THE EFFECT OF VOLUNTARY EXERCISE, WITH/WITHOUT ANTIOXIDANTS, ON MEAL-INDUCED INSULIN SENSITIZATION (MIS) IN HEALTH AND IN PREDIABETES AND THE STUDY OF CELLULAR SIGNALING PATHWAYS ASSOCIATED WITH MIS IN SKELETAL MUSCLE

By

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... Dedicated to my parents

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Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution. It is, strictly speaking, a real factor in scientific research.

Albert Einstein

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ADA	American Diabetes Association
AICAR	Aminoimidazole-4-carboxamide ribonucleoside
AKT (PKB)	Protein kinase B
AMIS	Absence of meal-induced insulin sensitization
AMP	Adenosine mono-phosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
AUC	Area under the curve
AV	Arterio-venous
BCRCP	British Columbia Reproductive Care Program
BENAC	Bethanechol + N-acetyl cysteine
BMI	Body mass index
cAMP	Cyclic AMP
CDA	Canadian Diabetes Association
CDCP	Centers for Disease Control and Prevention
cGMP	Cyclic GMP
DECODE	Diabetes Epidemiology Collaborative analysis of Diagnostic criteria in Europe
DME	Diabetic macular edema
DPP-4	Dipeptidyl peptidase-4
ED	Erectile dysfunction
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
FPG	Fasting plasma glucose
%FM	Percent fat mass
G6P	Glucose-6-phosphate
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GMP	Guanosine mono-phosphate
GS	Glycogen synthase
GSH	Glutathione
GSK3	Glycogen synthase kinase 3
HbA_1C	Glycosylated hemoglobin

HDL	High density lipoprotein
HISS	Hepatic insulin sensitizing substance
HK	Hexokinase
HOMA-IR	Homeostatic model assessment for insulin resistance
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
INR- β	Insulin receptor- β
IRS-1	Insulin receptor substrate-1
IV	Intravenous
LDL	Low density lipoprotein
MANOVA	Multivariate analysis of variance
MCD	Malonyl-CoA decarboxylase
MIS	Meal-induced insulin sensitization
NAC	N-acetyl cysteine
NIDDKD	National Institute of Diabetes and Digestive and Kidney Diseases
NO	Nitric oxide
NOS	Nitric oxide synthase
NPDR	Nonproliferative diabetic retinopathy
OGTT	Oral glucose tolerance test
PBS	Phosphate buffer saline
PDR	Proliferative diabetic retinopathy
PHAC	Public Health Agency of Canada
PI3K	Phosphatidylinositol 3-kinases
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PPG	Postprandial blood glucose
RIST	Rapid insulin sensitivity test
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SAMEC	S-adenosylmethionine + vit E + vit C
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNRI	Serotonin-norepinephrine reuptake inhibitor
TG	Triglyceride
WHO	World Health Organization

ABSTRACT

Background: The augmented whole body glucose uptake response to insulin during the postprandial state is described as meal-induced sensitization (MIS). MIS occurs when the presence of food in the upper gastrointestinal tract (GIT) activates two feeding signals (activation of hepatic parasympathetic nerves and elevation of hepatic glutathione level), and causes insulin to release hepatic insulin sensitizing substance (HISS), which stimulates glucose uptake in peripheral tissues. The impairment of HISS release results in the absence of meal-induced insulin sensitization (AMIS), causing progression to a cluster of metabolic, vascular, and cardiac dysfunction, which we refer to as components of the AMIS syndrome. **Objectives**: The objective of my doctoral research was to study the manipulation of the HISS-pathway, in age- and diet-induced AMIS models, with exercise ± antioxidants. Also, in a separate project I studied the signaling pathways involved with the HISS action in skeletal muscle. **Methods**: The 7-day voluntary running was used as exercise intervention to manipulate the HISS pathway in healthy and prediabetic rats. The interaction of an antioxidant cocktail, SAMEC (S-adenosylmethionine + vitamin E + vitamin C), with the effects of exercise on postprandial insulin response was studied. Moreover, in the signaling studies the insulin and 5'-adenosine monophosphate activated protein kinase (AMPK) pathways were examined to test their possible involvement with the HISS action in skeletal muscle. **Results**: Voluntary running-wheel exercise for 7 days increases the postprandial glucose uptake response to insulin in health and in prediabetes through enhancement/restoration of HISS action. Supplementation with SAMEC during 7 days of exercise does not either harm or add benefits to the positive effects of exercise on insulin sensitivity. Finally, the signaling studies indicate that HISS increases the rate of glycogen synthesis in muscle through an insulin/AMPK-independent pathway.

SECTION I

INTRODUCTION

1. INTRODUCTION

1.1 Diabetes: from antiquity to the present

Diabetes mellitus has been known since ancient times. An early reference to diabetes was made by the Egyptians in 1500 BC, describing polyuria as a common symptom of the 'sugar disease' in the Ebers Papyrus, the oldest Egyptian medical book (King and Rubin, 2003). The term 'diabetes' has a Greek root meaning 'siphon' and a Latin root meaning 'honey', references to polyuria and glycosuria, two of its cardinal symptoms. In ancient India it was called 'madhu meha' or "sweet melody". Other names or malady dramatizing sad suffering of the disease were 'pissing evil', 'melting down of flesh and blood' etc. (Raju, 2006). The historical records described diabetes as a 'mysterious and savage disease', whereby patients complained not only of passing excessive amounts of urine, but also of very dry skin, being troubled with boils and carbuncles, having very little perspiration, dry bowel movements and basically wasting away. In fact "No one knew how to live with it, let alone correct it." (MacCracken and Hoel, 1997).

The strategies for diabetes management, based on its pathology, were not mentioned in medical literature until the 17th century (Bliss, 1982). In 1675, Thomas Willis (a British physician) tasted the urine of a patient with diabetes and found it to be sweet. For diabetes control he recommended a dietary management that mainly included meat, high-fat and high-protein diet and was low in carbohydrates (Raman, 2001; King and Rubin, 2003). Apart from the discovery of urine to be sweet, and with some

individuals having benefited from a low calorie diet, very little was known about the disease (King and Rubin, 2003).

In 1776, Dr. Matthew Dobson of Manchester crystallized sugar and discovered the presence of sugar not only in the urine but also in the blood of diabetic patients, realizing the condition to be a systemic disorder (Roszler, 2001). The primary school of thought for treatment/management of diabetes was limited to the dietary strategies, and indeed the intention was for the patients to withdraw from all types of food containing sugar and starch, which were to be replaced with meat and some green vegetables (King and Rubin, 2003). Beverage containing sugar was forbidden and it was also suggested that in order to alleviate the craving for liquid, all drinks should be taken warm (Gull, 1886). This close attention to diet based on the empirical findings at that time was deemed appropriate, in that it did offer some reduction in blood glucose levels but did little to relieve the suffering and extend life of the patients with diabetes (Suleyman, 1998).

Up to the 1800s, the pharmacological treatment often included opium given as compound pills three times a day and was considered to be of benefit in reducing the amount of urine passed (Bliss, 1982). Opium was used because ‘dope dulled the despair’ and made the sufferings tolerable (Raju, 2006). Phosphoric acid, bromide of potassium and nitrate of uranium were also thought to be useful in reducing urine and restoring general demeanors. Extract of ergot was used particularly when other treatments failed (King and Rubin, 2003). In this early time, the knowledge about diabetes and its

treatment were based on superstitions and ritualistic practices. Indeed, early writings indicated that the cause of diabetes mellitus could be attributed to either drinking cold water when one was hot, or drinking an excess amount of alcohol, or suffering from an intense amount of anxiety as a result of being a victim of act of crime, or a fit of anger, or as a direct result of a physical attack to the body (Gull, 1886).

One of the greatest discoveries in the history of diabetes was the discovery of the islet cells in the pancreas by a German medical student, Paul Langerhans, in 1869. He died in 1888 not having explained the cells and their significance (Pyke, 2001). Later, two fellow Germans, Joseph von Mering and Oskar Minkowski (von Mering and Minkowski, 1890) discovered that the removal of the pancreas from a dog led to the dog developing diabetes (Bliss, 1982; Pyke, 2001). In 1893, Gustave Laguesse, a French doctor, finally suggested that the islet cells in the pancreas were involved in a role other than secretion to aid digestion, and named them the islets of Langerhans after their discoverer (Hammonds, 2000).

Moses Barron, while undertaking the autopsy of a patient with diabetes, discovered by chance that the islets of Langerhans were damaged in that patient. Barron subsequently suggested (Barron, 1920) that a substance is released from islets preventing diabetes, and damage to the islets must be the cause of human diabetes (King and Rubin, 2003; Vivisection Information Network, http://www.vivisectioninformation.com/index.php?p=1_7_Diabetes-and-Insulin). In 1910, Sir Edward Albert Sharpey-Schafer, an English physiologist, named this substance ‘insuline’ named after the Latin word ‘insula’

meaning island (Gerritsen, 2001; Catania, 1995). Later, the anti-diabetic treatment became known as insulin (Brar, 2001). Fredrick Banting and Charles Best, in collaboration with John Macleod at the University of Toronto, using Barron's hypothesis, reproduced the works of previous scientists through isolating and extracting the substance, insulin, from healthy dog's pancreas and injecting it into diabetic dogs, thus at least temporarily controlling diabetes (Banting et. al., 1922a). Further, a biochemist, James Collip, managed to extract a reasonably pure form of insulin from the pancreas of cattle (Brar, 2001), and the extract was used by Banting and Best to treat a 14-year old diabetic patient, Leonard Thompson (Banting et. al., 1922b). Banting and Macleod received the Nobel Prize in 1923 in Physiology/Medicine for their work in the discovery of insulin, and they further shared their prize with Best and Collip (Lilly, 2002; King and Rubin, 2003).

The drug company Eli Lilly offered assistance for the purification of insulin and was able to produce large quantities of highly refined insulin. Insulin was offered for sale shortly thereafter. Purified animal-sourced insulin was the only type of insulin available to diabetics until the genetic breakthroughs occurred later in medical research. The amino acid sequence of the two polypeptide chains of insulin was characterized in 1951 by Frederick Sanger (Sanger and Tuppy, 1951; Sanger, 1959). In 1959, Solomon Berson and Rosalyn Yalow developed a radioimmunoassay technique to measure the insulin level in blood, and identified diabetes of two major categories, type 1 diabetes (insulin dependent) and type 2 diabetes (non-insulin dependent diabetes) (Patlak, 2002). The first genetically-engineered synthetic human insulin was produced in a laboratory in 1977 by

Herbert Boyer using E. Coli (Genentech, 1978; Tof, 1994). Eli Lilly went on in 1982 to sell the first commercially available biosynthetic human insulin under the brand name Humalin. Most of the insulin currently used worldwide is now biosynthetic recombinant human insulin or its analog.

Over the last few decades our knowledge of diabetes has advanced significantly through the development of various technologies for diagnosis of the disease, and the establishment of various guidelines and interventions for diabetes management. Diabetes is a chronic and progressive disease which is difficult to cure. As diabetes is a prime risk factor for cardiovascular, renal, eye, and neurological complications, controlling other risk factors which may give rise to secondary conditions, as well as diabetes itself, is one of the facets of diabetes management. While hyperglycemia is an important prognostic parameter, other biological markers have been introduced as the basis of diagnosis and treatment of diabetes and its associated complications. Diabetes management concentrates on keeping blood glucose level as close to normal (euglycemia) as possible with various interventions including dietary management, exercise, and medications. Though there is a significant advancement in diabetes interventions, the global burden of diabetes is anticipated to be increased significantly over next decades as a consequence of population aging and urbanization (Wild et. al. 2004). The next section covers some statistics of diabetes and diabetes-attributable mortality in global and regional perspective, and may help to shed light on the progressive crisis and considerable health burden of diabetes.

1.2 Diabetes fact-sheet: present context to future projection and the Canadian perspective

The global prevalence of diabetes is increasing due to population growth, aging, urbanization, and increase in obesity and physical inactivity. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and projected to be 4.4% in 2030. The total number of people with diabetes is estimated to be increased from 171 million in 2000 to 366 million in 2030 (Wild et. al. 2004). The prevalence of diabetes is higher in men than women. The urban population in developing countries is projected to double between 2000 and 2030. The most important demographic change to diabetes prevalence across the world appears to be due to the increase in proportion of people >65 years of age (Fig. 1). The diabetes epidemic will continue even if levels of obesity remain constant. Given the increase in obesity, it is likely that these figures provide an underestimate of future diabetes prevalence (Fig. 2) (Wild et. al. 2004).

The burden of mortality attributable to diabetes is significant. Unmanaged diabetes can progress to multiple complications and lead to premature mortality, accounting for at least 10% of total health care expenditure in many countries (Diabetes Atlas, 2003). However, routinely reported statistics based on death certificates seriously underestimate mortality from diabetes (Fuller et. al., 1983; Roglic et. al. 2005), because individuals with diabetes most often die of cardiovascular and renal diseases and not from a cause uniquely related to diabetes (Morrish et. al. 2001). The proportion of undiagnosed diabetes is considerable and is often higher than 50% (World Health Organization, 2003). The application of complex methods to estimate cause-specific

mortality attributable to diabetes speculates that diabetes related mortality is likely to be considerably higher than previous global estimates based on death certificates. This moves diabetes from the 8th to the 5th place in cause of death ranking, after communicable diseases, cardiovascular disease, cancer, and injuries (Roglic et. al. 2005). The global mortality attributable to diabetes in the year 2000 was estimated to be 2.9 million deaths, equivalent to 5.2% of all deaths. Mortality attributable to diabetes accounted for 2-3% of deaths in poorest countries and over 8% in the US, Canada, and the Middle East. In people 35-64 years old, 6-27% of deaths were attributed to diabetes (Roglic et. al. 2005).

Diabetes is a chronic, often debilitating disease that imposes immense financial burden to the individual and the country. According to the current statistics of the Canadian Diabetes Association, more than 9 million Canadians live with diabetes and prediabetes. The personal costs of diabetes may include a reduced quality of life and the increased likelihood of complications such as heart disease, stroke, kidney disease, blindness, amputation and erectile dysfunction. Approximately 80% of people with diabetes will die as a result of heart disease or stroke. Diabetes is a contributing factor in the deaths of approximately 41,500 Canadians each year. People with diabetes incur medical costs that are two to three times higher than those without diabetes. A person with diabetes can face direct costs for medication and supplies ranging from $1,000 to $15,000 a year (Canadian Diabetes Association, The prevalence and costs of diabetes, Link: http://www.diabetes.ca/diabetes-and-you/what/prevalence).

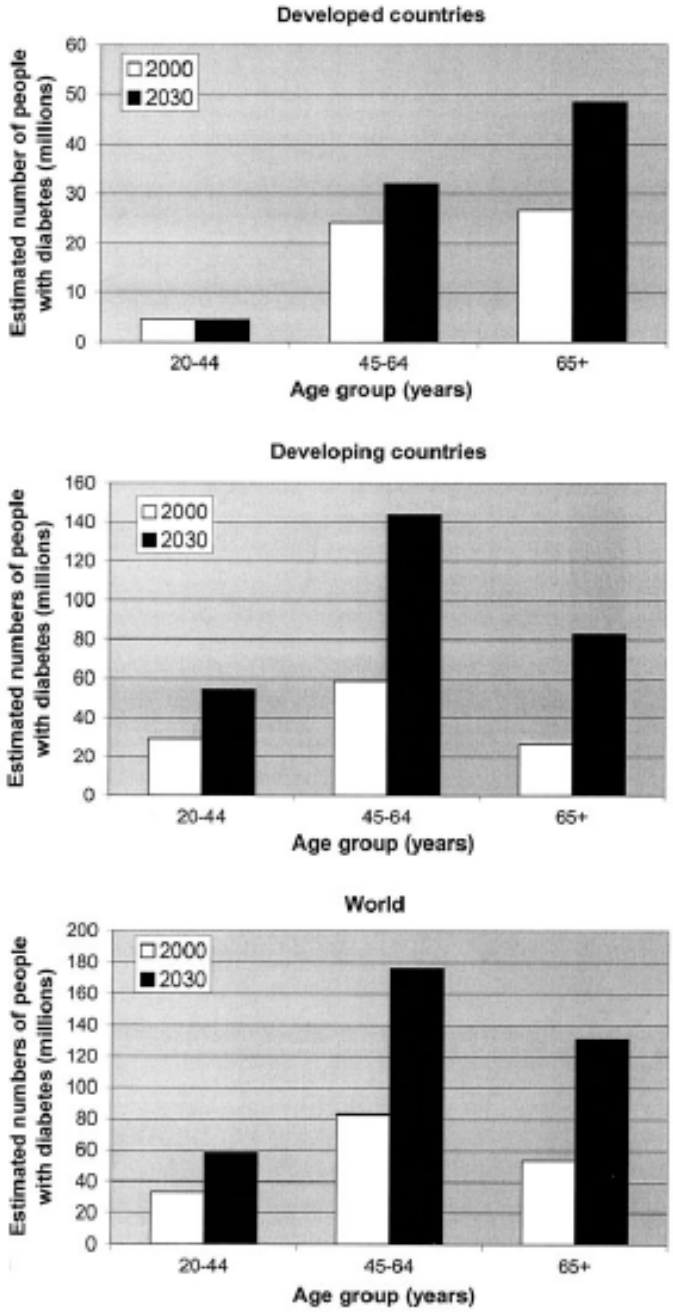


Fig. 1: The estimated prevalence of diabetes in 2000 and its projection to 2030 in different age groups in the developed and developing countries. [*Adapted from Wild et. al., (2004), Diabetes Care, Vol. 27, 2004; 1047-1053, Reprinted with permission from the American Diabetes Association*]

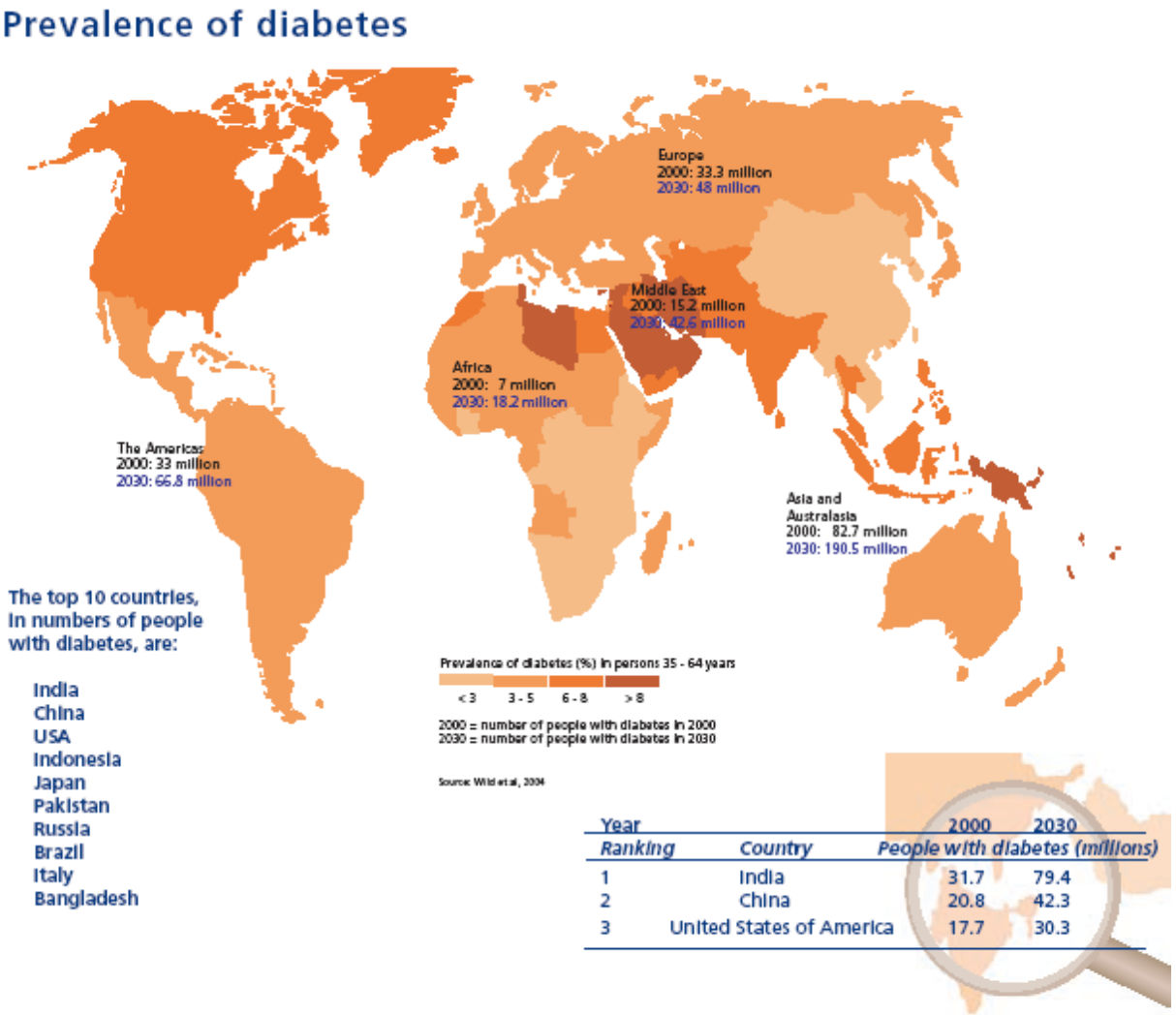


Fig. 2: The regional and global prevalence of diabetes in 2000 and its projection to 2030. [*Adapted from Wild et. al., (2004), Diabetes Care, Vol. 27, 2004; 1047-1053, Reprinted with permission from the American Diabetes Association*]

According to the Public Health Agency of Canada, the economic burden of diabetes alone is estimated at $1.6 billion in 1998; $0.4 billion (25%) in direct costs and $1.2 billion (75%) in indirect costs. Direct costs are defined as the value of goods and services for which payment was made and resources used in treatment, care and rehabilitation by governments (federal, provincial and territorial) as well as by individual Canadians. The direct cost estimate for diabetes includes only hospital care and drug expenditures, at $203.5 million and $181.0 million, respectively.

