated in an ECL solution (a type of chemiluminecense substrate which can convert by HRP into a light emitting itself) for 1-2 minutes.
8. Then, in the darkroom, PVDF was placed in the vicinity of X-ray film special for western

blot for 1-5 minutes.

9. Again, washing like step 4.

10. In the next stage, film processing was done. For this purpose X-ray film was placed in developing solution until the bands appear. Then rinsed in water for 1-2 minutes and put in a fixing solution.

11. The film was scanned and semi-quantitative analysis of bands was performed with a software TotalLab.

2.8. Treatment with palmitate (Seahorse Bioscience)

Given that the cell culture medium is a aqueous environment, fatty acids and lipids generally are insoluble. So to access the cells to fatty acid palmitate is essential that to make it soluble. For this purpose, a mechanism similar to the transport of fatty acids in the blood by albumin was used and palmitate was conjugated with free fatty acid-BSA according to below steps:

1- Free fatty acid-BSA with ratio 1% w / v was added to DMEM culture medium without antibiotics and FCS then dissolved.

2. The necessary amounts of palmitate to prepare the desired concentrations (0.25, 0.5, 0.75 mM) were weighed and dissolved in ethanol 50% and 50 ^{o}C.

3. alcohol-based palmitate solution was added to the culture medium containing free fatty acid-BSA.

4. Then solution was incubated at 37 ^{o}C for 2 hours with shaking (rpm 100).

5. after 2 hours this culture medium was sterilized with filter and cells were treated with.

2.9. Compare the key elements of insulin signaling pathway in normal cells with decreased TNF-α expression

To investigate the effect of decreased TNF-α expression on insulin signaling pathways, positive phosphorylation (which leads to increase of activation) of two key molecules, IRS- and Akt, was analyzed semi-quantitavely in the presence and absence of palmitate. To analyze the amount of phosphorylation, Western blotting was performed in the manner described above. The difference is that for phosphorylation analysis of IRS-1, a primary antibody against phosphotyrosine 632 and for Akt of primary antibodies against phosphoserine 473 were used. In addition, in this experiment to remove the error caused by loading the sample rather than for β- actin, western blot analysis was performed for proteins IRS and Akt. All other stages are quite similar to the Western blot method.

2.10. Glucose Uptake Test

Radioactively-labeled 2-deoxy-D-glucose was applied to determine glucose uptake in myotubes. After incubation, radioactivity was measured in the cell lysates. 2-deoxy-D-glucose is a glucose analogue where the 2-hydroxyl group has been replaced by hydrogen. Like glucose, 2-deoxy-D-glucose is taken up by cells and phosphorylated by hexokinase, but not further metabolized. Thus, the molecule is trapped inside the cell and by measuring the accumulation of the radiolabeled isotope, an estimate of glucose uptake can be determined.
Cells were grown and differentiated in 6-well plates as described above. After overnight serum starvation cells were pre-incubated with or without 120 nmol/l insulin in Krebs buffer (140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l MgSO4, 1 mmol/l CaCl2, 20 mmol/l HEPES, pH 7.4) with 0.1 % BSA for 60 min. Following four repeated washes, cells were incubated for 10 min with 0.67 μCi/well 2-deoxy-D-glucose [1,2-3H(N)] (1 mCi/ml, final specific activity 50 Ci/mmol; Amersham) in Krebs buffer supplemented with 10 μmol/l 2-deoxy-D-glucose. After washing with ice-cold PBS cells were lysed in 0.4 mol/l NaOH and radioactivity was

determined in a liquid scintillation counter. To determine non-specific uptake parallel incubations were performed in the presence of 50 μmol/l cytochalasin B (Sigma) and non-specific uptake was subtracted from the total. Cytochalasin B exposure reduced glucose uptake approximately 15%. Each experiment was performed in triplicate and normalized by protein concentration, as assessed by the Pierce method (BSA Protein Assay Kit). Since the concentration of glucose in the incubation media is known the results can be expressed either as counts per minute, or calculated to nmol glucose taken up by the cells per min and per mg of proteins. In the latter cases, radioactivity was measured in 20 μl of radioactive media with a known concentration of glucose (triplicate samples), giving the amount glucose taken up in each assay.

Optimization of glucose uptake protocol: The rate of insulin-stimulated glucose uptake in TNF-α down-regulated cultured cells is low compared to intact normal cells. For example, in normal cultured cells insulin increases glucose uptake 1.2-1.4-fold (Al-Khalili et al., 2003), whereas in TNF-α down-regulated cultured cells insulin increases glucose uptake more than 2-fold (Karlsson et al., 2006, Krook et al., 2000). One possible explanation for the different fold-increase in glucose uptake between them may be the lower GLUT4 expression in TNF-α down-regulated cells(Sarabia et al., 1992). In an attempt to optimize the glucose uptake assay, and possibly increase insulin-stimulated values, different protocols were tested. The following parameters were analyzed:

- Changes in the concentration of non-radioactive 2-deoxy-D-glucose (10 mmol/l instead of 5 mmol/l).
- Changes in the concentration and time of radioactive labeled 2-deoxy-D-glucose (1 μCi for 15 min instead of 0.67 μCi for 10 min).
- Use of glucose free DMEM for incubations instead of Krebs buffer.
- Changes in the pre-incubation time with insulin (30 min instead of 1h).

2.11. STATISTICAL ANALYSIS

All statistical analyzes were performed using SPSS software. Each experiment was repeated at least three times. The comparison between all groups was performed by using ANOVA. If there were differences statistically significant, then T-Test was applied. The values of $p < 0.05$ were considered statistically significant.

2.12. Upstream and downstream fragments of MIG1 gene cloning, MIG1 disruption and recombinant screening

Both forward and reverse primers provided by Oligo and Blast software for each of N-fragment (before starting codon of MIG1) and C-fragment (after stop codon of MIG1) included restriction sites for EcoRI and BamHI. Primers were made by Clontech Company according to the N- and C- terminal sequences of MIG1 gene. PCR amplification of N and C terminal fragments was performed by thermocycler according to instructions followed: initial single denaturation at 94 ^{o}C for 5 min, 30 cycles of denaturation at 94 for 30 s, annealing at 45 ^{o}C for 45 s, extension at 72 ^{o}C for 1 min and final extension at 72 ^{o}C for 5 min. The correct amplification of PCR products (N-fragment of 400 bp, Cfragment of 600 bp) was confirmed by gel electrophoresis (Alipourfard et al., 2018).

The amplified N and C fragments were cleaned up by PCR product purification kit (Biogen Company). The purified products enabled restricted digestion of fragments on suitable restriction sites which existed within the primers. Namely, N fragments were digested with EcoRI and BamHI enzymes and C fragments were digested with XbaI and BamHI enzymes. The restriction digestion reactions were carried out for overnight at 37 ^{o}C. The enzymes were inactivated in 10 min at 65 °C. After gel electrophoresis of digested fragments, 400 and 600 bp DNA bands corresponding to N-and C- fragments, respectively, were extracted from gel 16. The gel purified N- and C- fragments were inserted into plasmid pBluescript II SK (Stratagene) using Fermentase DNA cloning kit (Thermo Fisher Scientific Company). The resulted constructed plasmid pMIG1 consists of BamHI as midline restriction site between inserted N- and C-

fragments. Furthermore, plasmids were transferred to Escherichia coli (E. coli) DH5α (Bethesda Research Laboratories). E. coli was transformed by electroporation using a Gene Pulser (Pulse Controller, Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. The growth of transformants in LB culture media containing ampicillin (100 mg/ml) and X-α-gal (as screening agent) and IPTG (as galactose metabolism inducing factor) allowed selection of white colonies consisting of pMIG1 plasmid. The recombinant pMIG1 plasmid consisted of plasmid pBluescript, N- and C- fragments digested with BanHI and inserted with selection marker, URA3 (Alipourfard et al., 2019b)..

The wild strain of yeast *Saccharomyces cerevisiae* was transformed by PEG/Li acetate method to replace to produce MIG1 disrupted strains. URA3 enables recombinant yeast to synthesize required uracil for growing in uracil-lacked medium (SC-URA). To measure sugars and extracellular metabolites, cultivation medium was sampled, immediately filtered through a 0.45 mm pore size cellulose acetate filter. Glucose, galactose, maltose, ethanol, glycerol, acetate and pyruvate were separated on an Aminex HPX-87H ion exclusion column HPLC and were detected refractometrically or spectrophotometrically (Alipourfard et al., 2019b).

3. RESULTS

3.1. Effect of TNF-a down-regulation on insulin signaling and metabolic syndrome processes of hepatic cell lines

The overall goal of this research work is to dissect pathways regulating glucose and lipid metabolism in human liver cell. Investigation of these pathways will give further insight into the action of insulin, and could reveal novel molecular targets for treatment of insulin

resistance in obesity or type 2 diabetes. In this regard the specific aims are to explore following aspects:

A) Whether palmitate can induce insulin resistance in HepG2 cells.

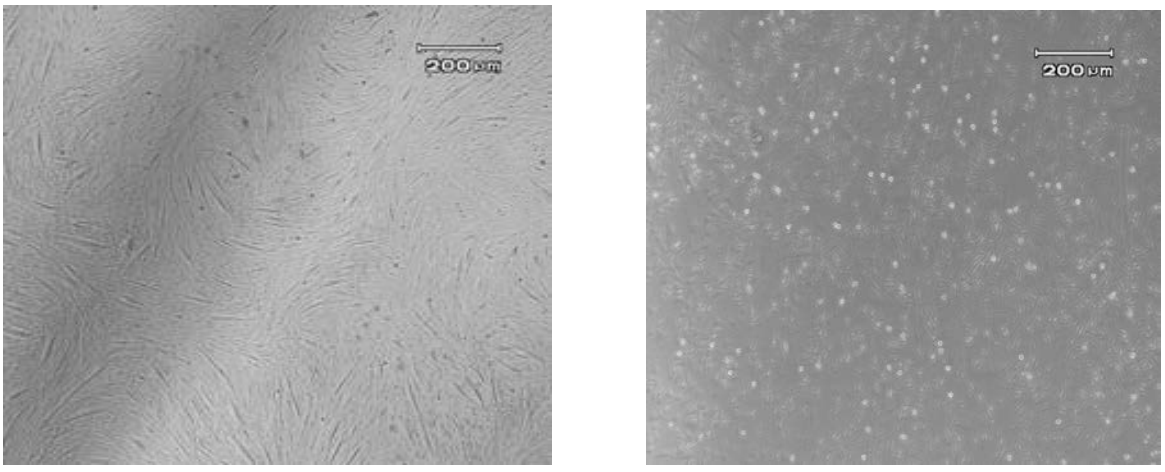
B) The role of palmitate in stimulation of TNF-α protein expression in HepG2 cells.

C) Whether the decreased expression of TNF-α can promote positive phosphorylation of key insulin signaling molecules, Akt, and IRS-.

D) Does the reduction of TNF-α expression leads to palmitate induced insulin resistance in HepG2 cells?

3.1.1. Cell Cultures

Cells were grown in complete DMEM culture medium (containing FBS10%, antibiotics 1%) and in terms of contamination and daily growth rate observed under inverted microscope, if necessary, replaced the culture medium and photographed. The cells in the early stages are spherical form, after 2-3 days, and covering 70-80% of the culture surface are ready for trypsination (**Figure 9**).



(A) (B)

Figure 9. Cell culture in complete DMEM medium.

(A) HepG2 after one day are spherical form. (B) HepG2 after 3 days are ready to passage.

This process has been observed and photographed with inverted microscope.

3.1.2. Transfection with Calcium Phosphate Method

In order to evaluate the efficacy of calcium phosphate method, GFP (green fluorescent protein) plasmids were used for transfection. 48 hours after transfection of the plasmids into cells, GFP would be expressed throughput to be seen in green when exposed to UV light inverted fluorescent microscope. The higher number of green cells, its efficiency is more approach. As seen in **Figure 10** evenly, calcium phosphate method is a highly efficient method to transfect cells HepG2.