Indirect costs refer to the dollar value of lost production due to illness, injury, disability or premature death. In terms of the principal indirect cost components that are estimated for diabetes in 1998, the value of lost production due to premature mortality represents the largest indirect cost at $732.8 million. The morbidity costs due to long-term disability represent $529.1 million (Fig. 3) (Public Health Agency of Canada, Economic burden of diabetes in Canada, http://www.phac-aspc.gc.ca/publicat/dic-dac2/english/45chap5-eng.php). By 2020, it is estimated that diabetes will cost the Canadian healthcare system approximately $16.9 billion a year (Canadian Diabetes Association, The prevalence and costs of diabetes, http://www.diabetes.ca/diabetes-and-you/what/prevalence).

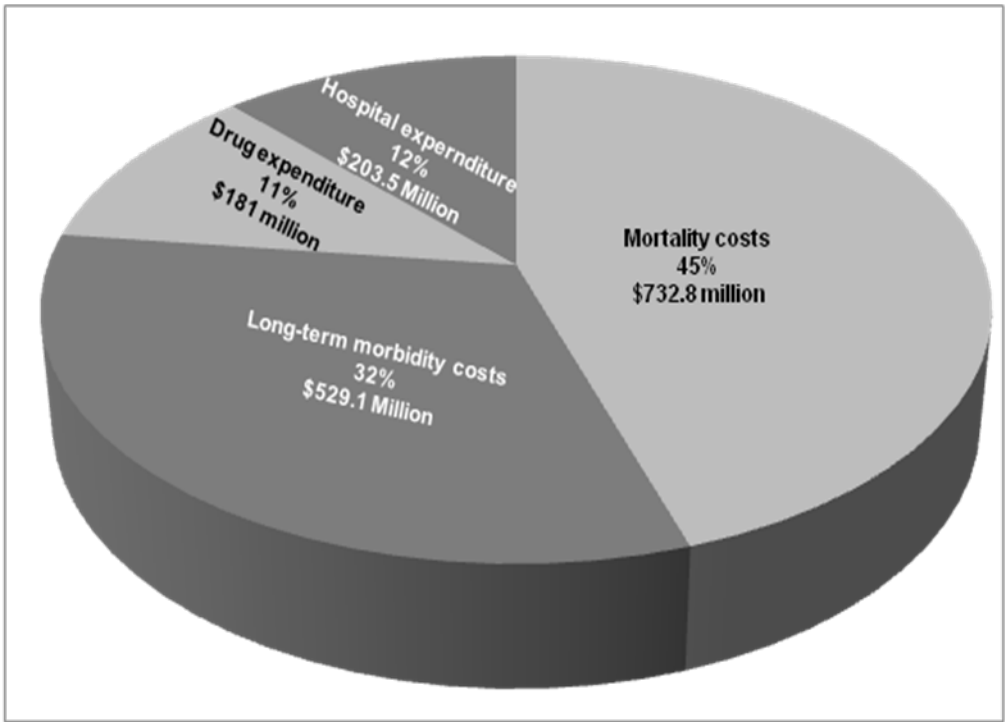


Fig. 3: Economic burden of diabetes in Canada by cost component, 1998: The relative magnitude of the major cost components of diabetes in Canada. [*Adapted from Public Health Agency of Canada, Economic burden of diabetes in Canada, http://www.phac-aspc.gc.ca/publicat/dic-dac2/english/45chap5-eng.php*]

1.3 Categories and diagnosis of diabetes and intermediate hyperglycemia

Diabetes is characterized by abnormally high plasma glucose levels (Rendell and Jovanovic, 2006). In current descriptions diabetes is referred to as more than a single disease, rather it is described as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The long-term hyperglycemia leads to dysfunction and damage of multiple organs including eyes, kidney, heart, blood vessels and nerves (The American Diabetes Association, 1997). The World Health Organization (WHO) has been reviewing the guidelines for diagnosis and classification of diabetes since 1965. According to the latest recommendation by WHO and International Diabetes Federation (IDF), the criteria for diabetes should be a maintained fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or 2-hour postprandial plasma glucose ≥ 11.1 mmol/l (200 mg/dl) (World Health Organization, Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia, http://www.idf.org/webdata/docs/WHO_IDF_definition_diagnosis_of_diabetes.pdf).

There are three main types of diabetes. Type 1 diabetes, usually diagnosed in children and adolescents, occurs when the pancreas is unable to produce insulin. Insulin is a hormone that controls the amount of glucose in the blood. Approximately 10 per cent of people with diabetes have type 1 diabetes. The remaining 90 per cent have type 2 diabetes, which occurs when the pancreas does not produce enough insulin or when the body does not effectively use the insulin that is produced. Type 2 diabetes usually develops in adulthood, although increasing numbers of children in high-risk populations

are being diagnosed. A third type of diabetes, gestational diabetes, is a temporary condition that occurs during pregnancy. It affects approximately 2 to 4 per cent of all pregnancies (in the non-Aboriginal population) and involves an increased risk of developing diabetes in future for both mother and child (Canadian Diabetes Association, Gestational diabetes: preventing complications in pregnancy, http://www.diabetes.ca/diabetes-and-you/what/gestational).

Detecting individuals at risk of future diseases and implementing programs to reduce risk of progression to the disease is a fundamental objective of reducing the burden of diabetes. High-risk states for future development of diabetes have been officially recognized for many years and were given different names. In 1965, one of the first formal definitions for intermediate hyperglycemia was proposed by WHO, which recommended the term 'borderline diabetes' (WHO, http://www.idf.org/webdata/docs/WHO_IDF_definition_diagnosis_of_diabetes.pdf; Colagiuri, 2011). At that time prediabetes was considered a retrospective diagnosis and was applied to people with normal glucose tolerance that later developed diabetes. Over time the term prediabetes has evolved, and different definitions are available to refer to prediabetes (Colagiuri, 2011). According to the American Diabetes Association, three different tests can be utilized to identify prediabetes. Those are fasting plasma glucose test (FPG), oral glucose tolerance test (OGTT), and glycosylated hemoglobin test (HbA_1C). The criteria and tests for diagnosis of diabetes and intermediate hyperglycemia are presented in Table 1.

TABLE 1: American Diabetes Association Risk Test for Diabetes: to determine if there is an increased risk for diabetes or prediabetes. A high score may indicate that there is a greater risk of predisposition to diabetes and associated complications. [*Adapted from: American Diabetes Association, http://www.diabetes.org/diabetes-basics/prevention/pre-diabetes/how-to-tell-if-you-have.html*]

	FPG	**OGTT**	**HbA_1C**
Normal	<100 mg/dl	<140 mg/dl	<5.7%
Prediabetes	100-126 mg/dl	140-200 mg/dl	5.7-6.4%
Diabetes	≥126 mg/dl	≥200 mg/dl	≥6.5%

The intermediate state between normal blood glucose and diabetes is termed as intermediate hyperglycemia or prediabetes. This stage is characterized by impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). IFG is considered when the fasting glucose level is higher than 100 mg/dl but lower than 126 mg/dl. IGT is considered when blood glucose is measured 2h after an OGTT and is higher than 140 mg/dl but lower than 200 mg/dl (Ryden et. al., 2007). Sometimes patients show both IFG and IGT, and it can progress more rapidly towards type 2 diabetes (de Vegt et. al., 2001; Meigs et. al., 2003; O'Rahilly et. al., 1994).

There is a high correlation between fasting plasma glucose (FPG) levels and the magnitude of postmeal glucose excursions (Erlinger and Brancati, 2001). Measurement of plasma glucose level 2h after oral administration of a standard amount of glucose, typically 75 g as oral glucose tolerance test (OGTT), has been widely used to measure postprandial glucose response and body insulin sensitivity. Certainly, the response to pure glucose does not adequately relect the effects of protein and fat ingestion during a typical meal (Rendell and Jovanovic, 2006). However, there has been no generally accepted standardization of a characteristic meal used to assess glucose response, so most studies on postprandial glucose rely on OGTT. Individuals with IGT manifest abnormalities in both insulin action and early insulin secretion similar to those seen in patients with type 2 diabetes (Pratley and Weyer, 2002; Abrahamson, 2004).

It is estimated that approximately 344 million people worldwide, or 7.9% in the age group of 20 to 79 years, have impaired glucose tolerance (IGT) (Diabetes Atlas, 2009, Link; http://www.diabetesatlas.org; Colagiuri, 2011). There are 79 million people in the United States who have prediabetes. Recent research has shown that some long-term damage to the body, especially to the heart and circulatory system, may start occurring during prediabetes (The American Diabetes Association, Prediabetes, http://www.diabetes.org/diabetes-basics/prevention/pre-diabetes/how-to-tell-if-you-have.html).

1.4 Diabetes Complications

The metabolic dysregulation associated with diabetes causes changes in multiple organs leading to morbidity and mortality, and imposing remarkable burden on the patient and on the healthcare system (Pectasides and Kalva, 2011). Diabetes mellitus is a leading cause of cardiovascular disease, end-stage renal disease, neuropathy, retinopathy, and limb amputations. The prevalence of diabetes is similar in men and women throughout most age ranges, but is slightly greater in men over 60 years (Powers, 2008; Pectasides and Kalva, 2011). In the female population, patients with diabetes mellitus lose the cardioprotective effects of the female sex and have rates of coronary heart disease identical to those of men. In patients with diabetes mellitus, women have a higher risk for myocardial infarction than men (Huxley et. al., 2006).

1.4.1 Diabetes and Vascular Diseases:

Diabetes mellitus increases the cardiovascular death rate by two folds in men and four folds in women (Powers, 2008; Pectasides and Kalva, 2011). Cardiovascular disease accounts for approximately 70% of total mortality. All forms of vascular diseases, including cardiovascular disease, coronary artery disease, stroke and peripheral vascular disease, are substantially higher in patients with type 2 diabetes (Pyörälä et. al., 1987; Laakso and Lehto, 1997; Haffner et. al., 1998). Cardiovascular complications may be present even at the time of diagnosis of type 2 diabetes, and in fact subjects with IGT (i.e. intermediate hyperglycemia) have about a twofold increase in the risk of macrovascular diseases (Pyörälä et. al., 1987; Laakso and Lehto, 1997). Hyperglycemia increases the risk of cardiovascular consequences, and surprisingly the excess risk starts at levels of glycemia that are considered lower than the threshold in current diagnostic criteria (Laakso, 1999). Hyperglycemia induces several harmful effects that could potentially contribute to atherothromobotic events. High blood glucose can cause increased oxidative stress (Baynes, 1991), enhanced leukocyte-endothelial interaction (Morigi et. al., 1998) and glycosylation of lipoproteins and clotting factor (Vlassara, 1997). The precipitation of these complex events leads to an increased risk of progression to cardiovascular pathologies in type 2 diabetes.

The risk of atherosclerosis is increased in diabetic patients and this pathology occurs earlier and progresses more severely in the diabetic population. Diabetes predisposes to the risk for carotid artery stenosis, and the prevalence of cerebrovascular disease and stroke is increased by 3 fold in patients with diabetes mellitus (Powers, 2008;

Laing et. al., 2003). Diabetic patients more commonly develop symptomatic peripheral arterial disease. Diabetes increases the risk of intermittent claudication by 3.5 fold in men and 8.6 fold in women (Kannel and McGee, 1985), and is an independent predictor of vessel wall calcifications in patients with peripheral arterial disease (Ouwendijk et. al., 2006).

1.4.2 Diabetes and Nephropathy:

Diabetes mellitus increases the risk of development of renal diseases. Diabetic nephropathy is a progressive kidney disease and the most common cause of end-stage renal disease (ESRD) (McGill, 2009), which is a leading cause of morbidity and mortality in diabetic patients (Powers, 2008). Diabetic nephropathy is characterized by proteinuria (>200 µg/min or >300 mg/day of urinary albumin excretion), diminished glomerular filtration rate (GFR), and elevated renal arterial blood pressure (McGill, 2009; Krentz et. al., 2007; American Diabetes Association, 2004). Lifetime risk for development of diabetic nephropathy with progression to ESRD is roughly equivalent in type 1 and type 2 diabetes (Ritz and Orth, 1999). Approximately 30% of patients with either type 1 or type 2 diabetes develop diabetic nephropathy (Dalla Vestra et. al., 2000; Choudhury et. al., 2010). While diabetic nephropathy progresses to ESRD, it also imposes significant risk for the development of cardiovascular diseases and associated mortalities (National Institute of Diabetes and Digestive and Kidney Diseases, 2010). Diabetes mellitus also has strong association with renal papillary necrosis, with up to 24% patients with renal papillary necrosis being diabetic patients (Groop et. al., 1989).

Diabetic patients are also predisposed to contrast-induced nephrotoxicity (Gleeson and Bulugahapitiya, 2004).

1.4.3 Diabetes and Retinopathy:

Damage to the eyes is one of the major microvascular complications of diabetes mellitus. In the United States, diabetic retinopathy is the third leading cause among all cases of blindness. The percentage of cases of blindness attributed to age-related macular degeneration, glaucoma, and diabetic retinopathy is 50%, 18%, and 17% respectively (Resnikoff et. al., 2004). Approximately 60% of patients with type 2 diabetes and almost all patients with type 1 diabetes develop retinopathy during the first 20 years of the disease (Fong et. al., 2004; Morello, 2007). Approximately 40% of adults 40 years of age and older with diabetes have retinopathy, and the condition is vision-threatening in 20% of this patient population (Kempen et. al., 2004). Diabetic patients aged 50 or older have twice the risk of vision impairment as people of the same age without diabetes (Centers for Disease Control and Prevention, 2004).

There are three mechanisms by which diabetic retinopathy causes blindness: macular ischemia, retinal and vitreous hemorrhage, and retinal detachment (Rosenblatt and Benson, 2004). However, the primary mechanism of vision loss is centrally involved diabetic macular edema (DME), characterized by vascular leakage and subsequent edema that affects the center of the macula, and it can occur at any level of diabetic retinopathy (Davidson et. al., 2007). Diabetic retinopathy is divided into 2 categories: nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy

(PDR) (Fong et. al., 2004; Wilkinson et. al., 2003). NPDR produces increased capillary permeability, hemorrhage, and DME. PDR causes neovasculization on the vitreous surface of the retina, in the vitreous cavity, and on the iris (McGill, 2009). Vision loss occurs as a consequence of the progression of NPDR to PDR, or the development of DME at any stage of retinopathy (Davidson et. al., 2007).

1.4.4 Diabetes and Neuropathy:

The microvascular complications of diabetes mellitus lead to nerve damage that accounts for roughly 60-70% of the diabetic population, and it can lead to ulcer formation and non-traumatic limb amputations (Centers for Disease Control and Prevention, 2005; Morello, 2007). Diabetic peripheral neuropathy causes pain, tingling, numbness, or impaired sensation in the feet (Morello, 2007), and may lead to neuropathic arthropathy with subsequent deformity, and impaired immunity in healing lower extremity wounds or injury (Pinzur, 2011). Foot ulcers, infections, and deformity are the major causes of morbidity and mortality, and 15% of individuals with diabetes develop foot ulcers in their lifetime. Foot ulcers may lead to lower extremity amputations in 85% diabetic patients (Reiber et. al., 1999; Apelqvist and Agardh, 1992; Diabetes Control and Complications Trial Research Group, 1993; McNeeley et. al., 1995; Rith-Najarian et. al., 1992; Veves et. al., 1994; Ramsey et. al., 1999). After amputation, the 2-year mortality was reported to be as high as 36% (Moulik et. al., 2003; Boyko et. al., 1996; Pinzur et. al., 1993; Reiber et. al., 1998). These patients are at greater risk for premature death, even if they do not undergo an amputation (Moulik et. al., 2003; Robbins et. al., 2008).

The mechanism behind the development of peripheral neuropathy in diabetes is not clear. It is suggested that microvascular disease process within peripheral nerves is responsible for the peripheral nerve dysfunction (Tesfaye et. al., 1992; Ram et. al., 1991). There might be both vascular and metabolic origins for the genesis of the pathology. It has been suggested that accumulation of complex metabolic and vascular pathologies, including advanced glycation of arterial vessel walls, basement membrane thickening, multifocal ischemic proximal nerve lesions, epineural vessel atherosclerosis, increase ROS generation, reduced endothelial nitric oxide activity, nerve hypoxia, sorbitol accumulation, and various enzymes deficiency, lead to the development of peripheral neuropathy in diabetic patients (Pinzur, 2011).

1.4.5 Diabetes and Pregnancy Outcomes:

Diabetes during pregnancy or gestational diabetes mellitus (GDM) is defined as glucose intolerance of variable severity with onset or first recognition during pregnancy (Langer, 2006). In developed countries there is an epidemic increase in GDM, which might cause progression to obesity and type 2 diabetes in mother and the child. In the United States, GDM affects about 4% of all pregnant women, and approximately 135,000 cases are reported each year (American Diabetes Association, GDM)). In Canada, GDM is higher than previously thought, varying from 3.7% in non-Aboriginal women to 8-18% in Aboriginal women (Canadian Diabetes Association, Gestation diabetes, http://www.diabetes.ca/diabetes-and-you/what/gestational). In Manitoba, the prevalence of GDM has increased significantly during a 20-year period. The incidence of gestational diabetes was 2.3% in 1985-1989, and 3.7% in 1999-2004 (Aljohani et. al., 2008a).

Macrosomia (excessive body weight of newborn) was detected in 15.3% of newborns in Manitoba, and the incidence was higher in pregnant women with GDM than in those without GDM (Aljohani et. al., 2008b).

Pregnancy is normally attended by progressive insulin resistance that begins near mid-pregnancy, and progresses through the third trimester to a level seen in clinical type 2 diabetes (Buchanan and Xiang, 2005). Insulin resistance is a normal phenomenon during pregnancy, and is thought to secure glucose supply to the growing fetus. In the greater majority of pregnancies, the demand is readily met, and the balance between the insulin resistance and insulin supply is maintained (Ben-Haroush et. al., 2004). In GDM more insulin is needed to overcome the resistance, and about 1.5-2.5 times more insulin is produced than that in a normal pregnancy (Carr and Gabbe, 1998). Placental hormones, and to a lesser extent increased fat deposition during pregnancy, seem to mediate this insulin resistance. The unmodifiable risk factors associated with insulin resistance during pregnancy include age, genetic background, ethnicity, and number of previous pregnancies. The modifiable known risk factors are obesity, lack of exercise, dietary fat, and lifestyle habits that adversely influence insulin resistance; such as smoking and certain drugs (Bo et. al., 2001).

During pregnancy, because of insulin resistance, the maternal blood glucose level is increased and it is diffused across the placenta. So, the fetus is exposed to a high glucose concentration in the placental fluid. Since insulin itself cannot cross the placental barrier, the higher glucose level leads to an increase in fetal insulin concentration. The

growth-stimulating effects of insulin might lead to excessive growth and a large body weight in the newborn (macrosomia) (Kelly et. al., 2005). Fetal hyperglycemia may also lead to traumatic delivery, hypocalcemia, hyperbilirubinemia, and polycythemia. Maternal hyperglycemia is often associated with an increase in perinatal mortality and congenital anomalies (British Columbia Reproductive Care Program: Gestational diabetes, http://www.perinatalservicesbc.ca/sites/bcrcp/files/Guidelines/Obstetrics/MasterDibetesMellitusOctober.pdf.). GDM is also linked to maternal obesity, and future diabetes in mothers and their offspring. Approximately 50% women diagnosed with GDM have chance of developing type 2 diabetes within 5 years of delivery (Kjos, 2000).

1.4.6 Diabetes and Cancer:

In individuals with type 2 diabetes, there is a higher prevalence of some forms of cancers including cancers of the breast (Larsson et. al., 2007), colorectum (Larsson et. al., 2005), endometrium (Friberg et. al., 2007), liver (El-Serag et. al., 2006), and pancreas (Huxley et. al., 2005). The prevalence of diabetes in newly diagnosed cancer patients is high ranging from 8% to 18% (Ko and Chaudhry, 2002). Moreover, patients diagnosed with cancer who have preexisting diabetes are at increased risk for long-term, all-cause mortality compared with those without diabetes (Barone et. al., 2008). This may be partly because cancer patients with diabetes might receive less aggressive cancer treatment or less aggressive diabetes care, both of which could compromise survival (Richardson and Pollack, 2005).

The entanglement of the common risk factors in diabetes and cancer may provide a possible explanation of pathological links between these two common diseases. Diabetes and cancer share several common nonmodifiable risk factors including age, sex, race, and ethnicity. The modifiable risk factors include overweight, obesity, diet, physical inactivity, tobacco, and alcohol consumption (Mitka, 2010). It is hard to speculate clearly how the risk factors would trigger specific metabolic derangements that lead to insulin resistance and increased risk of certain types of cancers. However, there are many proposed hypotheses that might explain the links between diabetes and cancer. Cancer cells depend on glycolysis for their energy metabolism and high level of blood glucose in diabetes, therefore, may foster the proliferation of the neoplastic cell. Hyperinsulinemia in diabetes might provide increased growth and progression of breast cancer through growth promoting functions of insulin. Increased circulating insulin can also displace sex hormones (especially estrogen) from sex-hormone binding globulin, leading to a higher level of bioavailable estrogen that increases the risk of postmenopausal breast and endometrial cancers. Increased adiposity and chronic inflammation of adipose tissue in diabetes can cause elevation of inflammatory markers that could influence the regulation of malignant transformation or cancer progression. Additionally, it has been suggested that chronic treatment of diabetic patients with exogenous insulin, particularly insulin glargine, might promote the risk of cancer (Mitka, 2010).

1.4.7 Diabetes and Erectile Dysfunction:

Diabetes and obesity independently increase the risk of erectile dysfunction (ED). More than 10 million men in the United States are affected by ED, with an estimated 100 million men affected worldwide (NIH Consensus Development Panel on Impotence, 1993; Zusman et. al., 1999). In men over the age of 50 years, increasing duration of diabetes is positively associated with increased risk of ED relative to nondiabetic subjects (Bacon et. al., 2002). Type 1 diabetic patients are more likely than type 2 diabetes to have ED (Fedele et. al., 1998; Fedele et. al., 2000; Romeo et. al., 2000; Bacon et. al., 2002). The risk of ED is approximately 2-fold greater in men with type 2 diabetes compared with men without diabetes. In type 1 diabetes the risk of ED is nearly 3 times higher than that in men without diabetes (Bacon et. al., 2002). Moreover, ED shares same etiology and pathology of vascular dysfunction with cardiovascular diseases, and the degree of erectile dysfunction correlates with the severity of cardiovascular diseases (Thompson et. al., 2005). Patients with cardiovascular disease frequently develop ED (Montorsi et. al., 2003), and the risk factors for both diseases include diabetes, obesity, tobacco use, physical inactivity, hypertension, and hyperlipidemia (Ponholzer et. al., 2005). There is a 2-fold greater risk of cardiovascular disease in men with ED than men without ED (Thompson et. al., 2005), and approximately 75% increased risk of peripheral vascular disease with preexisting erectile dysfunction (Blumentals et. al., 2003).

There are several potential mechanisms that can describe the higher prevalence of ED in the diabetic population. Microangiopathy of the cavernosal artery, corporal veno-occlusive dysfunction, and autonomic neuropathy are the primary pathological pathways

for ED (Hakim and Goldstein, 1996). Advanced glycation end products, as a result of insufficient glycemic control in diabetes, are elevated in the collagen of the penile tunica and corpus cavernosum of diabetic penile tissue and inhibit nitric oxide production (Seftel et. a., 1997). Smooth muscle tone and relaxation may be disturbed by reduced nitric oxide action (Burnett, 1997). The dysfunction in nitric oxide production and its action in the penile vasculature in diabetic patient may be associated with a higher prevalence of ED in these patients.

1.5 Management and treatment of diabetes

Type 2 diabetes is a progressive disease that proceeds with a period of insulin resistance and IGT (Nyenwe et. al., 2011). The conversion from IGT to type 2 diabetes may take 9 to 12 years, unless there are lifestyle modifications and other therapies that may reduce the risk (Nyenwe et. al., 2011; Bergenstal et. al., 2001). The insulin resistance in muscle, adipose tissue, and liver causes gluco- and lipo-toxicity that lead to beta cell dysfunction characteristic of the advancement to type 2 diabetes (DeFronzo, 2009). The treatment and management plan for type 2 diabetes comprise the combined approach of various non-pharmacological and pharmacological strategies. The non-pharmacological managements for prevention and treatment of type 2 diabetes target the modifiable risk factors including body weight or BMI, central adiposity, diet profile, and sedentary life style.

Although the nutritional compositions in dietary strategy of diabetes management remain a major subject of interest, it has been suggested that dietary measures are

effective in weight reduction irrespective of the composition, provided that there is adequate energy restriction, reduction in saturated fat to less than 7% and adequate provision of dietary fiber (Foster et. al., 2003; Stern et. al., 2004). Low-carbohydrate and low-fat diets are both effective to cause weight loss, however the effect on lipid profile management may differ. Low-carbohydrate diet may yield a greater reduction in triglyceride with higher improvement in HDL. Low-fat diet causes higher reduction in LDL compared to that achieved with low-carbohydrate diet (Nordmann et. al., 2006). Lower consumption of total and saturated fat and processed foods, and higher consumption of fibers, whole grains, fruits, and vegetables have been shown to improve glycemic control in patients with diabetes (Nyenwe et. al., 2011).

Sedentary lifestyle is one of the most important modifiable risk factors behind the development of insulin resistance and its progression to type 2 diabetes, and physical activity contributes strong metabolic benefits in the prevention and management of the disease (Look AHEAD Research Group, 2007; Knowler et. al., 2002; Pan et. al., 1997). Improvement in peripheral insulin sensitivity contributes to the clinical efficacy of physical exercise in diabetes (Kirwan et. al., 2009; Praet and van Loon, 2009). The inverse relationship between physical activity and type 2 diabetes was quantified (Helmrich et al., 1991; Venables and Jeukendrup, 2009), demonstrating that for every increase in leisure time energy expenditure of 500 kcal the age-adjusted risk for developing type 2 diabetes is decreased by 6%. It is also shown that one week of vigorous exercise program in patients with insulin resistance resulted in improvements in insulin action in the absence of weight loss (Kirwan et al., 2009). The gluco-regulatory

benefits of prolonged exercise may result from the structural adaptive responses, including increased mitochondrial enzyme activity and oxygen uptake capacity in skeletal muscle (Mandroukas et. al., 1986; Henriksson, 1992).

The choice of pharmacotherapy for the treatment of type 2 diabetes is primarily aimed to obtain glycemic control and to manage obesity, which is guided by multiple factors including medical needs of the patients, treatment goals, potency of the pharmacological agent(s), tolerability, side effect profile, mode of administration, dose compliance, cost effectiveness etc. (Nyenwe et. al., 2011). Since obesity is the strongest modifiable risk factor for type 2 diabetes, measures directed at weight reduction provide metabolic benefits in obese diabetic patients. Surgical interventions for gastric reduction (i.e. bariatric surgery) include gastric banding and gastric bypass surgery that are effective in weight reduction and significant improvement in glycemic control. Patients undergoing bariatric surgery still need to consider life-style management for adequate control of diabetes. Surgery remains as the last resort for the patients who have BMI > 40 kg/m^2 or >35 kg/m^2 plus diabetes, and in whom diabetic control cannot be achieved with life-style management and pharmacotherapy alone (American Diabetes Association, 2010; Nyenwe et. al., 2011). The pharmacotherapy for weight management includes appetite suppressants (e.g. sibutramine), lipid-absorption inhibitors (e.g. orlistat), glucagon-like peptide-1 (GLP-1) analogs (e.g. exanetide), dipeptidyl peptidase-4 inhibitors (DPP-4, responsible for degradation of GLP-1) (e.g. sitagliptin) and cannabinoid receptor blockers (e.g. rimonabant). Sibutramine is a serotonin-norepinephrine reuptake inhibitor (SNRI) that induces satiety and prevents a diet-induced

decline in metabolic rate. However, it can elevate heart rate and blood pressure, and patients may regain weight rapidly after discontinuation of therapy (James et. al., 2000). Orlistat is a lipase inhibitor that reduces dietary fat absorption in the intestine, and causes significant weight loss in obese and diabetic patients (Torgerson et. al., 2004). Exanetide and sitagliptin are also effective for weight loss of 3.0 and 1.1 kg respectively, compared to a weight gain of 0.6 kg with insulin in type 2 diabetic patients (Horton et. al., 2010). The cannabinoid receptor blocker, rimonabant, decreases appetite and has been shown to reduce weight gain and HbA_{1C} and improve lipid profile, but affects mood and increases suicidal ideation (Nyenwe et. al., 2011).

The glycemic management in type 2 diabetes is usually done with insulin, and various hypoglycemic (e.g. sulfonylureas) and antihyperglycemic (e.g. metformin) agents. Patients with type 2 diabetes primarily develop peripheral insulin resistance that progressively leads to pancreatic beta-cell failure. This in turn results in deficient insulin secretion and consequent hyperglycemia and elevated free fatty acid level. The resulting glucotoxicity and lipotoxicity initiate a vicious cycle that further compromises the ability of beta cells to secrete insulin in response to oral hypoglycemic agents, particularly insulin secretagogues (e.g. sulfonylureas). This decline in beta-cell function in type 2 diabetes might cause therapeutic failure of certain classes of oral hypoglycemic agents over time (Klein et. al., 2004; Gerstein et. al., 2006). The major classes of oral agents that are used to manage hyperglycemia in diabetes include α-glucosidase inhibitors (e.g. acarbose), Biguanides (e.g. metformin), Thiazolidinediones (e.g. rosiglitazone, pioglitazone), incretins (e.g. exenatide), sulfonylureas (e.g. glimerpiride, glipizide) and

meglitinides (e.g. repaglinide) (Nyenwe et. al., 2011). The antihyperglycemic efficiency of various therapeutic agents is usually determined by measuring the degree of reduction in HbA_{1C} level following the course of treatment. As monotherapy, most antidiabetic agents are able to reduce HbA_{1C} level by 0.5-2.0%, except insulin which can reduce HbA_{1C} by more than 3% (Nathan et. al., 2009). Therefore, it is unlikely that any single agent will achieve the glycemic target in a patient with HbA_{1C} >8.5%. However, some drug combinations appear to be synergistic and can reduce the HbA_{1C} level by up to 3.5% (Nyenwe et. al., 2011).

1.6 Glucoregulation and cellular energy homeostasis: role of insulin and AMPK in normal vs. energy deprived (or high demand) state

The physiological mechanisms for the energy metabolism of cells in resting and high demand state (e.g. exercise) are regulated through complex neuronal, hormonal and chemical interactions. The dynamic regulatory controls of cellular energy balance assist to overcome the challenge between the high and deprived energy states. The whole-body and cellular glucoregulation involves various pathways which enable the cells to utilize carbohydrates, lipids, and proteins, and to store them for future energy availability. Though numerous mechanisms have been proposed acting independently and/or interdependently in maintaining glucose homeostasis, this section details the well-established cellular pathways where insulin and AMP-activated protein kinase (AMPK) play the major roles in facilitative glucose uptake with the aid of various glucose transporters.

1.6.1 Facilitated Glucose Transport:

Glucose enters into the cell by facilitated diffusion. Facilitative glucose transport is mediated by members of the Glut protein family that belongs to a superfamily of 12-transmembrane-spanning proteins with intracellular located amino- and carboxyl-termini (Wood and Trayhurn, 2003). These proteins are expressed in a tissue- and cell-specific manner and exhibit distinct kinetic and regulatory properties that reflect their specific functional roles (Mueckler, 1994). The primary function of facilitative glucose carriers is to help in the exchange of glucose between blood and the cytoplasm of the cell. Glucose in the circulation attaches to the extracellular face of the transporter, causing conformational change of the protein transporter, and hence glucose uptake in cell cytoplasm. The net uptake of glucose into the cell depends on the cell type, its metabolic state and the metabolic state of the organism (Mueckler, 1994). Most of the mammalian cells lack significant levels of glucose-6-phophatase and therefore are incapable of producing free glucose and glucose efflux. The hepatocytes, on the other hand, become a net producer of blood glucose in the post-absorptive state. Glycogenolysis and gluconeogenesis increase the intracellular free glucose concentration, resulting in net efflux of glucose from the cells into the plasma. Conversely, in the postprandial state the hepatocytes take up blood glucose and use it to replenish its glycogen storage, thus completing the cycle (Mueckler, 1994). Although skeletal muscle represents the greatest depot of glycogen, it is unable to release glucose into the blood stream. Muscle glycogen is utilized during fasting and physical exercise, and replenished in the post-exercise period as a high metabolic priority in conditions when sufficient carbohydrate is consumed with meals (Kiens and Richter, 1998).

There are 12 different types of Glut transporters that have been identified and characterized in term of their structure, function, and tissue distribution (Table 2). In the current discussion I will confine the focus on the major glucose transporters, Glut 1 – 4. Glut1 is expressed particularly in the brain (including blood-brain-barrier) and erythrocytes. Moderate level of expression is also found in adipose tissue, muscle, and liver (Wood and Trayhurn, 2003). In human erythrocytes Glut1 comprises approximately 5% of the total red cell membrane, by far the highest concentration density for any glucose transporter (Kasahara and Hinkle, 1977; Mueckler, 1994). Glut2 is expressed in hepatocytes, pancreatic β cells, absorptive epithelial cells of intestinal mucosa, and kidney (Fukumoto et. al., 1988; Thorens et. al., 1988; Thorens et. al., 1992). In β cells, Glut2 plays a role in the glucose sensing mechanism that regulates insulin secretion. In liver it is expressed on the sinusoidal membrane of hepatocytes and allows bi-directional transport of glucose (Wood and Trayhurn, 2003). Glut3 has a high affinity for glucose and it is expressed in parenchymal cells of adult brain (Kayano et. al., 1988; Nagamatsu et. al., 1992).

Glut4 is the insulin-responsive transporter found in heart, skeletal muscle, and adipose tissues. It is responsible for glucose uptake in peripheral tissues and reduction in postprandial blood glucose level. Although these tissues express other glucose transporter isoforms, most notably Glut1, Glut4 is primarily responsible for insulin stimulated glucose uptake in these tissues. Glucose disposal by skeletal muscle in humans accounts for approximately 20% and 75-95% of whole body glucose uptake under basal and hyperinsulinemic conditions respectively (Baron et. al., 1988). Most of the glucose

entering the muscle is converted into glycogen (Shulman et. al., 1990), and the transport step by Glut4 is rate-limiting for glucose uptake into muscle under most conditions (Ziel et. al., 1988).

In absence of insulin, Glut4 resides in a subcompartment of the trans-golgi reticulum. It may recycle through the plasma membrane via coated pits in this state, but the externalization is much slower than the rate of internalization. This causes a significant decrease in transporter concentration at the cell surface. Insulin increases the rate of exocytosis and/or decreases the rate of endocytosis, therefore bringing about a steady-state increase in the level of plasma membrane Glut4 concentration (Mueckler, 1994).

The possible role of altered Glut4 expression in development of insulin resistance and diabetes has been studied. No alterations were found for the level of Glut4 expression in fat or muscle tissues of obese, insulin resistant, and diabetic (db/db) mice relative to their lean litter mates (Koranyi et. al., 1990), and no changes were observed in Glut4 gene expression in skeletal muscle of insulin resistant fatty Zucker rats (Mueckler, 1994). These findings suggest that insulin resistance in skeletal muscle is not caused by a decrease in Glut4 content or its expression. However, Glut4 content in skeletal muscle is increased by chronic exercise training in rat (Rodnick et. al., 1990) and in human (Ebeling et. al., 1993). The increase in Glut4 correlates with the augmented insulin-stimulated glucose uptake in isolated muscles and improved insulin sensitivity in vivo.