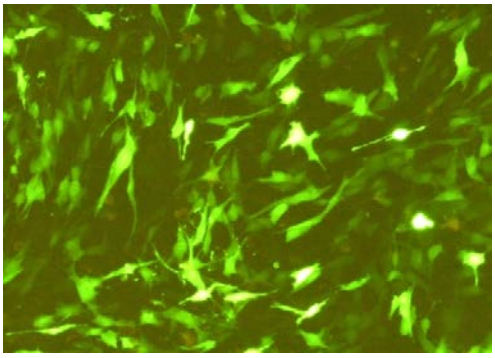


Figure 10. HepG2 cells transfection with GFP plasmids using calcium phosphate method. Cells inserted GFP seen green under UV light of inverted microscope.

3.1.3. Temporary Transfection

Since HepG2 cells were transfected with plasmids shRNA TNF-α (TNF-α Knockdown cells), plasmids lacking shRNA TNF-α (control cells) and plasmids containing shRNA which interferes with none of the intracellular mRNA (Scramble Cells) under using calcium

phosphate method. Equal amount of each protein sample was loaded and then Western blotting was performed after determining the protein concentration. The results of Western blotting showed that the TNF-α gene expression compared to control cells, in cells transfected with shRNA TNF-α has reduced approximately 53% compared to scramble and normal cells which suggests that the plasmid containing shRNA against TNF-α causes the highest rate of TNF-α silencing **(Figure 11).** It is noted that due to T-test, the value rate of gene expression reduced to p < and considered to be significant.

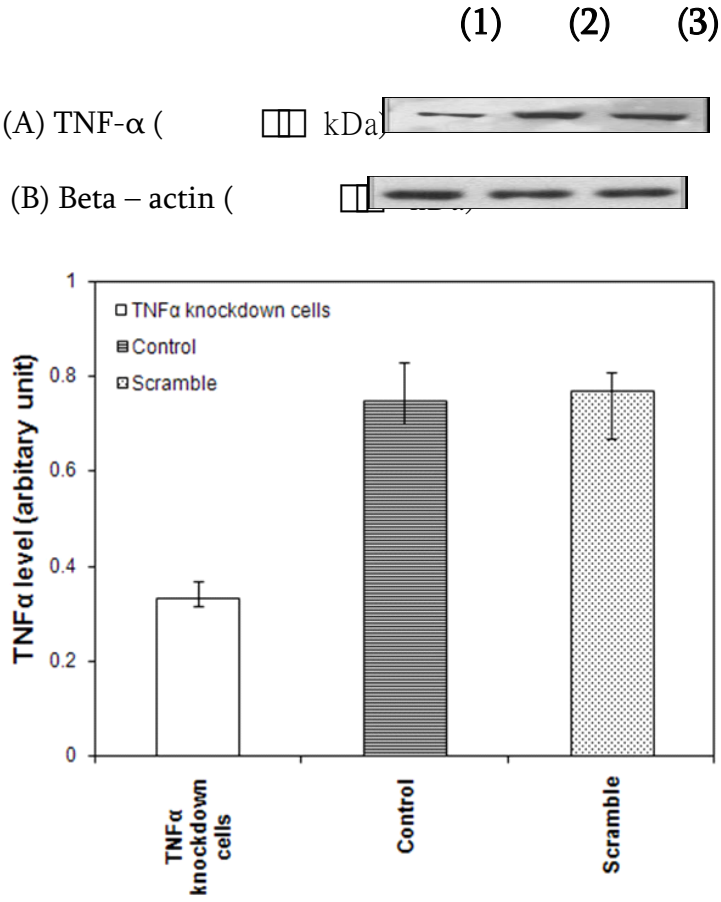


Figure 11. Reduction of TNF-α expression in HepG2 cells following transfection with, (1) shRNA TNF-α plasmid, (2) plasmid lacking shRNA TNF-α, (3) Nonsense plasmid, using Calcium Phosphate method. HepG2 cells transfected with μ20g of each plasmids 1-3. 48 hours later western blot performed for TNF-α and β-Actin as internal control. The amounts of TNF-α protein normalized by

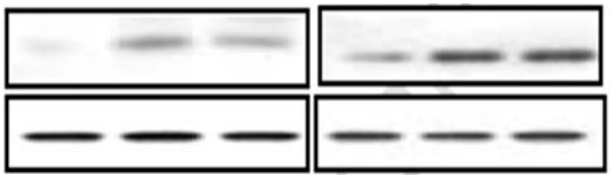
considering the amounts of β-Actin protein. The date achieved of three independent experiments and shown as mean ± SD, $p<0.05$

3.1.4. Increase of insulin signaling pathway in cells with reduced TNF-α expression affected by palmitate.

As mentioned in the Materials and Methods, to evaluate insulin signaling, the positive phosphorylation of two key molecules in this pathway, namely tyrosine 632 in molecule IRS-1 and serine 473 analyzed by Western blot testing. The results showed the phosphorylation of tyrosine 632 of IRS-1 (in the case of stimulation with insulin) in the treatment with palmitate, both in normal and down-regulated cells decreased (**Figure 12**). In terms of stimulation with 100 nM insulin and treated with mM palmitate tyrosine 632 phosphorylation of IRS- molecules in TNF-α down-regulated expression cells of 29% ($p < 0.05$) and in normal cells 58% ($p < 0.05$), although insulin stimulation of 100 nM and absence of palmitate the phosphorylation of tyrosine 632 of molecule IRS-1 findings 33% ($p <0.05$) in TNF-α down-regulated expression compared to normal cells of 50% ($p <0.05$) has been reduced.