1.6.2 Insulin Signaling:

When the blood glucose level rises significantly in a normal fed state, it causes a burst of postprandial insulin release from pancreatic β cells in response to the meal. In contrast, the fasting state is characterized by a lower and constant basal level of insulin (Charpentier et. al., 2006). The postprandial insulin response occurs in two distinct phases: a rapid phase which lasts 5 to 10 minutes followed by a prolonged phase that lasts as long as the glycemic stimulus is present (Tibaldi, 2009). The high blood glucose level in the postprandial state inhibits glucagon secretion from pancreatic α cells, while in the fasting state low glucose level stimulates the secretion of glucagon, which in turn increases the mobilization and synthesis of glucose from the liver to the circulation (Cryer, 2008).

The demonstration that insulin acts through binding with a plasma membrane receptor in the early ‘70s (Fröjdö et. al., 2009) led to the intensive investigation aiming to understand the intracellular steps of insulin signaling and glucose transport. Insulin action is mediated through intracellular signal transduction when insulin binds with the transmembrane insulin receptor. The insulin receptor belongs to the super-family of tyrosine kinase receptors and consists of 2 extracellular alpha subunits and 2 intracellular beta subunits. Insulin binding to the alpha subunits results in a conformational change of the receptor, leading to autophosphorylation of the intracellular tyrosine kinase domain located in the cytoplasmic face (De Meyts and Whittaker, 2002; White, 2003; Glund and Zierath, 2005). The activated receptor initiates a cascade of reactions through phosphorylation of a panel of substrate molecules and causes intracellular signal

transduction. Among the substrate molecules, insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) are the adapter molecules playing a major role in activation of the downstream kinases (White, 2002). Tyrosine phosphorylated IRS-1/2 recruit the heterodimeric p85/p110 phosphatidylinositol 3-kinases (PI3K) at the plasma membrane, where it produces lipid second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 in turn causes phosphorylation and activation of a number of serine/threonine protein kinases including protein kinase B (PKB)/Akt (Alessi and Downes, 1998; Vanhaesebroeck and Alessi, 2000; Beeson et. al., 2003; Fröjdö et. al., 2009). One of the major targets for Akt is glycogen synthase kinase 3 (GSK-3). Upon Akt-mediated phosphorylation on the serine-9 residue, GSK-3 is inactivated (Cross et. al., 1995). The inactivation of GSK-3 relieves the inhibitory phosphorylation of glycogen synthase (GS), which becomes activated and promotes glycogen synthesis in cells (Brady et. al., 1998). Akt also promotes the insulin-stimulated translocation of glucose transporter, GLUT-4, at the plasma membrane and favors glucose uptake in the cell. This pathway involves inhibitory phosphorylation of the RabGTPase activating protein AS160, therefore relieving its inhibitory effect towards GLUT-4 translocation from the intracellular compartment to the plasma membrane (Sano et. al., 2003). The overall process of insulin-mediated signal transduction leads to glucose uptake and storage as glycogen in the cell, and helps in maintaining whole body glucose homeostasis (Fig 4).

TABLE 2: Facilitative glucose transporters (Glut) and their tissue localization [*Adapted from Wood and Trayhurn (2003), Br J Nutr., 89(1):3-9, reprinted with the permission of Cambridge University Press*]:

Type	Localization
Glut1	Ubiquitous. Erythrocytes, brain etc.
Glut2	Liver, pancreas, intestine, kidney
Glut3	Brain
Glut4	Heart, muscle, adipose tissues, brain
Glut5	Intestine, testes, kidney
Glut6	Brain, spleen, leucocytes
Glut7	Not determined
Glut8	Testes, brain
Glut9	Liver, kidney
Glut10	Liver, pancreas
Glut11	Heart, muscle
Glut12	Heart, prostate, muscle, small intestine, adipose tissues

1.6.3 AMPK Signaling

AMP activated protein kinase (AMPK) works as a critical signaling molecule for regulating multiple metabolic processes in skeletal muscle. It is a major cellular energy sensor and a master regulator of metabolic homeostasis (Zhang et. al., 2009), and acts as a fuel-gauge monitoring cellular energy levels (Sakamoto and Goodyear, 2002). AMPK senses decreased energy storage and it acts to switch off ATP consuming pathways and switch on ATP regeneration pathways (Sakamoto and Goodyear, 2002). The AMPK pathway is primarily activated by various conditions that lead to alterations of the intracellular AMP/ATP ratio (hypoxia, ischemia, inhibition of glycolysis, glucose deprivation, muscle contraction etc.) and calcium concentration (Sakamoto and Goodyear, 2002; Zhang et. al., 2009; Towler and Hardie, 2007). AMPK pathway is also activated through the action of various hormones, cytokines, and adipokines (Zhang et. al., 2009).

AMPK is a heterotrimeric enzyme composed of a catalytic (α_1 or α_2) subunit and two regulatory (β_1 or β_2 and γ_1, γ_2 or γ_3) subunits, all of which are encoded by separate genes making it possible to form a total of 12 complexes (Hardie, 2008). Binding of AMP to the γ subunit leads to allosteric activation of AMPK and protection of Thr172 from dephosphorylation, and therefore maintaining the enzyme in the active state (Zhang et. al., 2009). The activated AMPK stimulates translocation of GLUT4 to the plasma membrane, resulting in increased glucose uptake into the cell (Kurth-Kraczek et. al., 1999). The AMPK pathway can be activated experimentally using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). AICAR is taken up by muscle and metabolized by

adenosine kinase to form a monophosphate derivative, ZMP, that mimics the effects of AMP on AMPK and increases glucose uptake in skeletal muscle (Hayashi et. al., 1998; Merrill et. al., 1997). AICAR-stimulated glucose transport is not inhibited by wortmannin, an inhibitor of PI3K, an important substrate of insulin signaling (Sakamoto and Goodyear, 2002), suggesting that AMPK-pathway is independent of the insulin signaling pathway. There is no additive effect on glucose transport with the combination of AICAR plus muscle contraction (Hayashi et. al., 1998).

Contractile activity of muscle, e.g. physical exercise, can alter the fuel status of skeletal muscle and can stimulate AMPK-dependent glucose uptake. Exercise is a physiological stimulus to elicit activation of AMPK in working skeletal muscle and the degree of activation depends on the intensity of muscle contraction (Sakamoto and Goodyear, 2002). The AMPK activity is significantly increased with exercise in vivo (Rasmussen et. al., 1998; Rasmussen and Winder, 1997), sciatic nerve-stimulated muscle contraction in situ (Hutber et. al., 1997; Vandenburgh, 1992; Vavvas et. al., 1997), and contraction of isolated muscles in vitro in the absence of systemic factors (Hayashi et. al., 2000; Hayashi et. al., 1998; Ihlemann et. al., 1999). Greater the force of contraction, there is a higher activation of AMPK (Ihlemann et. al., 1999). The contraction-mediated activation of AMPK may provide multiple metabolic benefits of exercise. In addition to the increased glucose uptake and utilization by the muscle, activation of the AMPK pathway also leads to an increased fatty acid oxidation by decreasing malonyl-CoA concentration through inhibition of acetyl-CoA carboxylase (ACC) and activation of

malonyl-CoA decarboxylase (MCD). Increased mitochondrial fatty acid oxidation results in decreased intramyocyte lipid accumulation (Ruderman et. al., 2003).

In addition to the metabolic importance of the AMPK pathway, it has been suggested that AMPK signaling plays role to maintain peripheral vascular tone. Activation of the AMPK pathway in endothelial cells causes increased phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser1177 and increased NO production (Morrow et. al., 2003). Phosphorylation of Ser1177 on eNOS increases the Vmax of the enzyme and its sensitivity to Ca^{+2} and calmodulin (Chen et. al., 1999). Activation of eNOS by AMPK and resultant increase in NO production cause relaxation of the vascular smooth muscle and increased blood flow to hypoxic tissues (Towler and Hardie, 2007). Blockade of AMPK inhibits eNOS phosphorylation at Ser1177 in response to hypoxia (Nagata et. al., 2003).

1.6.4 Insulin vs. AMPK Signaling: Are they complementary or counter-regulatory?

The insulin signaling mechanism is activated when nutrients are available, whereas the AMPK pathway is activated when cells have energy/nutrient deprivation. Therefore, one would expect these 2 pathways to oppose each other, and this is often the case. Insulin promotes lipid, protein, and glycogen synthesis, whereas AMPK inhibits these biosynthetic pathways (Towler and Hardie, 2007). In some tissues, such as cardiac muscle, insulin antagonizes activation of AMPK (Gamble and Lopaschuk, 1997; Beauloye et. al., 2001). In other cases, the insulin and AMPK signaling pathways work in the same direction, particularly during glucose metabolism in skeletal muscle. The

activation of both of these pathways stimulate glucose uptake through increased translocation of GLUT4 to the plasma membrane. However, the fate of glucose taken up through these pathways is different; insulin signaling promotes glycogen synthesis, whereas AMPK signaling promotes glycolysis (Towler and Hardie, 2007).

Insulin and AMPK pathways provide complementary actions in liver. Both of the pathways suppress the enzymes of gluconeogenesis, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Lochhead et. al., 2000). This process of inhibition of gluconeogenesis makes obvious sense for insulin, which is released in response to high blood glucose, to suppress hepatic glucose production (Towler and Hardie, 2007). However, this is in contrast to the general anti-anabolic action of AMPK. Suppression of gluconeogenesis has been suggested to be the major cause of the plasma glucose-lowering effects of adipokines, adiponectins, and the antidiabetic drug metformin, which involve activation of the AMPK pathway (Yamauchi et. al., 2002; Shaw et. al., 2005). The parallel and complementary effects of insulin and AMPK on muscle glucose uptake and hepatic glucose production introduced the idea that activators of the AMPK pathway could be used to treat type 2 diabetes and obesity (Winder and Hardie, 1999). This idea was further supported by the finding that activation of AMPK underlies the glucose-lowering effects of metformin (Zhou et. al., 2001; Shaw et. al., 2005).

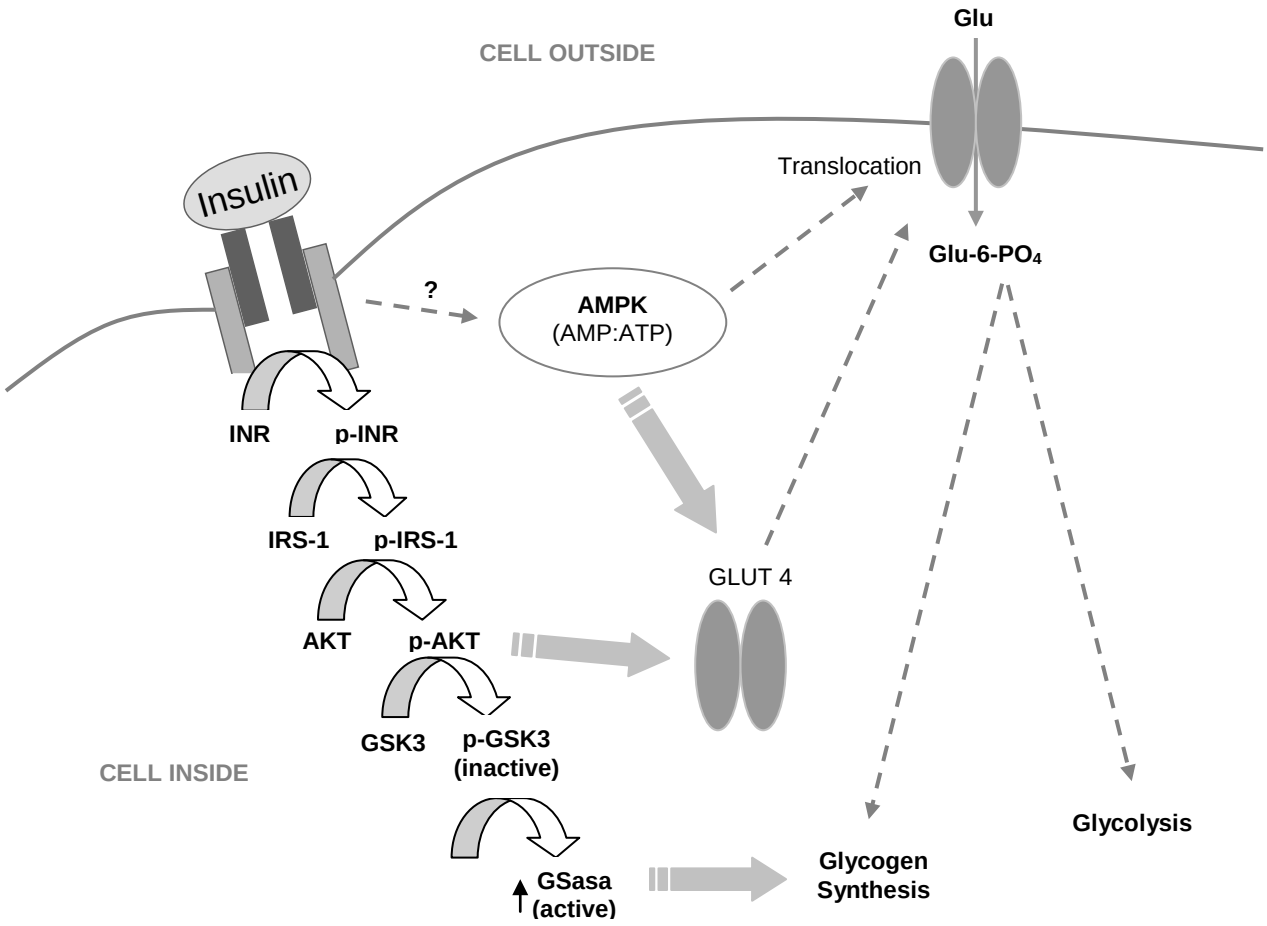


Fig. 4: The glucose uptake and energy homeostasis in a cell involving the insulin- and AMPK-pathways: Binding of insulin with the α-subunits of the tetrameric insulin receptor causes autophosphorylation of the β-subunits, and initiates a series of intracellular reactions leading to the activation of downstream regulators (IRS-1, AKT etc). The consequence of the reaction cascade causes translocation of glucose transporter (GLUT 4) in the cell membrane, cellular glucose uptake and glucose storage as glycogen. The activation of AMPK pathway mostly takes place under stress (e.g. exercise), when the ATP level declines. The major role of AMPK-pathway is increased recruitment of GLUT4 in the plasma membrane and glucose uptake into the cell. The inter-dependency of AMPK- and insulin-pathways is somewhat predicted, but still not well established.

In adipocytes, insulin and AMPK act also in the same direction through suppression of the hormone-sensitive lipase, and hence inhibition of lypolysis (Sullivan et. al., 1994; Daval et. al., 2005). The mechanism of anti-lypolytic action of these 2 pathways is different. Insulin causes phosphorylation and activation of phosphodiesterase 3B by Akt, thus lowering cAMP and inhibiting lypolysis (Wijkander et. al., 1998). AMPK phosphorylates hormone-sensitive lipase at a site (Ser565) that antagonizes activation by cAMP-dependent protein kinase (Garton et. al., 1989). The anti-lypolytic action of insulin is straightforward, since it represents one of the major anabolic actions of insulin. However, AMPK inhibits lypolysis to preserve cellular ATP concentration through an indirect mechanism. If fatty acids released by lypolysis are not removed from the cell rapidly enough, they are known to recycle into triglyceride, thus consuming ATP and causing cellular ATP depletion (Brooks et. al., 1983). It has been proposed that AMPK inhibits lypolysis as a mechanism to limit this recycling, ensuring that the rate of lypolysis does not exceed the rate of fatty acid utilization/oxidation (Hardie and Carling, 1997; Towler and Hardie, 2007).

1.7 Fasting vs. postprandial insulin sensitivity: Dynamic postprandial glucoregulation via meal-induced insulin sensitization (MIS) process

Diabetes is conventionally diagnosed by abnormal increase in fasting blood glucose level ≥126 mg/dl (or 7 mmol/L) (Rendell and Jovanovic, 2006). Since the postprandial state accounts for the major part of the time during a day, assessment of the metabolic status of an individual based on the fasting parameters cannot accurately predict the metabolic abnormalities and associated cardiovascular diseases. Though there is a high correlation between the fasting blood glucose (FBG) and postmeal glucose

excursion (Erlinger and Brancati, 2001), the 2 hr postprandial blood glucose (PPG) level greater than 200 mg/dl (11 mmol/L) confirms diabetes, even in absence of FBG elevation (Rendell and Jovanovic, 2006). The prediabetic state can be diagnosed by measuring the postprandial insulin sensitivity with the glucose tolerance test, which is accepted as a surrogate for postmeal glucose utilization. In this test 75 g of glucose is administered orally, and impaired glucose tolerance (IGT) is characterized by a normal FPG level, but 2 hr value of glucose tolerance test between 140 and 199 mg/dl. Although the response to pure glucose does not accurately reflect the effects of a typical meal containing carbohydrate, protein, fat, and other nutrients, it is the most widely accepted technique, since there is no standardized meal yet available to assess the postprandial glucose utilization response to insulin.

There are observational studies that support a greater importance of PPG in prognosis of metabolic and cardiovascular events. Studies have demonstrated that PPG levels can better predict overall diabetic mortality (Balkau et. al., 1998; DECODE Study Group, 2001; de Vegt et. al., 2001), cardiovascular mortality (Balkau et. al., 1998; Barrett-Connor and Ferrara, 1998), and heart disease (Barrett-Connor and Ferrara, 1998; Hanefeld et. al., 1996; Donahue et. al., 1987; Jackson et. al., 1992). Atherosclerotic changes start to develop in the prediabetic state when PPG is only modestly elevated above the normal (Haffner, 1998). The Diabetes Epidemiology Collaborative analysis of Diagnostic criteria in Europe (DECODE Study Group, 2001) compared the predictive value of FBG and 2 hr PPG measures on mortality in 10 prospective European cohort studies (approximately 21,000 individuals) over 8.8 years of follow-up. It showed that

elevated 2 hr PPG excursions were associated with an increased risk of atherosclerosis and cardiovascular diseases. PPG was a better predictor of all-cause and cardiovascular deaths than FBG. Elevation of 2 hr PPG is also correlated with the development of serious long-term manifestations such as retinopathy and nephropathy (American Diabetes Association, 2003; Fonseca, 2003). It is also suggested that drugs targeting to manage PPG may reduce the risk of cardiovascular complications (Tibaldi, 2009).

There are numerous potential mechanisms that may contribute to the strong association between postprandial hyperglycemia and cardiometabolic abnormalities. Postprandial hyperglycemia has several metabolic consequences, including increased oxidative stress, abnormal vascular reactivity, protein glycation, hypercoagulability, and increased endothelial inflammation. It is suggested that these pathologies may be linked to a single mechanism induced by hyperglycemia: the overproduction of reactive oxygen species (ROS) (Brownlee, 2005). The resulting postprandial oxidative stress triggers numerous changes leading to atherogenesis and other associated complications such as inflammation, vasoconstriction, thrombogenicity, and oxidation of low-density lipoprotein (LDL) (O'Keefe and Bell, 2007). Postprandial glucotoxicity and oxidative stress may also promote the progressive decline in β cell function and advancement to type 2 diabetes (Wajchenberg, 2007). Since the assessment of postprandial parameters provides a better prognosis of the metabolic and vascular dysfunctions associated with insulin resistance, it is important to understand the dynamic postprandial glucoregulatory mechanism that is controlled through the response to individual meals. The phenomenon

of augmented glucose utilization response to insulin by a meal can be described with the meal-induced insulin sensitization (MIS) process.