	Control Cells			TNF-α Knockdown Cells		
Insulin(100Nm)	-	+	+	-	+	+
Palmitate(0.5 mM)	-	-	+	-	-	+

P-(Tyr632)-IRS-1



IRS-1

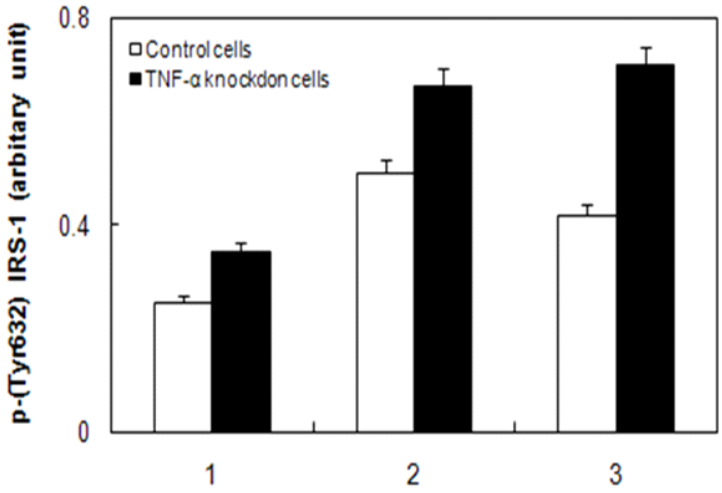
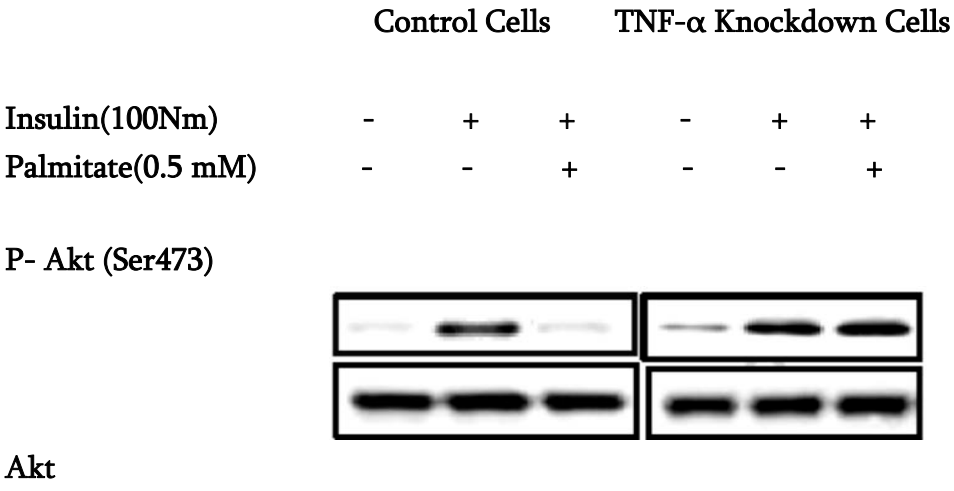


Figure 12. Effect of palmitate on amount of Tyr 632 phosphorylation of IRS-1 molecule in HepG2 cells TNF-α down-regulated expression and normal ones. Cells treated with palmitate and before harvest incubated in presence and absence of insulin conditions. Western blot test performed by using antibody against phosphotyrosine 632 of IRS-1 and IRS-1(as internal control). The amount of phosphorylation of tyrosine 632 of IRS-1 normalized with adjacent IRS-1 control molecules. The date has been achieved of three independent experiences and demonstrated as mean ± SD, $p<0.05$.

In conditions without treatment with insulin and absence of palmitate there was not significant differences in tyrosine 632 phosphorylation of IRS-1 molecules in TNF-α decreased expression and normal cells ($p <0.05$). However, the treatment with palmitate 0.5 Mm reduced the tyrosine 632 phosphorylation of molecule IRS-1 in both down-regulated TNF-α expression and normal cells, the amount of phosphorylation in the presence of palmitate mM in cells with decreased expression of TNF-α approximately 1.3 times (p

<0.05) is higher than normal cells. **(Figure 12)**
In this study, the effect of palmitate on phosphorylation of serine 473 of Akt molecules in TNF-α down-regulated cells and of the normal cells was examined (Alipourfard et al., 2019a).

The results showed that the phosphorylation of serine 473 of Akt molecule (in the case of insulin stimulation) with treatment of palmitate, has been decreased in both TNF-α down-regulated expression and normal cells. In terms of stimulation with 100 nM insulin and treated with mM palmitate the serine 473 phosphorylation of Akt molecules in TNF-α down-regulated expression cells of 18% ($p < 0.05$) and in normal cells 69% ($p < 0.05$), although insulin stimulation of 100 nM and absence of palmitate the phosphorylation of serine 473of molecule Akt 20% ($p < 0.05$) in TNF-α down-regulated expression compared to normal cells of 23% ($p < 0.05$) found to be reduced. In non-insulin treatment conditions and lack of palmitate there was not significant differences in serine 473 phosphorylation of Akt molecules in TNF-α decreased expression and normal cells ($p < 0.05$).



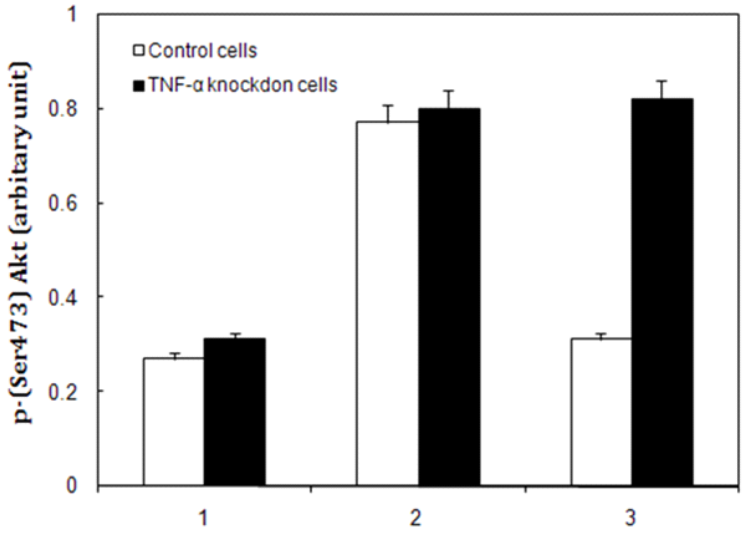


Figure 13. Effect of palmitate on amount of Ser 473 phosphorylation of Akt molecule in HepG2 cells TNF-α down-regulated expression and normal ones. Cells treated with palmitate and before harvest incubated in presence and absence of insulin conditions. Western blot test performed by using antibody against phosphoserine 473 of Akt and Akt (as internal control). The amount of phosphorylation of Serine 473 of Akt normalized with adjacent Akt control molecules. The date has been achieved of three independent experiences and demonstrated as mean ± SD, $p<0.05$.