1.7.1 Meal-induced Insulin Sensitization (MIS): The Hepatic Insulin Sensitizing Substance (HISS) -hypothesis

1.7.1.1 The Feeding Signals for MIS

The MIS process is described by the HISS-hypothesis. Following a meal the dynamic glucose disposal response to insulin is approximately doubled in rats (Lautt, 1999; Latour and Lautt, 2002; Sadri et. al., 2006) and tripled in young healthy men (Patarrão et. al., 2008). This phenomenon of potentiation of the insulin response by a meal is called meal-induced insulin sensitization (MIS), and has been demonstrated in animal models (Lautt, 2004) and in humans (Patarrão et. al., 2008). MIS results when food in the upper gastrointestinal tract causes two permissive feeding signals to be delivered to the liver. A reflex feeding signal involves activation of the hepatic parasympathetic nerves (Lautt, 1999), and a chemical signal is mediated by an increase in the hepatic glutathione (GSH) level (Guarino et. al., 2003). In the presence of these two feeding signals, insulin causes the release of a putative hormone called hepatic insulin sensitizing substance (HISS) from the liver. HISS is released into the blood stream and works primarily in skeletal muscles to increase glucose uptake. HISS does not sensitize the response to insulin but rather provides a proportional additive effect. Both of the feeding signals play a purely permissive role in that they have no direct effect on the glucose utilization process, but they are required in order for pulses of insulin to release pulses of HISS from the liver. Either signal alone is not sufficient to activate the HISS-pathway (Guarino and Macedo, 2006; Lautt et. al., 2011) (Fig 5).

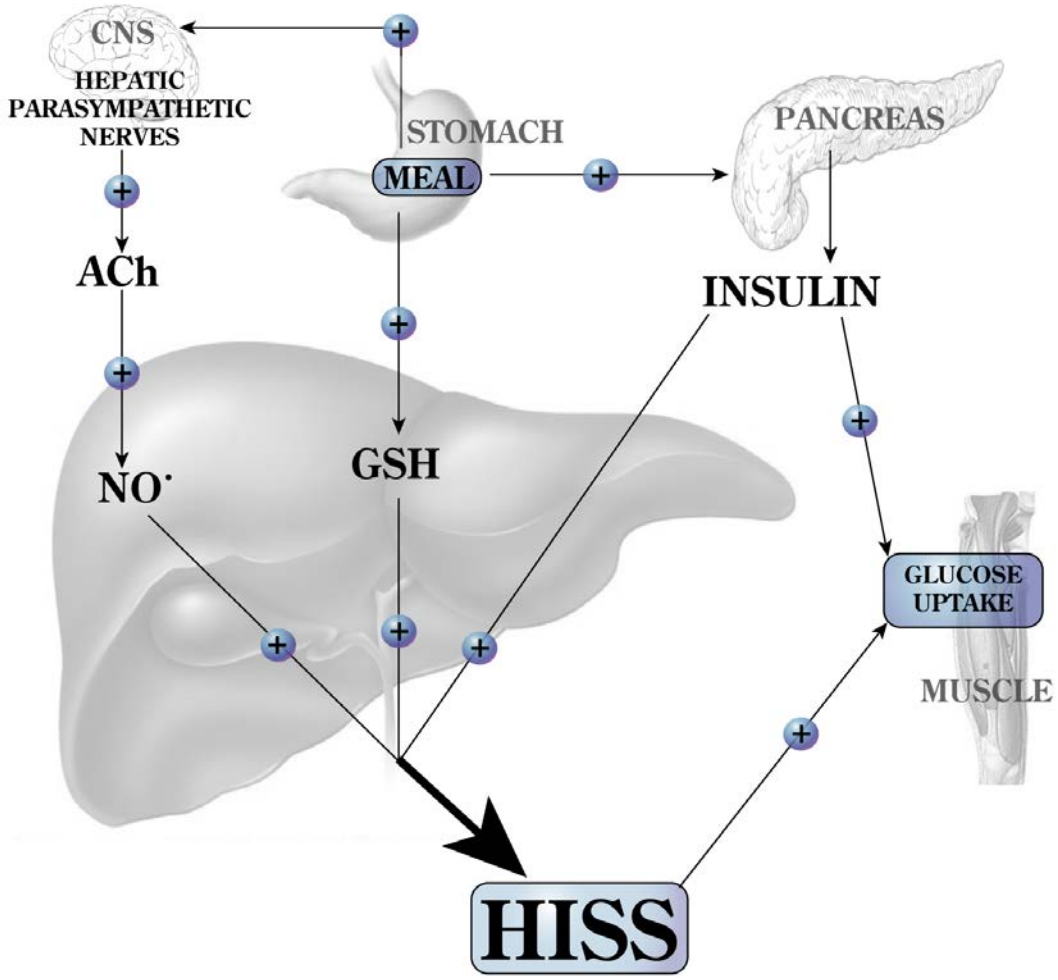


Fig. 5: The MIS process and HISS pathway: Presence of food in upper GIT initiates two feeding signals, including activation of the hepatic parasympathetic nerves and elevation of the hepatic GSH concentration, which causes insulin to release HISS from the liver. HISS works in skeletal muscle to cause glucose uptake, and it represents approximately 50% of glucose utilization response to insulin during the postprandial state. [*Adapted from: Lautt WW, Ming Z, Legare DJ (2010), Attenuation of age- and sucrose-induced insulin resistance and syndrome X by a synergistic antioxidant cocktail: the AMIS syndrome and HISS hypothesis; Can J Physiol Pharmacol., 88:313-23, © 2008 Canadian Science Publishing or its licensors. Reproduced with permission*]

1.7.1.2 The Parasympathetic Feeding Control of MIS

Parasympathetic control in the liver for MIS process involves Ach/NO/cGMP pathway (Guarino et. al., 2004). The type(s) of cholinergic receptors contributing to the initiation of this process has not yet been identified. The muscarinic acetylcholine receptors (mAchRs) mediate most of the actions of the neurotransmitter Ach in the end organs of parasympathetic nerves (Koppen and Kaiser, 2003). There are 5 distinct mAchR subtypes, $M_1 - M_5$. The mAchR belongs to the superfamily of seven-transmembrane G proteins coupled receptors. The M_1, M_3, and M_5 receptor subtypes are coupled with G_q subtype G proteins, leading to activation of phospholipase C (PLC), and therefore inositol triphosphate and intracellular calcium as a signaling pathway. M_2 and M_4 receptors are coupled with G_i subtype G proteins, which causes a decrease in cAMP in the cell, inhibition of voltage-gated Ca^{+2} channels, and efflux of K^+, resulting in inhibitory-type of effects (Caulfield, 1993; Rümenapp et. al., 2001).

Although there is a big body of physiological evidence on the involvement of Ach in hepatic control of glucose metabolism, the mechanism of signal transduction pathway from the nerves to the organ remains unclear (Vatamaniuk et. al., 2003). It is suggested that nerves act directly on only a few parenchymal cells from which the signal is transmitted in several possible ways: via gap junction, via an overflow of neurotransmitter from the vasculature into the sinusoids, or via hemodynamic changes (Hartmann et. al., 1982). It is known that Ach mediates its effects on hepatic glucose metabolism through muscarinic receptors. Metabolic effects of Ach on liver metabolism result in the increase of glucose production and glycogen content in perfused liver under

gluconeogenic conditions. These effects can be eliminated by the muscarinic antagonist, atropine. It is suggest that these metabolic effects are mediated through M_3 cholinoreceptors expressed in hepatocytes (Vatamaniuk et. al., 2003). However, it is shown that M_1 receptor subtype is responsible for the synergistic effect of the hepatic parasympathetic nerves and for insulin to release HISS from the liver (Xie and Lautt, 1995b). In our studies, atropine has been used as an effective pharmacological tool for measurement of the HISS-action in fed animals. Atropine blocks the hepatic parasympathetic feeding signal, and therefore inhibits insulin to release HISS from the liver. Various aspects of atropine pharmacology and its use for quantification of MIS are discussed under ``technical consideration`` in the Discussion section.

1.7.1.3 Manipulation of the Feeding Signals

In animals the HISS-dependent glucose uptake accounts for approximately 50% of the total glucose sequestering response to insulin during the postprandial state, and the rest represents HISS-independent glucose uptake (i.e. the direct action of insulin) (Lautt, 2004). In humans the HISS-action constitutes approximately 2/3rd of the postprandial glucose uptake response to insulin (Patarrão et. al., 2008). Both of the feeding signals are needed for insulin to cause the release of HISS, and blockade of any portion of these pathways leads to blockade of HISS release. The HISS-dependent component of postprandial glucose utilization decreases progressively from maximal activation after a meal to insignificance after 24 hours of fasting with neither signal being delivered (Lautt et. al., 2001; Lautt et. al., 2011). The HISS-pathway can be blocked experimentally by fasting or denervation of the hepatic parasympathetic nerves or pharmacologic blockade

of the nerves with atropine (Lautt et. al., 2001), and pathologically as in aging or diabetes (Ming et. al, 2009; Ribeiro et. al., 2008; Lautt et. al., 2008). The parasympathetic feeding signal in the HISS-pathway involves generation of nitric oxide (NO), which is activated by muscarinic receptor agonists (Xie and Lautt, 1996a; Xie and Lautt, 1996b) and inhibited by NO synthase blockers (Sadri et. al., 1997; Sadri and Lautt, 1999; Guarino et. al., 2003; Guarino et. al., 2004). Pathological impairment of either or both of the feeding signals prevents the MIS process from occurring, and causes precipitation of a cluster of metabolic abnormalities characterized as the absence of MIS or AMIS syndrome (see section 5). Pharmacological manipulation of either or both of the feeding signals is discussed in section 6.

1.7.1.3 Quantification of MIS

The degree of MIS can be quantified by conducting the rapid insulin sensitivity test (RIST), which is a rapidly sampled transient euglycemic clamp in response to a bolus administration of insulin. Insulin sensitivity is measured using the RIST in fed animals, where both the HISS-dependent and HISS-independent components are present. When HISS release is blocked, the response to insulin represents only the HISS-independent component. The difference between the two tests represents the MIS process, attributable to the action of HISS. The HISS action can also be measured by comparing the RIST before and after intragastric administration of a liquid test meal. The operating procedure for the RIST is described in section 2: ‘Materials and Methods’.

1.7.2 Role of MIS in control of postprandial metabolic and vascular dynamics, and attenuation of the process by pathologies

1.7.2.1 Metabolic role of MIS and its regulation by the nutrient composition of a meal

The postprandial phase involves complex neurohormonal, metabolic, and cardiovascular interactions (Kearney et. al., 1995). A pivotal role in regulation of the postprandial metabolic and hemodynamic actions is played by insulin (Baron, 1994; Steinberg et. al., 1996; Kearney et. al., 1996); while almost 50% of the metabolic (Lautt, 2004) and 100% of the vascular (Ming and Lautt, 2011) response to insulin is mediated through the meal-induced insulin sensitization (MIS) process and HISS action.

However, the metabolic dynamic following a meal relies on the gastrointestinal feeding reflex generated in response to the complex interaction between the nutritional ingredients and the nutrient-sensing mechanisms. The epithelial cells lining the inner surface of the gastrointestinal tract (GIT) are in direct contact with a luminal environment that varies dramatically with diet. It is suggested that the epithelial layer carries different nutrient-sensing molecules that can sense the nutrient composition of the luminal contents (Shirazi-Beechey et. al., 2011).

Sadri et. al. demonstrated that the nutrient compositions of a meal can significantly influence the postprandial glucose utilization response to insulin. They showed that a mixed meal is able to produce meal-induced insulin sensitization (MIS), causing doubling of the glucose utilization response to insulin during the postprandial state; conversely glucose and sucrose were ineffective to induce appropriate feeding

signals to cause MIS (Sadri et. al., 2006). Sucrose diets are not only incapable of inducing MIS response, but chronic ingestion of high-sucrose diets also impairs the MIS process. If MIS does not occur in the postprandial phase it results in a progressive series of metabolic abnormalities referred to as the AMIS syndrome (see section 1.7.2.3). Therefore, an appropriate nutritional composition in a balanced meal is important to regulate the postprandial feeding signals.

1.7.2.2 Coupling of the metabolic response of MIS with the postprandial vascular dynamics

The postprandial state is characterized by fluctuations of circulating metabolites that are tightly coupled with variations in hemodynamics (Vigili et. al., 2011). Pathological alterations in this hemodynamic-metabolic coupling lead to a predisposition to various cardio-metabolic events (Rendell and Jovanovic, 2006; Gill et. al., 2004). In diabetes, in conjunction with the early metabolic abnormalities, the vascular reactivity and circulatory response to insulin may be impaired during the postprandial state (Ming and Lautt, 2011; Vigili et. al., 2011), even in absence of subclinical atherosclerosis (Scognamiglio et. al., 2005; Wascher et. al., 2005; Rask-Madsen and King, 2007). The vasodilation response after insulin administration is proposed to be an indirect effect caused by HISS action. Absence of HISS has vascular as well as metabolic consequences.

In diabetes there is an increased prevalence of microvascular dysfunctions (Anfossi et. al., 2009; Muniyappa et. al., 2007; Singleton et. al., 2003) that may cause polyneuropathy, retinopathy, nephropathy and limb amputations (Ming and Lautt, 2011).

Impairment of the vasodilatory response to insulin has been suggested to be the cause of vascular pathologies associated with diabetes. However, the prandial status regulates the action of insulin in controlling the vascular dynamics. The vasodilatory response to insulin is present only in the fed state (Utriainen et. al., 1995; Yki-Jarvinen and Utriainen, 1998; Zhang et. al., 2004; Ming and Lautt, 2011). This postprandial vasodilation represents an indirect action of insulin as shown from the observation that local forearm hyperinsulinemia does not result in vasodilation, but it is caused only when insulin is administered systemically (Tack et. al., 1998; Cardillo et. al., 1998). These observations are consistent with the HISS-hypothesis and signify that the metabolic dynamics of the MIS process is coupled with the postprandial hemodynamic response to insulin. Insulin action itself does not cause hind-limb vasodilation. In postprandial state, almost 100% of the vascular response to insulin results from the action of HISS (Ming and Lautt, 2011).

The hormonal nature of HISS was shown first for its metabolic action, and confirmed with its vascular action (Ming and Lautt, 2011). Hepatic parasympathetic denervation blocks the metabolic and vascular actions of HISS. Continuous intraportal infusion of acetylcholine mimics the nerve signal and can restore insulin-mediated release of HISS, and therefore the metabolic and vascular response in the hindlimbs. Intraportal acetylcholine is rapidly metabolized and does not recirculate to cause direct effects on the hindlimbs, and the baseline glucose and blood flow remain constant. The same dose of acetylcholine given intravenously is rapidly metabolized and does not restore HISS release from the liver, and therefore it does not have any effect on the hindlimbs. Intraportal acetylcholine acting on the liver is able to restore the ability of

insulin to stimulate HISS release. These findings suggest that the postprandial metabolic and vascular dynamics of the hindlimbs are tightly coupled through the action of a substance that is released from the liver and is hormonal in nature, providing metabolic and vascular actions.

1.7.2.3 Absence of MIS (or AMIS): An early metabolic abnormality of insulin resistance and diabetes

MIS is documented by a dramatic increase in the glucose disposal response to insulin during the postprandial state and results through a greatly amplified response to insulin secondary to HISS acting on skeletal muscle (Xie and Lautt, 1996a; Moore et. al., 2002). The concept of MIS is derived from the observation that the dynamic response to insulin, determined after a 24 hour fast, is at least doubled following intragastic administration of a mixed meal in rats (Sadri et. al., 2006). MIS was quantified also in humans suggesting that approximately 2/3rd of the postprandial response to insulin was a result of the MIS process (Patarrão et. al., 2008). The process of MIS is a result of the action of HISS, which is released from the liver in response to a pulse of insulin and acts selectively to stimulate glucose uptake in skeletal muscle (Xie and Lautt, 1996a, Fernandes et. al., 2011). The impairment of HISS release prevents the development of MIS in response to a meal and causes progression to an AMIS (absence of meal-induced insulin sensitization) syndrome. When MIS does not occur, feeding causes postprandial hyperglycemia and compensatory increase in serum insulin concentration. Since insulin is lipogenic in nature, it causes conversion of glucose into lipids in adipose tissues and mobilization of it from liver to the peripheral tissues. By this process, AMIS results in progressive increase in whole body adiposity. We have suggested that AMIS is the

earliest metabolic abnormality of insulin resistance, and it leads to progressive and predictable metabolic and vascular dysfunctions (Lautt et. al., 2008; Lautt et. al., 2010; Ming and Lautt, 2011). The cluster of abnormalities, as a consequence of the impaired HISS-pathway, is collectively described as the AMIS syndrome, which constitutes major cardiometabolic risks associated with insulin resistance.

The development of the AMIS syndrome was studied in animal models of aging (Ming et. al, 2009) and type 2 diabetes (Afonso et. al., 2010; Ribeiro et. al., 2005). Chronic dietary interventions with high-fat diet (Afonso et. al., 2010) and 35% sucrose supplement (Ribeiro et. al., 2005) are capable of inducing the first stage of the AMIS syndrome as early as 2 weeks of exposure. The ability of age and dietary stress to impair the MIS process and to develop the AMIS syndrome results through impairment of the HISS-pathway, while the direct action of insulin remains mostly unaffected (Lautt et. al., 2010).

1.7.2.4 Hepatic glucose production (HGP), hypothalamic control of HGP, and their role in insulin resistance

After a meal, insulin is secreted into the portal vein to prevent a rise in blood glucose by increasing glucose uptake into the muscle and adipose tissue, and through suppression of HGP. The postprandial state switches the liver from a net producer to a net consumer of glucose (Cherrington, 1997). This physiological role of insulin is impaired in insulin resistance associated with type 2 diabetes or obesity. Under normal physiological condition, the postprandial state is characterized by increased secretion of insulin and suppression of glucagon. In type 2 diabetes, insulin and glucagon secretion are abnormal

following carbohydrate ingestion. Insulin secretion is decreased and delayed, and glucagon does not suppress (Butler and Rizza, 1991; Rizza, 2010).

The brain, specifically the hypothalamus, plays an important role in regulating food intake and nutrient partitioning. Hypothalamic insulin signaling plays a significant role in control of HGP, and it was supported by the observation that microinjection of insulin into the ventromedial nucleus lowers blood glucose and requires the vagus nerves (Szabo et. al., 1983; Buettner and Camacho, 2008). Hypothalamic insulin action is required for physiological suppression of HGP. This central control of HGP is regulated through KATP channels expressed in the hypothalamus, and are responsive to insulin and leptin (Spanswick et. al., 1997; Spanswick et. al., 2000). The hypothalamus senses hormones and nutrients, and causes inhibition of HGP through hyperpolarization of neurons by hypothalamic KATP channels (Pocai et. al., 2005; Obici et. al., 2002; Lam et. al., 2005). The possible association of abnormal HGP and impaired hypothalamic control of HGP with the AMIS syndrome is discussed under “technical consideration” in the Discussion section.