However, the treatment with palmitate 0.5 Mm reduced the serine 473 phosphorylation of molecule Akt in both down-regulated TNF-α expression and normal cells, the amount of phosphorylation in the presence of palmitate mM in cells with decreased expression of TNF-α approximately 3.9 times ($p <0.05$) is higher than normal cells. **(Figure 13)**

Furthermore, there was not a significant difference between the amounts of IRS-1 and Akt proteins in TNF-α down-regulated expression cells in compare to normal cells, in the presence of palmitate.

3.2. Key role of MIG1 gene of *Saccharomyces cerevisiae* in metabolic switching of fermentation /oxidation

3.2.1. Construction of disrupted mutant yeast

The haploid wild type strain 2805 of *Saccharomyces cerevisiae* was selected to test the feasibility of suppressing effect of *MIG1* by gene disruption and to simplify direct isogenic comparison of *MIG1* expressing strains with that of *MIG1* disrupted mutants. The confirmation of amplification of N and C fragments of *MIG1* gene sequence with 400 and 600 *bp*, respectively, were compared to size marker (**Figure 14).**

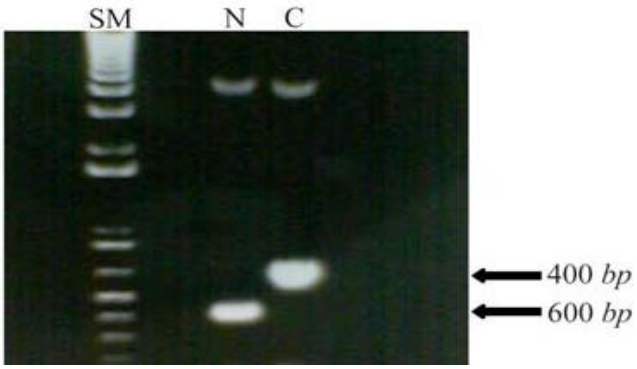


Figure 14. Amplified N fragment (400 *bp*) and C fragment (600 *bp*) of MIG1 chromosomal gene of Saccharomyces Cerevisiae. The size marker (SM) contains of bands for each equals to 100 *bp.*

Insertion of N and C fragments in pBlueskript II plasmid and transformation to *E. coli* DH5a leading to antibiotic resistance attributed to the bacterial aminoglycoside phosphotransferase-encoding *APT2* gene which is inserted between the *PGK* promoter and terminator. After replica plating to X-α-Gal medium under inducing conditions with galactose and glycerol, transformed bacterial strains were isolated (Data not shown). Upon

transformation of strain 2805 with pBluescript II integrated with N and C fragments of *MIG1* sequence interrupted with URA3, several clones of yeast able to grow in SC-URA were isolated .

3.2.2. Metabolic assessment after MIG1 disruption

For batch cultivations on glucose, each of the Saccharomyces strains 2805 and ΔMIG1 were grown on 40.0 g/L of glucose. The results were from the study of aerobic metabolism under defined conditions of glucose control. Each experiment was repeated at least three times. The achieved results were analyzed by using SPSS 21.0 (SPSS, Chicago, IL) software. All normally distributed continuous variables were demonstrated as mean.

3.2. 3. Glucose consumption, ethanol production, cell protein content and growth rate

Glucose consumption in ΔMIG1 mutant was proceeded to wild 2805 strain by 25.0% within several batch and lag phase measurements (**Figure15A)**. The maximum specific growth rate of 2805 and of ΔMIG1, calculated from nine simultaneous optical density measurements demonstrated 0.39 and 0.43 h^{-1}, respectively. Thus, MIG1 disruption led to 12.0% average increase of specific growth rate on glucose **(Figure15B).**

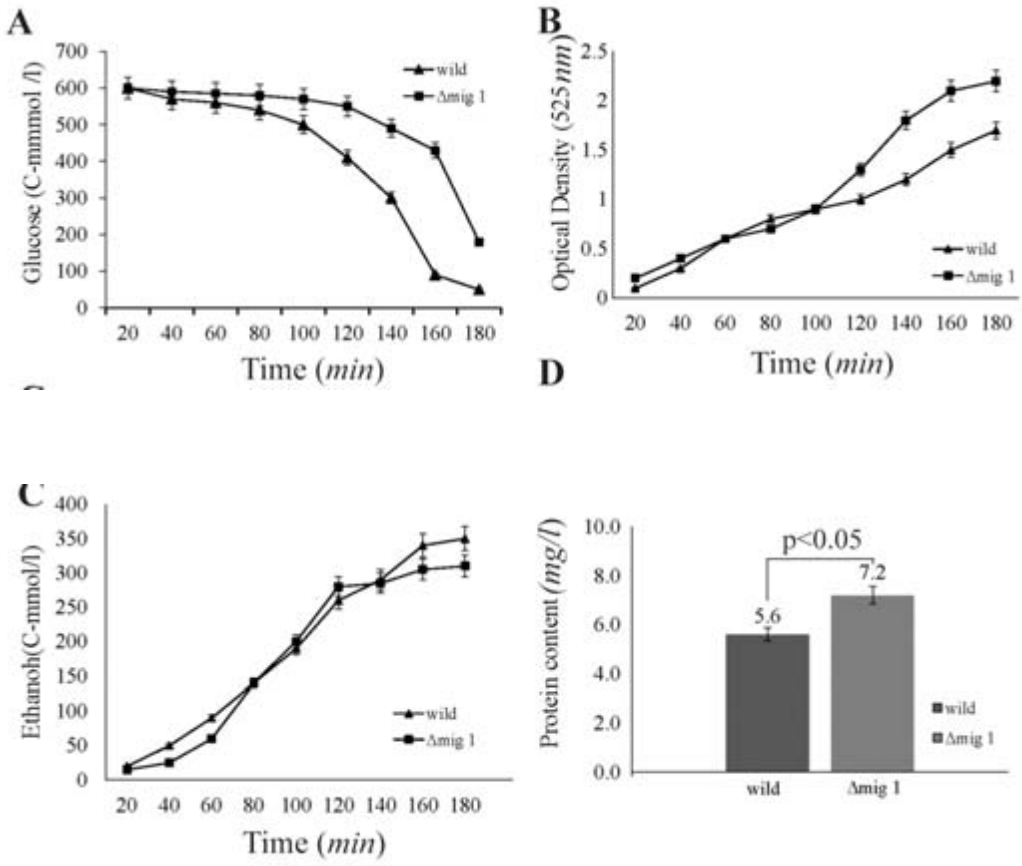


Figure 15. Concentration of A) glucose, B) cell mass, C) ethanol, and D) cell protein in batch cultivations of wild strains 2805 (■) and ΔMIG1 (MIG1 disrupted mutant) (▲) on a medium with glucose control conditions. The data achieved of three independent experiences and calculated mean values have been demonstrated for each amount on the related graph, (n=3), p<0.05, significantly different characteristics of mutant strain from respective wild strain.

In batch cultivation, the rate of carbon flux directed to ethanol production in Δ*MIG1* strain was reduced about 24.0% (*C-mmol/l*) of lag phase cultivation **(Figure 15C**). The less formation of ethanol and related anaerobic metabolites at a higher rate with Δ*MIG1* than with 2805 demonstrates considerable shifted metabolism from respiro-fermentative to respiratory pathway (Alipourfard et al., 2019b). Intracellular protein content accounted for

both strains showed 22.0% (w:w) difference to significant increase in Δ*MIG1* compared with 2805 (**Figure 15D**).

3.2.4. Glycerol, pyruvate and acetate formation

The carbon fluxes have been increased to respiratory metabolism pathway. It is indicated by more remarkable formation of oxidative metabolites markers, i.e glycerol and pyruvate which have been produced 122.86 (C-mmol/L) and 40.0% (C-mmol/L) in ΔMIG1 prominent than strain 2805, respectively. Conversely, the decrease of fermentative metabolite of acetate by 34.58% (C-mmol/L) has been observed. The differences between the rate of metabolite production within cultivation period have been calculated and expressed as percentage (Δ%) **(Figure 16).**

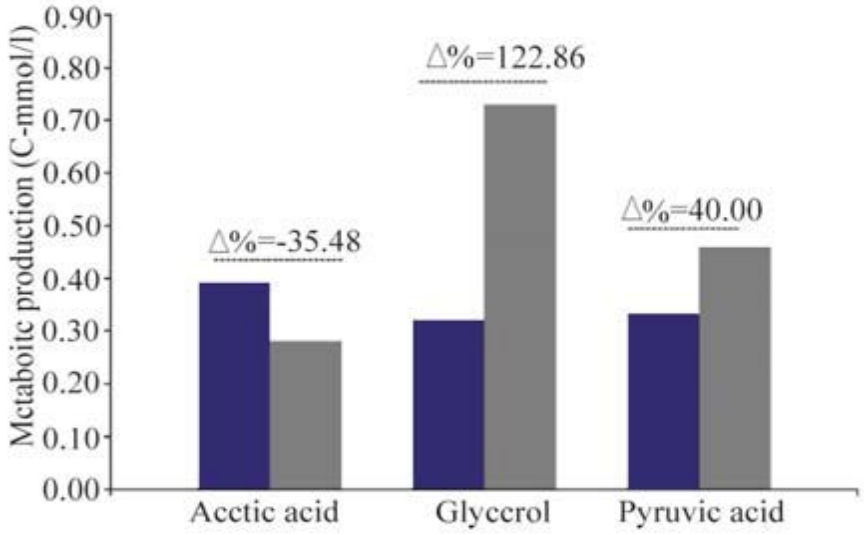


Figure 16. Different production ratio (Percent variation Δ%) of metabolite parameter (Acetic acid, Glycerol, Pyruvic acid) in batch cultivations of wild strains 2805 (■) and ΔMIG1(MIG1 disrupted mutant) (■) on a medium with glucose control conditions.

The comparison between all continuous variables was performed by using one-way analysis of variance (ANOVA). If there were statistically significant differences, then Tukey's PostHoc test was applied. The values of $p<0.05$ were considered statistically significant (Alipourfard et al., 2019b).

4. DISCUSSION

4.1. TNF-α Downregulation Modifies Insulin Receptor Substrate 1 (IRS-1)

Several genetic and environmental factors have been determined to involve in insulin resistance, of which TNF-α has taken much concentration recently. Studies have shown that insulin resistance is associated with elevated plasma levels of TNF-α and causes a higher level of expression in tissues such as adipose and skeletal muscle. On the other hand, it has been frequently reported that the elevated level of saturated fatty acids, namely palmitate as the most abundant one in plasma, leads to insulin resistance in insulin target tissues. In order to understand the possible role of TNF-α as an inflammatory mediator cytokine on insulin signaling inhibition, we designed the current course of TNF-α levels in the simulated obese HepG2 cells under treatment with palmitate. The idea is consistent with the previous studies regarding elevated expression of TNF-α in the liver and skeletal muscle in palmitate-induced insulin resistance condition. Our results demonstrated the highest induced TNF-α protein levels on 16-hour treatment with palmitate (0.5 mM). This indicates the etiological role of elevated palmitate on TNF-α overexpression in obese insulin resistance hepatocytes. The molecular mechanisms of insulin resistance have been demonstrated extensively. Multiple