1.7.4 Manipulation of the MIS process

The development of the AMIS syndrome can be prevented and the progression of the disease can be slowed or reversed by adopting strategies of manipulating the feeding signals involved in the MIS process. Benac, a repurposed pharmaceutical that is a combination of bethanechol and N-acetylcysteine, is able to mimic the feeding signals pharmacologically. Bethanechol mimics the parasympathetic signal and N-acetylcysteine

mimics the GSH-signal. Neither of the agents alone is capable to initiate the MIS process. Provision of the two feeding signals with Benac in fasted rats can cause sensitization of the whole body glucose uptake response to insulin, to levels similar to those seen in fed rats (Lautt et. al. 2011). The ability to cause HISS release in fasted rats using Benac indicates that only those two feeding signals are required for MIS. Benac is also capable of restoring the MIS process in rat models of diabetes that is produced through chronic supplementation of 35% sucrose. The 35% sucrose solution induces insulin resistance, impairing both feeding signals involved in the HISS-pathway. Benac mimics both signals and therefore restores the postprandial insulin sensitivity (Lautt et. al. 2011). As Benac is a repurposed pharmaceutical, it rapidly entered proof of principal phase 2 clinical trials (carried out by Diamedica Inc., Winnipeg, Manitoba, Canada).

A partially blocked HISS-pathway (using submaximal dose of atropine, ED_{75}) can also be restored through potentiation of the cholinergic signal with neostigmine, an acetylcholinesterase antagonist. By preventing the degradation of endogenous acetylcholine, neostigmine provides cholinomimetic effects, and therefore potentiates the MIS process (Schafer et. al., 2010). The parasympathetic feeding signal in the liver for HISS release involves Ach/NO/cGMP pathway. MIS can be blocked by nitric oxide synthase (NOS) inhibitors, and reversed by NO donors. However, the MIS process can be potentiated by elevating cellular cGMP concentration with the use of a phosphodiesterase antagonist (Guarino et. al., 2004).

The progression to the AMIS syndrome can also be prevented by chronic supplementation of a balanced antioxidant cocktail, SAMEC (S-adenosylmethionine + vit E + vit C) (Ming et. al., 2009). The increased oxidative stress associated with aging and diabetes targets the MIS process and induces postprandial insulin resistance. Chronic SAMEC supplementation protects the HISS-pathway from oxidative damage, and therefore prevents the development of the AMIS syndrome.

The MIS process can also be manipulated by non-pharmacological strategies including physical exercise. A major part of my doctoral research was aimed to test the interaction of voluntary exercise with the MIS process in presence/absence of antioxidant, therefore to provide a rational mechanistic explanation of the postprandial metabolic benefits of physical exercise in physiology and in pathophysiology. The studies of exercise interaction with the MIS process were published/accepted/submitted (Chowdhury et. al., 2011; Chowdhury et. al., 2012a; Chowdhury et. al., 2012b).

SECTION II

RESEARCH OBJECTIVES

2. RESEARCH OBJECTIVES

This doctoral research dealt with two independent objectives: study of the interaction of exercise +/- antioxidant with the meal-induced insulin sensitization (MIS) process in healthy rats and in diet-induced animal models of AMIS, and study of the possible cellular signaling pathways that cause increased postprandial glucose uptake in skeletal muscle through MIS. Each of the objectives incorporated testing a set of research hypotheses as follows:

Objective 1: Interaction of voluntary running-wheel exercise (7-days) with MIS

Hypothesis 1: Voluntary running wheel exercise for 7 days improves the postprandial response to insulin in normal aging rats through potentiation of the MIS process (*Protocol 1*).

Hypothesis 2: Voluntary running wheel exercise for 7 days recovers the postprandial insulin response in diet-induced insulin resistant rats through restoration of the MIS process (*Protocol 2*).

Hypothesis 3: Supplementation with antioxidant cocktail, SAMEC (S-adenosylmethionine + Vit E + Vit C), for 7 days does not affect the insulin sensitizing benefits of voluntary exercise in normal and insulin resistant rats (*Protocol 3*).

Objective 2: Cellular signaling pathways involved with MIS

Hypothesis 1: The increased skeletal muscle glucose uptake through MIS involves a cellular signaling pathway that is independent of the intracellular insulin signaling and AMPK signaling (*Protocol 4*).

Hypothesis 2: The increased skeletal muscle glucose uptake through MIS is manifested through enhanced cellular kinetics of glucose uptake and storage (*Protocol 5*).

SECTION III

STUDY DESIGN & METHODS

3. STUDY DESIGN AND METHODS

3.1 Animals and Groups

Animals were maintained in accordance with guidelines of the Guide to the Care and Use of Experimental Animals and protocols were approved by the Protocol Management and Review Committee at the University of Manitoba. Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) were housed in a climate-controlled animal care facility. All rats were housed in pairs in order to minimize stress. The group design and description, and the allocation of animals in the protocols follow.

3.1.1 Exercise Studies: Interaction of voluntary running-wheel exercise with MIS

The animals used for this study were allocated into 3 protocols (Protocol 1 – 3).

3.1.1.1 Protocol 1: Impact of voluntary exercise in normal aging rats

The rats were randomly assigned to the 9, 14, and 21-week groups, and maintained on standard laboratory rat chow (Prolab RMH300, PMI feeds, St. Louis, USA) and normal water. Insulin sensitivity test and biochemical sampling were done for each individual rat on a separate day, once the assigned group-age (4 weeks on arrival + 4/9/16 weeks of housing + 1 week with/without exercise) was met (Fig 6).

3.1.1.2 Protocol 2: Impact of voluntary exercise in diet-induced insulin resistant rats

The rats were randomly assigned to the high-fat diet (9-week, Hf-9) or 35% sucrose supplement (14-week, Sc-14 and 21-week, Sc-21) groups. The rats had a free

access to the intervention diets for assigned time periods to induce AMIS. Experiments were pre-scheduled and conducted for each individual rat on a separate day, when the rats reached the specified group-age {4 weeks on arrival + 4 weeks on high-fat diet or 9-16 weeks on 35% sucrose supplement + 1 week with/without exercise while on the normal diet} (Fig. 7).

3.1.1.3 Protocol 3: Impact of SAMEC and voluntary exercise in normal and insulin resistant rats

Rats were randomly assigned to one of the eight study groups: H-NC: healthy rats on normal (no-SAMEC) diet without exercise, H-NE: healthy rats on normal (no-SAMEC) diet with exercise, H-SC: healthy rats on SAMEC-supplemented diet without exercise, H-SE: healthy rats on SAMEC-supplemented diet with exercise, IR-NC: insulin-resistant rats on normal (no-SAMEC) diet without exercise, IR-NE: insulin-resistant rats on normal (no-SAMEC) diet with exercise, IR-SC: insulin-resistant rats on SAMEC-supplemented diet without exercise, and IR-SE: insulin-resistant rats on SAMEC-supplemented diet with exercise. Rats of all experimental groups reached the age of 14 weeks (4 weeks on arrival + 9 weeks on a specific diet protocol + 1 week with/without exercise while on the normal or SAMEC-supplemented diet) at the time of experimentation (Fig. 8).

3.1.2 Signaling Studies: Cellular signaling pathways involved with MIS

Rats (250 gm at arrival) were randomly assigned to the control (HISS positive, N=6) and atropine treated (HISS negative, N=6) groups. The group distribution and protocol design is illustrated in Fig. 9.

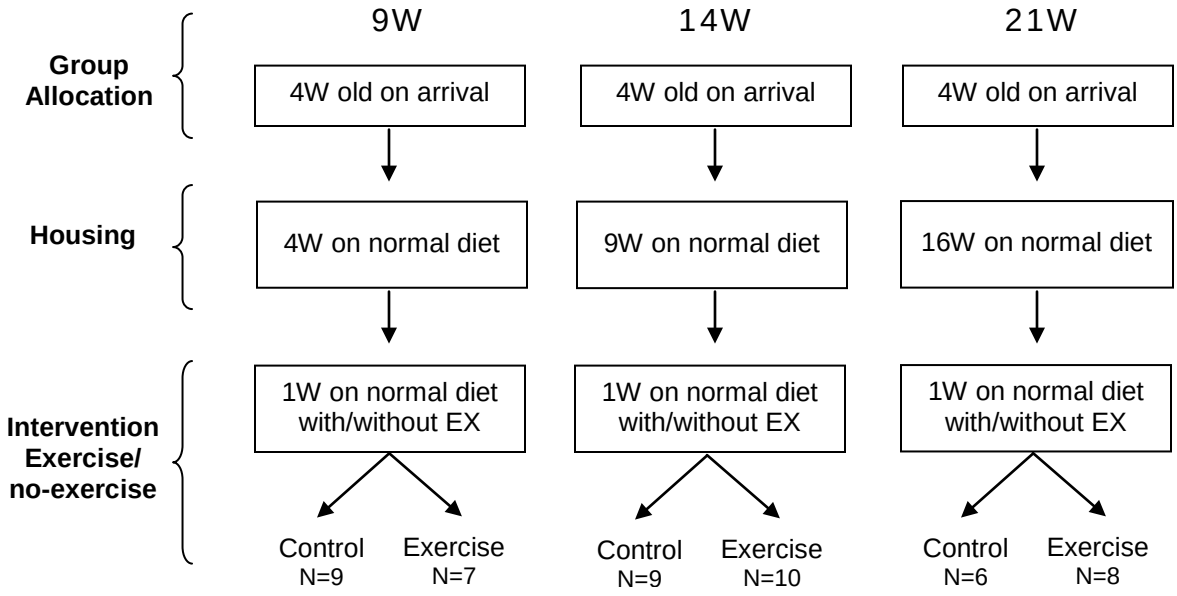


Fig. 6: Flow-chart of protocol 1 design: The rats were maintained on normal diet and drinking water throughout the protocol period, and during the final week they received exercise/no-exercise intervention.

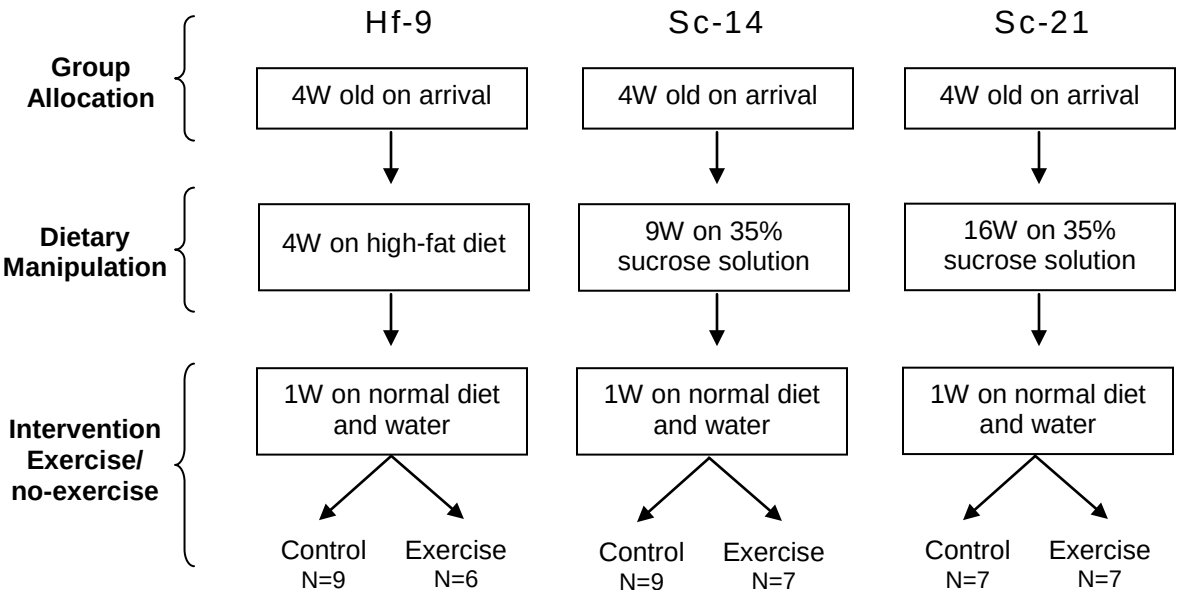


Fig. 7: Flow-chart of protocol 2 design: High-fat diet (Hf-9) or 35% sucrose supplement (Sc-14/Sc-21) was used in specific age groups to induce insulin resistance. Following the period of dietary insult, the rats were maintained on normal diet and drinking water for 1 week while they received exercise/no-exercise intervention. These dietary manipulations induce insulin resistance that does not recover spontaneously within a week upon withdrawal of the dietary stress. The longer duration of the sucrose insult resulted in a more severe set of dysfunctions.

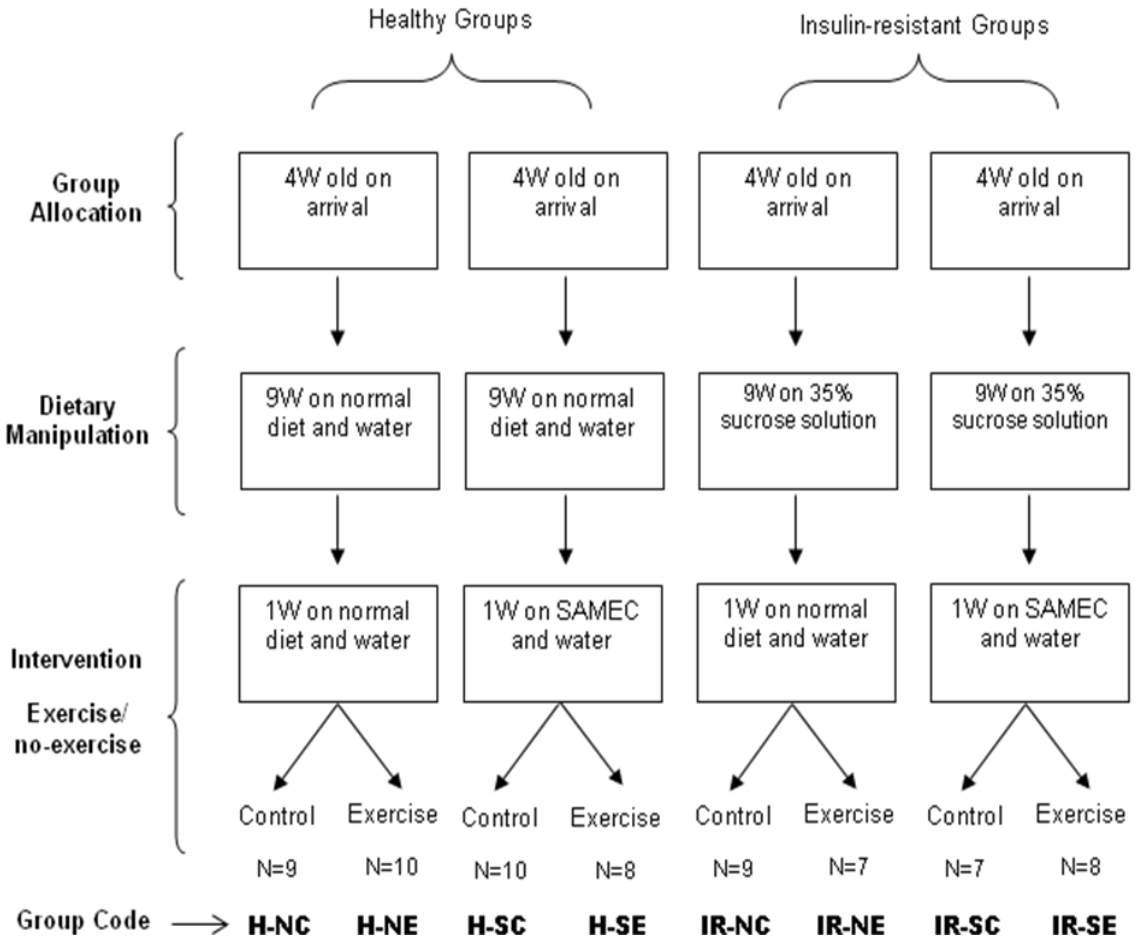


Fig. 8: Flow-chart of protocol 3 design: Abbreviations: H-NC: healthy rats on normal (no-SAMEC) diet without exercise, H-NE: healthy rats on normal (no-SAMEC) diet with exercise, H-SC: healthy rats on SAMEC-supplemented diet without exercise, H-SE: healthy rats on SAMEC-supplemented diet with exercise, IR-NC: insulin-resistant rats on normal (no-SAMEC) diet without exercise, IR-NE: insulin-resistant rats on normal (no-SAMEC) diet with exercise, IR-SC: insulin-resistant rats on SAMEC-supplemented diet without exercise, and IR-SE: insulin-resistant rats on SAMEC-supplemented diet with exercise.

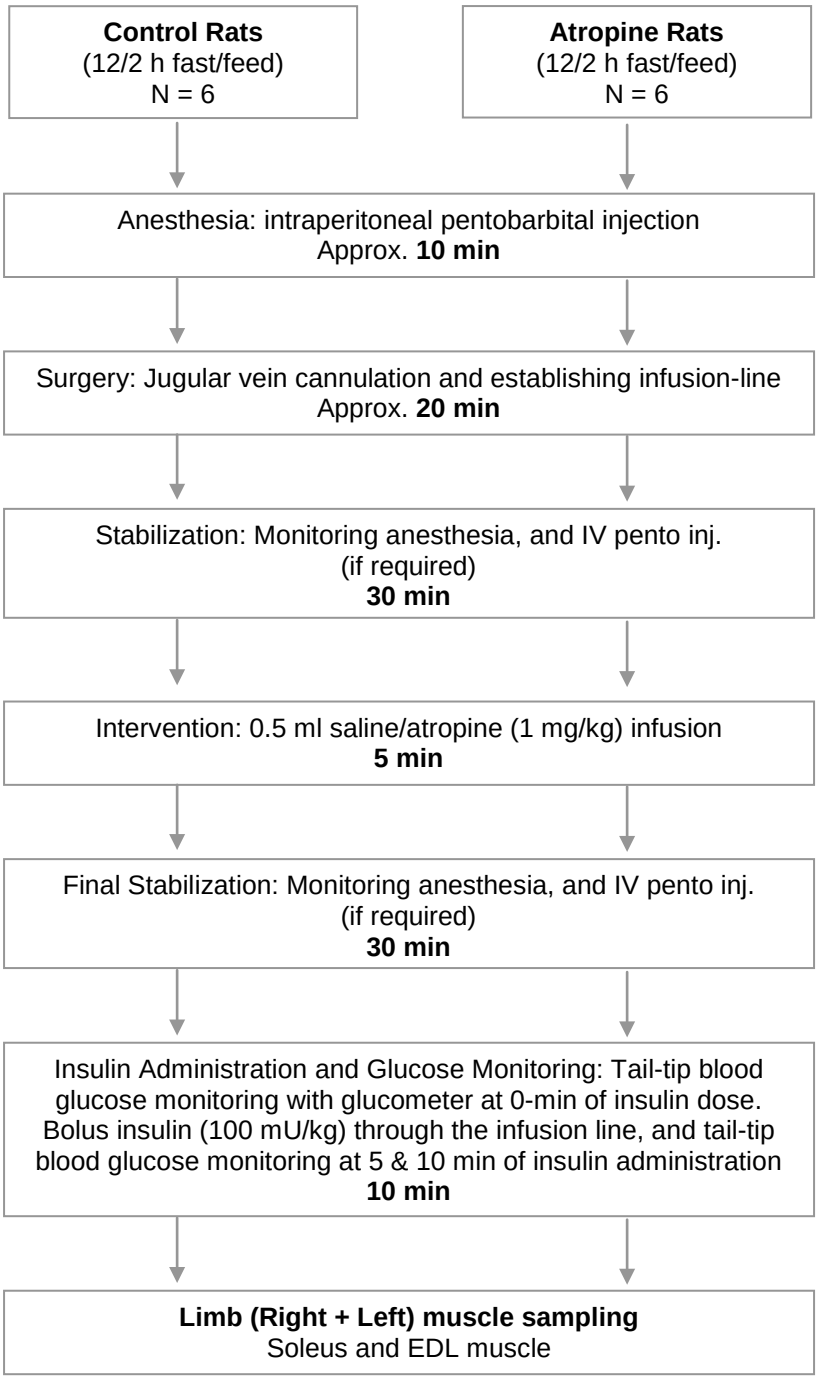


Fig. 9: Flow-chart of protocol 4 and 5 design: Hind-limb muscle samples were collected from HISS-positive (control) and HISS-negative (atropine) rats.

3.2 Dietary Intervention

In protocol 1 (*Impact of voluntary exercise in normal aging rats*) and signaling study, the dietary protocol was straightforward. The rats were maintained on normal rat chow and drinking water throughout the period of housing and during interventions.

In protocol 2 (*Impact of voluntary exercise in diet-induced insulin resistant rats*), 3 dietary intervention models were utilized. These animal models were previously shown to develop insulin resistance (Afonso et. al., 2010; Ribeiro et. al., 2005) that does not reverse spontaneously upon withdrawal of the dietary insults (pilot study, and confirmed in this study). The rats in the Hf-9 group were maintained on a high-fat diet (D12492, 60% kcal fat, Research Diet Inc, New Brunswick, NJ, USA) for 4 weeks to develop insulin resistance. During the final week prior to experimentation they were returned back to the normal diet (Prolab RMH300, PMI feeds, St. Louis, USA) while receiving exercise/no-exercise intervention. The rats in the Sc-14 and Sc-21 groups were maintained on the normal diet and normal drinking water while having free access to a 35% sucrose solution for a period of 9 and 16 weeks respectively. The sucrose supplementation was removed for the week prior to the experiments. At this time, the paired rats were randomly assigned to the non-exercise control or exercise subgroups (Fig. 7).

In protocol 3 (*Impact of SAMEC and voluntary exercise in normal and insulin resistant rats*) the dietary interventions were as follows. Healthy rats in the H-NC, H-NE, H-SC and H-SE groups were maintained on normal diet and water, with only the H-SC

and H-SE groups switched to the SAMEC-supplemented diet for 1 week prior to the experimentation. The healthy rats were randomly allocated to receive exercise/no-exercise. The rats in IR-NC, IR-NE, IR-SC and IR-SE groups had free access to 35% sucrose solution for 9 weeks to develop insulin resistance. The rats in these insulin resistant groups were withdrawn from the sucrose solution for a week before the experiments, and only the IR-SC and IR-SE groups were switched to the SAMEC-supplemented diet. Rats in insulin resistant groups were subjected to the exercise/no-exercise intervention in the final week. The interventions in the experimental groups are presented in the flow-chart (Fig. 8). The SAMEC supplemented diet contains normal chow supplemented with SAM (0.5 g/kg diet), vitamin E (1500 IU/kg diet), and vitamin C (12.5g/kg diet). The antioxidant supplemented chow was vacuum-purged with nitrogen and sealed in foil bags by the supplier (Research Diet Inc, New Brunswick, NJ, USA). Given the average daily food consumption of 20 g and considering the average body weight of approximately 0.5 kg in this age-group of rats, the approximate daily intake for SAM is 20 mg/kg body weight, vitamin E is 60 IU/kg body weight and vitamin C is 500 mg/kg body weight.

3.3 Exercise Intervention

The exercise intervention was identical in exercise studies (protocol 1 – 3) that employed voluntary running-wheel exercise for a week. Seven days before the experiment, a rat from the paired-cage was randomly selected and tail-marked for voluntary running. The other rat did not receive any exercise intervention and was used as a control. Both of the rats were maintained on the assigned diet and normal water

during this 1-week period. The exercised rat was kept in the voluntary running-wheel cage (Lafayette Instrument, Lafayette, Indiana, USA) for approximately 18 hours (15:00 to 09:00) with free access to the running wheel; for the rest of the day (09:00 to 15:00) the rat was returned to the cage-mate. This schedule was adopted to reduce the degree of stress on the rats. The revolutions run by the exercised rat were recorded each day. The 7-day average exercise performance was calculated as running distance (km/day) considering the circumference of the wheel of 143.7 cm. We did not set any standard range for exercise, or inclusion/exclusion criteria based on the exercise performance. This approach allowed us to perform correlation studies of different metabolic factors against variable degrees of exercise.

3.4 Fast-Feed Approach

The rats in all protocols (1 – 5), including that in the signaling studies, underwent a fast-feed procedure to study the MIS process. All rats underwent a fasting period of 12 hours (19:00 to 07:00) and a refeeding phase of 2 hours (07:00 to 09:00) before the acute experimentation. This fast-feed protocol maximizes food intake, necessary to elicit an optimal feeding signal for HISS-release. Experiments for exercised rats were done at least 24 hours after the last exercise session in order to avoid the acute effects of exercise.

3.5 Surgical Preparations

3.5.1 Exercise Studies (Protocol 1 – 3)

After feeding, the rat was weighed and anesthetized with an intraperitoneal injection of sodium pentobarbital (54.7 mg/kg, CEVA Sante Animal S.A., Libourne,

France). Whole body adiposity was determined using bioelectrical impedance method (Hall et al. 1989; Ming et al. 2009). The rat was then placed on its back and body temperature was monitored with a rectal probe thermometer. Temperature was maintained at 37.5º ± 0.5ºC using a heated surgical table and a heat lamp above the table. An arterio-venous (AV) shunt, which allows uninterrupted blood flow from the artery to the vein, was established by cannulating the right femoral artery and vein (PE60 polyethylene tubing, Becton Dickinson) and connecting them with silicon tubing (Lautt et al. 1998) (Fig. 10). The AV-shunt was connected to a transducer for monitoring heart rate and arterial blood pressure after briefly occluding the venous end of the shunt. Blood sampling for glucose measurements was done by puncturing the arterial side of the shunt. Infusion of pharmacological agents was done through the venous side. Supplemental anesthetic (5 ml/kg/h or 2.17 mg/kg/h of sodium pentobarbital in sterile-heparin solution) was infused throughout the experiment.

3.5.2 Signaling Studies (Protocol 4 – 5)

After feeding, the rat was weighed and anesthetized with an intraperitoneal injection of sodium pentobarbital (54.7 mg/kg, CEVA Sante Animal S.A., Libourne, France). The rat was then placed on its back and body temperature was monitored with a rectal probe thermometer. Temperature was maintained at 37.5º ± 0.5ºC using a heated surgical table and a heat lamp above the table. A catheter infusion-line was established into the jugular vein for supplemental anesthesia (pentobarbital sodium, 2.17 mg $kg^{-1}hr^{-1}$) and infusion of pharmacological agents.

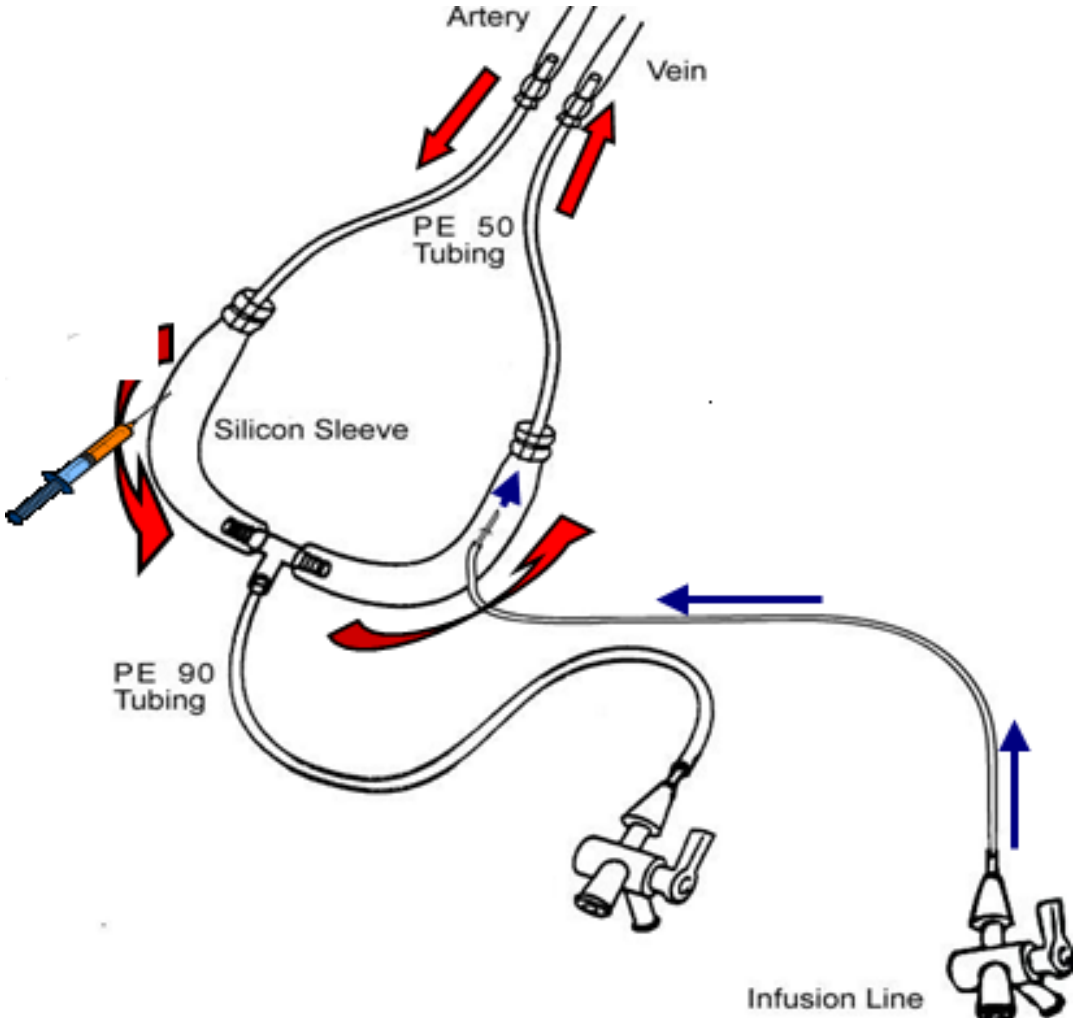


Fig. 10: Arterial-venous shunt: The arterial-venous shunt is established through cannulation of the right femoral artery and vein. The shunt was connected to the transducer for monitoring of physiological parameters like heart rate, arterial pressure and loop pressure. The arterial side of the shunt was used for blood sampling and the venous was used for infusion of various pharmacological agents. [*Adapted from: Lautt WW, Wang X, Sadri P, Legare DJ, Macedo MP (1998), Rapid insulin sensitivity test (RIST), Can J Physiol Pharmacol., 76: 1080-1086, © 2008 Canadian Science Publishing or its licensors. Reproduced with permission*]

3.6 Experimentations

The experiments and samplings varied according to the study design and objective, and are described below.

3.6.1 Exercise Studies (Protocol 1 – 3)

3.6.1.1 Rapid Insulin Sensitivity Test (RIST)

The RIST was used to measure insulin sensitivity (Lautt et al. 1998) (Fig. 11). Following surgery the rat was stabilized for 30 min. The baseline blood glucose was then determined through sampling of the arterial blood at 5-min intervals, until three successive stable values were obtained. An IV-bolus insulin infusion (50 mU/kg in 0.5 ml saline at 0.1 ml/min) was started and continued for 5 min. Approximately 1 min after the insulin infusion started, the first glucose sample was taken and glucose infusion was commenced through the venous line. Arterial glucose levels were determined by blood sampling (25 µl) every 2-min, while glucose was infused at a variable rate to maintain euglycemia. Arterial blood sampling and glucose infusion continued until the blood glucose level returned to control level and no further glucose infusion was required. The RIST index is the amount of glucose (mg/kg) infused over the test period to maintain euglycemia, following the single bolus infusion of insulin. A data acquisition system (National Instruments Lab-View, Austin, TX, USA) combined with application software (available on request from the authors) was used to record and analyze the mean arterial blood pressure, to calculate the RIST index, and to provide real-time monitoring of adherence to the euglycemic baseline. The software program calculated accuracy and precision for the maintenance of the euglycemic target baseline. If either deviated by more than 5%, the entire RIST was considered to be invalid, and was discarded. The

experimental protocol consists of two repeated RISTs, separated by a stabilization period of 30 min. The first RIST determined the dynamic glucose uptake response to both insulin and HISS. The second RIST was preceded by an IV infusion of atropine (1 mg/kg), which causes inhibition of HISS-release (Lautt 2004), thereby measuring only the direct insulin action. The difference between the two RISTs represents the HISS-dependent glucose uptake.

3.6.1.2 Biochemical Sampling and Analysis

A blood sample (80 µl) was drawn at the start of the RIST, and centrifuged at 16,000 g for 10 min. The serum was isolated, and preserved at -80ºC for further assay of insulin. The serum insulin measurement was done by ELISA (ALPCO Diagnostics, Salem, NH, USA). At the completion of both RISTs, liver samples were collected and stored at -80ºC for determination of the hepatic glutathione (GSH) level (Bioxytech GSH-420, OxisResearch, Foster City, CA, USA). After liver sampling the rat was euthanized and three regional fat pads (perinephric, epididymal, and perienteric) were collected, weighed, and compared with the bioimpedance estimate of total body fat content.

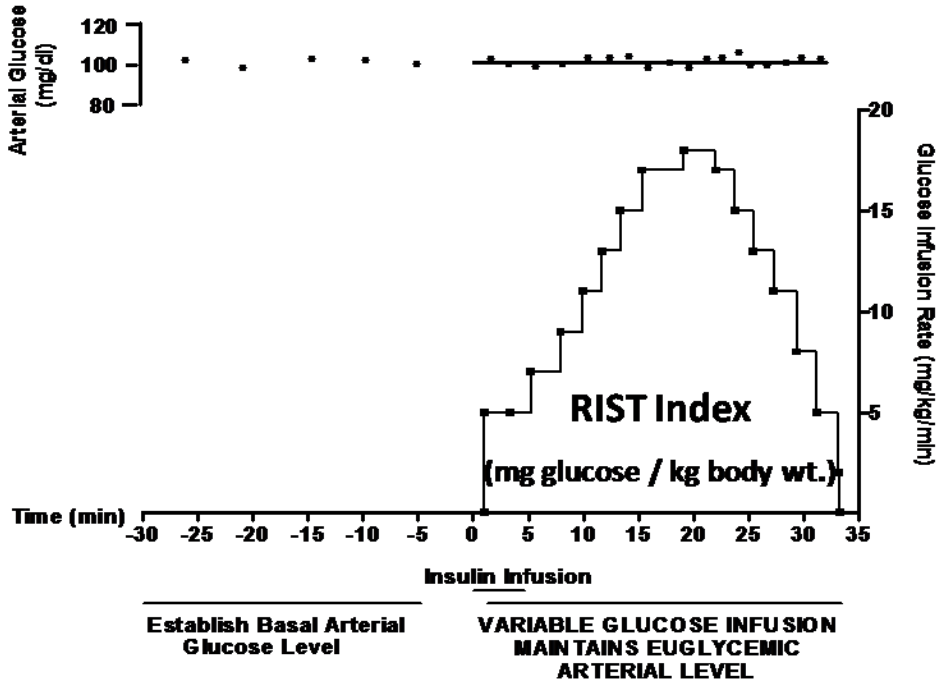


Fig. 11: A presentation of the rapid insulin sensitivity test (RIST): It is a hyperinsulinemic euglycemic clamp technique for measurement of insulin sensitivity. The baseline glucose was determined by sampling blood at 5 min interval after surgery and during 30 min stabilization period. The euglycemic baseline was set from 3 stable readings prior to starting the RIST. Insulin was infused at a dose 50 mU/kg, and glucose was infused at a variable rate to maintain the euglycemic baseline. The area under the curve (AUC) represents the RIST index, which is the amount of glucose (in mg) infused per kg body weight to maintain euglycemia during the RIST. [*Adapted from: Lautt WW, Wang X, Sadri P, Legare DJ, Macedo MP (1998), Rapid insulin sensitivity test (RIST), Can J Physiol Pharmacol., 76: 1080-1086, © 2008 Canadian Science Publishing or its licensors. Reproduced with permission*]

3.6.2 Signaling Studies (Protocol 4 – 5)

3.6.2.1 Tissue Sampling

The HISS-positive and HISS-negative rats were treated with saline and atropine (1 mg/kg) respectively through the jugular infusion line (Fig. 9). The rat was stabilized and an insulin-bolus (100 mU/kg) was administered through the jugular vein. Tail-tip blood sampling and glucose monitoring were done twice: before insulin administration and prior to the tissue sampling. Ten min after insulin infusion, the hind-limb muscles (soleus and EDL) were rapidly collected, frozen into liquid nitrogen and stored in the freezer (- 80º C).

3.6.2.2 Western Blotting

The experiments were conducted in Dr. Todd Duhamel's Lab. at Saint Boniface Research Center, Winnipeg. The tissues were homogenized in appropriate buffer, the protein content was estimated, and the whole homogenate was used for Western blots. Sample proteins (20-40 µg) were resolved by SDS-PAGE, transferred to the membrane, immunoblotted and detected. To quantify the signal activation pathways, the total and phosphorylated receptor/substrate (Table 3) were measured. Each of the blots was normalized by using an internal loading control and further standardized with β-actin. To study insulin signaling pathway, the insulin receptor- β (INR- β) and insulin receptor substrate- 1 (IRS-1) was targeted. AMPK signaling was also studied by determining the degree of activation of the pathway. The phosphorylation sites for INR- β, IRS-1, and AMPK were Tyr-1345, Ser-312, and Thr-172 respectively. Each signal was standardized by using an internal loading control, and further normalized with the respective β-actin

signal. The percent activation of the receptor/substrate was calculated from the ratio of the phosphrylated to the total form.

3.6.2.3 Glycogen Estimation

The protocol was received from Dr. Goodyear's lab, Joslin Diabetes Center, Harvard Medical School. Muscle tissue (10 mg) was dissolved in 300 ul of solution mix containing 30% KOH and 5% Na_2SO_4 at 70ºC for 15 min. Glycogen was then precipitated by adding 900 ul of absolute alcohol and stored overnight at - 20ºC. The precipitates were collected by centrifugation at 13,000*g* for 5 min. The glycogen was hydrolyzed in 50 ul of 6N H_2SO_4 at 100ºC for 45 min and cooled. Samples were neutralized with 150 ul of 2N NaOH and 25 ul of Tris buffer (pH 8.0). Glucose was measured using the glucose HK reagent (Sigma Chemical, catalog # 2820-1) and taking absorbance at 340 nm (Higaki et. al., 2001).