systemic factors associated with insulin resistance, for example, free fatty acids and TNF-α, can reduce IRS-1 serine phosphorylation and thus inhibits is associated with negative effects on the insulin signaling pathway and has been described in connection with TNF-α-induced insulin resistance. The elevated TNF-α levels have been reported in obesity and other insulin-resistant states. The experiment of TNF-α knockdown was carried out by shRNA-TNF-α lentiviral particles containing shRNA specifically for TNF-α. This process resulted in nearly 53.0% reduction in TNF-α protein levels (compared to normal cells). It is noted that due to *t*-test, the value rate of gene expression was reduced to $p < 0\ 05$ and considered to be significant. It suggests that the particles containing shRNA to silence TNF-α gene cause the efficient rate of TNF-α Mediators of Inflammation knockdown. The process of TNF-α downregulation was then followed by palmitate treatment to avoid coincidence reactions between TNF-α level changes and inhibition of insulin signaling. The tyrosine 632 phosphorylation of IRS-1 after treatment of cells with palmitate and stimulation with insulin showed less reduction of 55.0% ($p < 0.05$) in TNF-α-- downregulated cells compared to the normal cells of 71.0%($p < 0.05$). Although the treatment with palmitate 0.5mM generally reduced the tyrosine 632 phosphorylation of the molecule IRS-1 in both TNF-α-downregulated and normal cells, the amount of phosphorylation in TNF-α-downregulated cells is approximately 1.3 times ($p < 0.05$) higher than the normal cells. The achieved results confirm that the reduction of TNF-α expression in HepG2 cells improves insulin-stimulated Tyr632 IRS-1 phosphorylation. Our results were parallel to the other studies approaching the reduction of TNF-α through different strategies and have demonstrated improved insulin sensitivity in skeletal muscle, liver cells, and animal models. To sum up, according to our data, palmitate can dysregulate the insulin signaling pathway and causes diabetic insulin resistance by increased expression of TNF-α in hepatocytes (Alipourfard et al., 2019a).

The results demonstrated that palmitate (0.5 mM) induction can induce the highest TNF-α level on 16 hours of treatment. The molecular mechanisms of insulin resistance have been demonstrated extensively (Rothman, 1992). In the liver, increased levels of DAG

resulting from elevations in plasma FFA or suppressed β-oxidation activate PKC-ε leading to reduced IRS tyrosine phosphorylation (Ravichandran, 2001) This decreases activation of glycogen synthase and glycogen synthesis, and increases glucose output. The interaction between intracellular TNF-α signal and insulin cascade has been trialed. The role of certain serine kinases (e.g., JNK, IKK-β, and PKC-ζ) through knocked down process or inhibition by pharmacological inhibitors has been determined in insulin resistance induced by high fat diet. Regulation of serine phosphorylation of IRS proteins is major molecular mechanism of insulin resistance which is accomplished by activity of several serine kinases include of JNK, IKK, Akt, mTOR, ERK, PKC-ζ, glycogen synthase kinase 3, and casein kinase II. Akt is a key serine kinase activated by its own phosphorylation within TNF-α signal pathway. However, as a common component of both pathways its regulatory activation in term is not clear and has been focused by this research. We examined the Akt positive phosphorylation and its activity in TNF-deleted and non-deleted obese cells. The results show that TNF-α activates Akt serine kinase more in obese cells than normal cells. We consider that there are two biological meanings of Akt activation. First, activation of Akt may contribute directly to insulin resistance by induction of IRS-1 serine phosphorylation. Second, activation of Akt may account for part of acute inflammation events such as enhancement of glucose uptake by TNF-α. Although activity of Akt, in serine phosphorylation of IRS-1 have been reported (Paz, K, 1999), it remains to be studied how Akt serin kinase act in insulin resistance induced fatty acids. Additionally, its role in insulin sensitization by TNF-α romoval have not been reported. In this research, both issues were addressed. Akt phosphorylates IRS-1 at Ser267/270 as reported. Some studies mention that the phosphorylation leads to inhibition of IRS-1 (Ogihara, 1997) and some indicate the phosphorylation is required for activity of IRS-1. Akt serine phosphorylation is associated with effects on the insulin signaling pathway and has connection with TNF-α-induced insulin resistance. This led us to investigate the effect of TNF-α silencing on Akt serine phosphorylation in human obese cells. Our result supports that activation of Akt kinase promotes the activity of IRS-1 and subsequently leads to

improve of obesity condition in hepatocytes. The experiment of TNF-α knockdown carried by shRNA-TNF-α lentiviral particles that include shRNA specifically for TNF-α. This process resulted in nearly 48.6% reduction in TNF-α protein levels (compared to normal cells). The process of TNF-α gene knock-down and reduction in gene expression was considered significant ($p < 0.05$). Followind TNF-α down regulation, cells were treated with palmitate in order to avoid coincidence reactions between TNF-α changes and inhibition of insulin signaling. The serine 473 phosphorylation of Akt after treatment of cells with palmitate and stimulation with insulin showed increase of 58.7% in TNF-α down-regulated cells compared to the non down-regulated cells of 14.9% ($p < 0.05$). Although the treatment with palmitate 0.5 mM generally reduced the ser 473 phosphorylation of the molecule Akt in both TNF-α down-regulated and non down-regulated normal cells, the amount of phosphorylation in TNF-α down-regulated obese cells is approximately 3.9 times ($p < 0.05$) higher than non down-regulated obese cells (Alipourfard et al., 2019a.). The achieved results confirm that reduction of TNF-α protein expression in HepG2 cells improves insulin-stimulated ser473 Akt phosphorylation. Our results were parallel to the other studies approaching to reduction of TNF-α through different strategies which have been resulted in improved insulin sensitivity in skeletal muscle, liver cells and animal models. In summary, according to our data palmitate can dysregulate insulin signaling pathway and causes diabetic insulin resistance by increased expression of TNF-α in hepatocytes.

4.2. MIG1 gene has a role in metabolic switching of fermentation/oxidation

Saccharomyces cerevisiae can utilize a wide range of carbon sources; however, the presence of glucose suppresses the use of alternate carbon sources and also gluconeogenesis and respiration. These effects are exerted by several genes involved in the metabolic pathways. Among them, the zinc finger protein, Mig1 (multicopy inhibitor of GAL gene expression) plays important roles in glucose repression of *Saccharomyces cerevisiae*. To investigate whether the alleviation of

glucose effect would result in a switch to oxidative production pathway, MIG1 were disrupted in a haploid laboratory strain (2805) of *S. cerevisiae*. The impact of this disruption was studied under fully aerobic conditions when glucose was the sole carbon source.

In this research, the chromosomal metabolic gene, MIG1, disrupted by homologous recombination through antisense technique using upward and downward, N- and C-, fragments of MIG1 gene was applied to study the metabolic effects on yeast. Lateral fragments, N and C, after amplification were inserted in yeast specified vector, pTcURA3 alongside metabolic selection marker URA3. After transformation to yeast, MIG1 gene would be replaced with URA3 marker. It can be happened by recombination between its lateral N and C fragments and resembles sequences already inserted into the vector lateral to URA3 marker. This transformation enables the yeast to synthesize required nutrient uracil. In fact, through this method, MIG1 disrupted strain can be screened by growth on uracil negative medium. Then, further analyses of central and peripheral metabolic pathway have been done to approach variations between mutant and intact wild strain. The glucose accessibility is the most important environmental factor to regulate oxidation and fermentation metabolic pathways of Saccharomyces i.e., glucose concentration in batch inhibits the utilization of other carbon resources, the catabolic inhibition. Hexokinase PII starts glucose inhibition cascade and Snf1p as a signal transductor phosphorylates mig1p (regulatory zinc finger protein of MIG1 gene) which makes inhibitory complex with Ssn6 (cyc8)-tup1 components (Carlson, 1987) and binds to promoter of glucose controlled genes. The MIG1 gene was cloned in 1990 for the first time. Deletion of MIG1 demonstrates a greater impact on peripheral functions than on central metabolism. There are recent evidences which persist on the role of Hexokinase 2 (Hxk2) in regulation of DNA-binding repressor proteins of Mig1 protein and glucose repression signal in nucleus. Hxk2 acts as an intracellular glucose sensor that operates by changing its conformation in response to cytoplasmic glucose levels and regulates dephosphorylation of Mig1. In the present study, ΔMIG1 strain 2805 could utilize more glucose in comparison to the wild strain within the first 12 hr to reach to the zero point (Alipourfard et al., 2018.). When the glucose concentration is high,

Snf1p is inactivated and non-phosphorylated mig1p would remain in nucleus and represses transcription of dependent genes. Conversely, in low concentration of cultivation glucose, phosphorylated MIG1p can migrate to cytoplasm and leads to removal of repression status. The deletion of MIG1 can eliminate glucose repression partially from peripheral functions, rather than completely from central metabolic functions. The outcome of this deletion has been considered significant in industry because glucose is the most useful sugar among the carbon resources for yeast in industry process. This can delay uptake of other sugars resulting in elongation of production process. Therefore, an effective alleviation of glucose control would help to achieve a better process economy for the cultivation of baker's yeast, alcohol fermentation, bread-making and recombinant protein production. In some studies, it has been proved that disruption of MIG1 cannot remove the entire glucose repression effect from galactose, maltose and sucrose metabolism. Therefore, substantial MIG1-independent glucose control mechanisms exist for the GAL, MAL and SUC systems. On the other hand, glucose through MIG1 regulation process has several repression effects on the expression of GAL family genes like GAL2, GAL3, GAL4, and GAL80. They are involved in the metabolism of galactose and highly affected by MIG1 disruption. Imp2p is a MIG1 related regulator of GAL genes and has the positive effect on glucose derepression of the maltose, galactose and raffinose utilization pathways and in resistance to thermal, oxidative or osmotic stress of S. cerevisiae. The finding that CAT8 was derepressed in a ΔMIGl strain, whereas Cat8-controlled FBPl, PCKl and ZCLl were not, gives a clear hint of the existence of other repressors such as MIG2 or a post-translational modification of an effector such as the phosphorylation of the derepressor Cat8. The study of the effect of simultaneous deletion of MIG1/MIG2 genes on physiology of Saccharomyces exhibited that glucose control of maltose and sucrose metabolism was derepressed in disrupted MIG1/MIG2. However, the lag phase of galactose cultivation diminished and further deletion of MIG2 could not affect glucose dependent metabolism of galactose. The MIG1 gene was silenced by antisense MIG1 expression (Alipourfard et al., 2019b). The results of evaluation of glucose consumption, cell biomass production and intracellular protein contents by Optical Densitometry (OD) method and Invertase enzymatic activity assays demonstrated the more flexible growth of ΔMIG1 strains under the sufficient amount of oxygen and glucose. This is evidential for removal of glucose repression effects and impressive shift to more oxidative metabolism in comparison to wild strains. Yeast cell

cultivations are effectively stressed by chemical metabolites produced within growth phase namely acids and high percentage of ethanol which can suppress the yeast growth. Deletion of MIG1 can switch fermentative metabolism to aerobic pathway. It results in the production of less fermentative products like ethanol and acetate. Meanwhile, the promotion of Krebs cycle leads to more acidic derivative products such as glycerol and pyruvate (Alipourfard et al., 2019b). The data from the current research approved the similar concept. However, the increase of glycerol production in mutant strain is much prominent than what has been reported in similar studies. Such modifications in peripheral and central metabolic functions could satisfy industrial interests which attempt to have optimal engineered strains for recombinant production.

5. CONCLUSION

The data obtained support the evidence that downregulation of TNF-a and the related signalling has a potential possibility of targeting treatemnt. It can countribute to the improvement of insulin resistance in hepatic cells which is induced by palmitate. Taking these findings together, It could be a strong point in the management of metabolic and cardiovascular diseases, obesity and lipid disorders and diabetes T2DM.

This study showed the amount of ethanol measured by HPLC method in mutant (Δ*MIG1)* and wild strain of yeast *Saccharomyces cerevisiae* cultivations which determined less production of Δ*MIG1*. Enhancement of biomass production in Δ*MIG1* strains can lead to higher amount of intracellular protein content and carbohydrates. In the current research, results of strain Δ*MIG1* 2805 could confirm similar achievements rather than wild types. As to peripheral functions, efforts have already been exerted in production of partially derepressed galactose, maltose and sucrose metabolisms. For central functions, industrial interest could promote attempts in the metabolic engineering of strains that exhibit higher

specific growth rates for baker's yeast or recombinant protein production, or of distiller's yeast strains that give higher ethanol yields.

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