3.6.2.4 Glucose 6 Phosphate (G6P) Assay

The hindlimb tissue (Soleus and EDL) samples (30 mg) were homogenized in 500 ul of ice cold PBS (pH 6.5-8.0). The samples were centrifuged at 13,000*g* for 10 min. The supernatant was collected and G6P content was measured using G6P assay kit (Abcam, catalog # ab83426).

TABLE 3: The experimental conditions for testing different substrates with Western blots:

	Membrane Preparation			**Membrane Treatment/Imaging**					
Type	Gel	Protein load	Transfer	Blocking		1° Ab		2° Ab	Imaging
				Type	Incubation	Type	Incubation		
INR	7.5%	30-50 µg	22-24V, 2h, cold room	5% milk in TBST	Over night	1:1000 in Milk (Cell Signaling)	2 h	1:2000 in TBST (Cell Signaling)	5-10 min exposure
p-INR	6%	40-50 µg	22-24V, 2h, cold room	5% milk in TBST	Over night	1:1000 in TBST (Cell Signaling)	2 h	1:2000 in TBST (Cell Signaling)	5-10 min exposure
IRS-1	6%	40-50 µg	22-24V, 2h, cold room	5% milk in TBST	Over night	1:1000 in TBST (Abcam)	2 h	1:3000 or 5000 in TBST (Abcam)	5-10 min exposure
p-IRS-1	6%	40-50 µg	22-24V, 2h, cold room	5% milk in TBST	Over night	1:1000 in TBST (Abcam)	2 h	1:3000 or 5000 in TBST (Abcam)	5-10 min exposure
AMPK	7.5%	20-30 µg	Regular	5% BSA in TBST	Over night	1:1000 in TBST (Santa Cruz)	2 h	1:5000 in TBST (Santa Cruz)	5-10 min exposure
p-AMPK	7.5%	20-30 µg	Regular	5% BSA in TBST	Over night	1:200 in TBST (Santa Cruz)	2 h	1:5000 in TBST (Santa Cruz)	5-10 min exposure
β-Actin	12%	20-40 µg	22-24V, 1h, cold room	5% milk in TBST	Over night	1:1000 in TBST (Cell Signaling)	2 h	1:2000 in TBST (Cell Signaling)	5-10 min exposure

3.6.2.5 Glycogen Synthase Kinase β (GSK3β) and Phospho-GSK3β (pGSK3β) Assays

The hindlimb tissue (Soleus and EDL) samples (50 mg) were added to 500 ul cold 1X RIPA containing 0.1% SDS and protease inhibitors (Calbiochem cocktail III, catalog #539134). Each 10 mL of 1X RIPA was prepared from 10X RIPA (Millipore, catalog # 20-188) and adding 1 ul of protease inhibitor cocktail per ml of 1X RIPA. The tissue was homogenized and the lysate was centrifuged at 12,000 rpm for 10 minutes at 4ºC. The supernatant was collected and used to determine the content of GSK3β and pGSK3β (Ser-9) using ELISA kits (Millipore, catalog # 17-471 and 17-472 respectively).

3.7 Data Analysis

Values are presented as means ± SE. The comparisons between the control and intervention groups were done by using t-tests, one-way ANOVA and multivariate ANOVA (MANOVA) as appropriate. Statistical significance was considered at $p<0.05$. Linear regression analysis was performed to determine the correlations of any two variables. The signal intensity for Western blots was estimated by Bio-Rad Quantity 1 software. GraphPad Prism 5.0 and IBM SPSS Statistics 19 were used as statistical software to perform the analysis.

SECTION IV

RESULTS

4. RESULTS

4.1 Protocol 1: Impact of voluntary exercise in normal aging rats

4.1.1 Exercise Performance

In all age groups, there was an increase in the day-to-day exercise over the 7-day period. The average exercise in 9 (n=7), 14 (n=10), and 21- (n=8) week groups was 2.1 ± 0.20, 1.3 ± 0.16 and 0.68 ± 0.06 km day^{-1} respectively (Fig. 12).

4.1.2 Insulin/HISS Dynamic Action

Insulin sensitivity was quantified as the whole body glucose uptake after a bolus infusion of insulin (Fig. 13). The total glucose uptake has two components; HISS-dependent and -independent glucose uptake. The HISS-independent glucose uptake refers to the direct action of insulin. The total glucose uptake in the controls of 9, 14 and 21-week groups was 140.1 ± 11.0, 101.9 ± 6.3, and 89.3 ± 4.1 mg kg^{-1} respectively. The total glucose uptake in age-matched exercise groups was 185.3 ± 10.4, 142.3 ± 6.8, and 113.9 ± 3.8 mg kg^{-1} respectively. Voluntary exercise caused statistically significant improvements in total glucose uptake in different age groups by 32.3% ($p<0.05$), 39.6% ($p<0.001$) and 27.6% ($p<0.001$) respectively.

Exercise caused minor improvements in the HISS-independent glucose uptake in most of the study groups. In 9, 14 and 21-week control groups, the HISS-independent glucose uptakes were 84.5 ± 9.3, 61.3 ± 4.4, and 58.3 ± 3.4 mg kg^{-1} respectively. In exercised rats the HISS-independent glucose uptakes were 100.6 ± 7.0, 74.1 ± 3.6, and

59.3 ± 2.2 mg kg^{-1} respectively. The net changes in HISS-independent glucose uptake with exercise were 16.1 (p=NS), 12.8 ($p<0.05$), 1.0 (p=NS) mg kg^{-1} respectively.

The HISS-dependent glucose uptake in 9, 14 and 21-week control groups was 55.6 ± 10.7, 40.6 ± 4.6, and 31.0 ± 5.3 mg kg^{-1} respectively. The HISS-dependent glucose uptakes in exercised groups were 84.6 ± 6.9, 68.2 ± 4.5, and 54.6 ± 3.4 mg kg^{-1} respectively (Fig. 13). There were improvements in the absolute HISS-component by 29.1 ($p=0.052$), 27.6 ($p<0.001$), and 23.6 ($p<0.01$) mg kg^{-1} respectively. In order to compare the absolute impact of exercise among different age groups, we quantified the improvements in HISS-action against the unit (1 km/day) exercise (Fig. 13). Although aging caused progressive decrease in exercise performance, the therapeutic efficacy per unit exercise in improving HISS-action increased in older rats. The overall improvements in whole body insulin sensitivity were found to be correlated with the extents of exercise; and a linear relationship exists between exercise performance and HISS-dependent glucose uptake (Fig. 14).

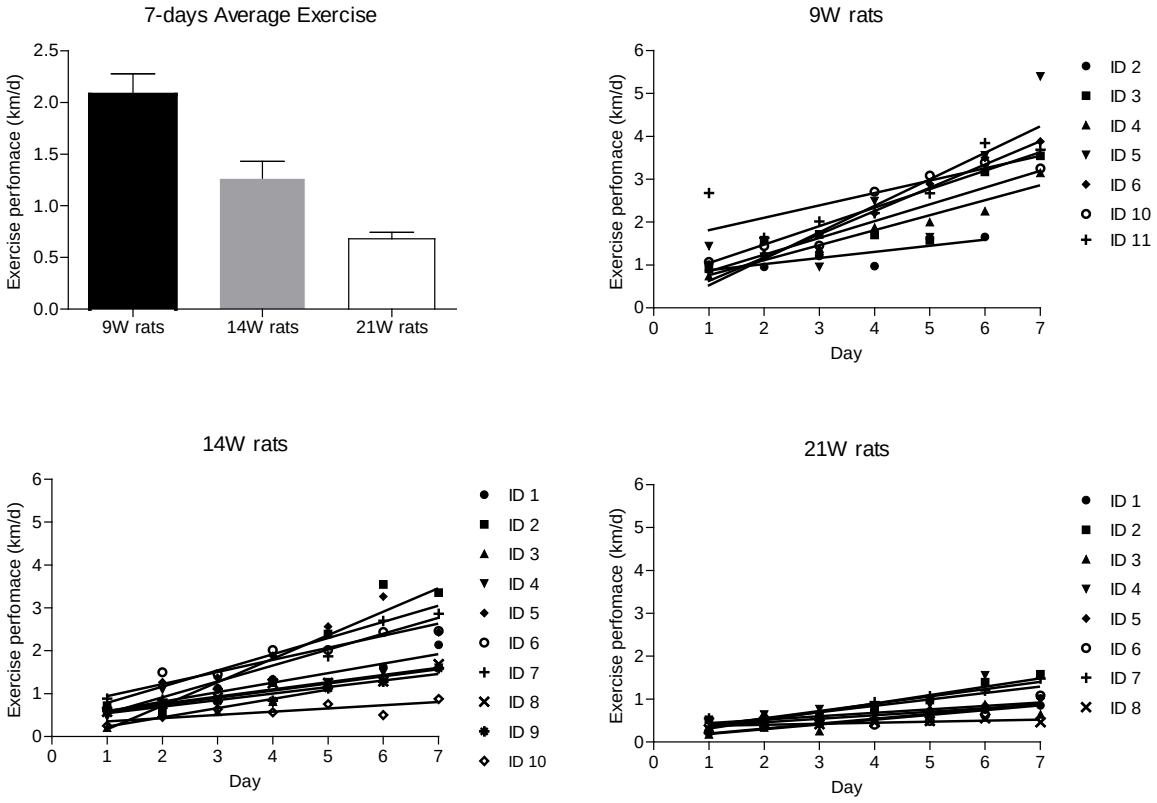


Fig. 12: Exercise performance (protocol 1): The 7-days average voluntary running-wheel exercise and day-to-day performance enhancement in 9 (n=7), 14 (n=10), and 21- (n=8) week age groups. Note: In 9W exercise group, a few rats were lost during surgery and therefore eliminated from the study. [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

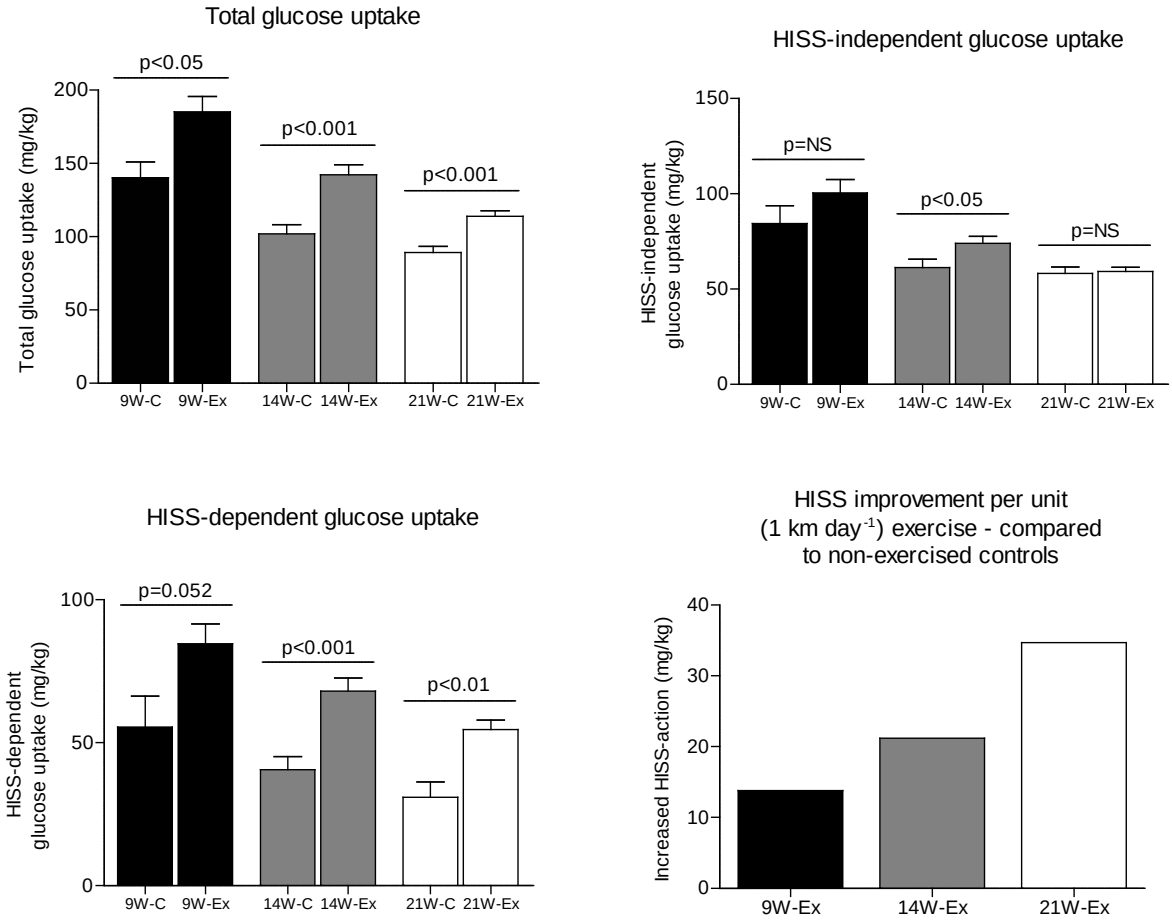


Fig. 13: Insulin sensitivity (protocol 1): The insulin sensitivity in rats of different age groups was measured by the rapid insulin sensitivity test (RIST), which represents the amount of glucose uptake (mg kg-1) in response to a bolus dose of insulin. The total glucose uptake in exercised rats is increased compared to the age-matched control rats. Exercise increased HISS-dependent glucose uptake, which contributes to the major improvements in insulin sensitivity. Quantification of the improvements in HISS-action per unit (1 km day-1) exercise shows that increased efficacy of exercise to improve HISS-action is attained among older rats. [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

4.1.3 Body Composition

Aging in rats caused progressive gain in body weight and whole body adiposity. The visceral fat content, measured as combined mass of perinephric, epididymal and perienteric fat pads, was also increased with age. The body weights of the control vs. exercise of 9, 14, and 21-week groups were 438.6 ± 11.4 vs. 399.7 ± 10.5, 556.9 ± 18.0 vs. 496.1 ± 8.2, and 671.5 ± 22.2 vs. 630.1 ± 14.8 gm respectively, representing an exercise-induced reduction in body weight by 8.9% ($p<0.05$), 10.9% ($p<0.01$), and 6.2% (p=NS) in the respective age groups.

We pooled data of all age control and exercise groups for the visceral fat content (in gm kg^{-1} body weight), and plotted against the whole body adiposity (measured by bioelectrical impedance) (Hall et. al., 1989). A linear and positive correlation ($r^2 = 0.65$) was found in the combined age groups. This suggests that there is a coordinated increase in visceral fat mass with the increase in whole body adiposity related to aging (Figure 15). Exercise decreased whole body adiposity and visceral fat mass in all age groups. An inverse relationship ($r^2 = 0.40$) was found between the visceral fat mass and the exercise performance in pooled age groups (Fig. 15). We also found a negative correlation ($r^2 = 0.25$) between the HISS-action and visceral fat mass, and negative correlation ($r^2 = 0.14$) between the HISS-action and serum insulin concentration (Fig. 16). The whole body adiposity (% fat mass) and visceral fat were increased with age, and decreased significantly by exercise in 9 and 14-week groups. The lean body mass was determined by subtracting total body fat (calculated from % fat mass) from body weight. The muscle-mass was increased with age, but not altered by exercise in corresponding age groups (Fig. 17).

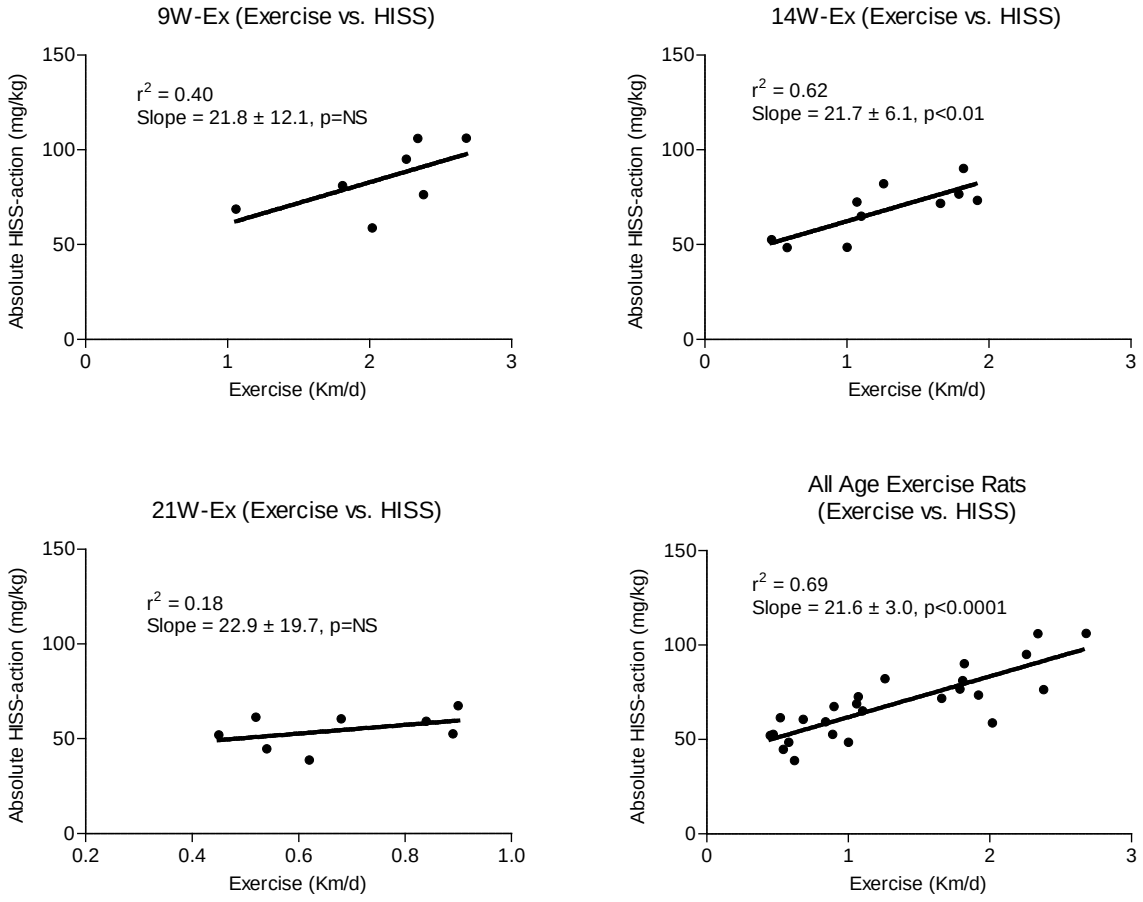


Fig. 14: HISS vs. exercise (protocol 1): The relationship between HISS-action and exercise performance among different age groups. Linear correlation was found between HISS-action and amount of exercise in 9, 14, and 21-week age groups. The slope of the regression curves is similar for all ages. Note that the exercise axis is on a different scale for the oldest rats, which exercised the least. When HISS action is plotted against the amount of exercise for pooled age groups, a statistically significant correlation is obtained. [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

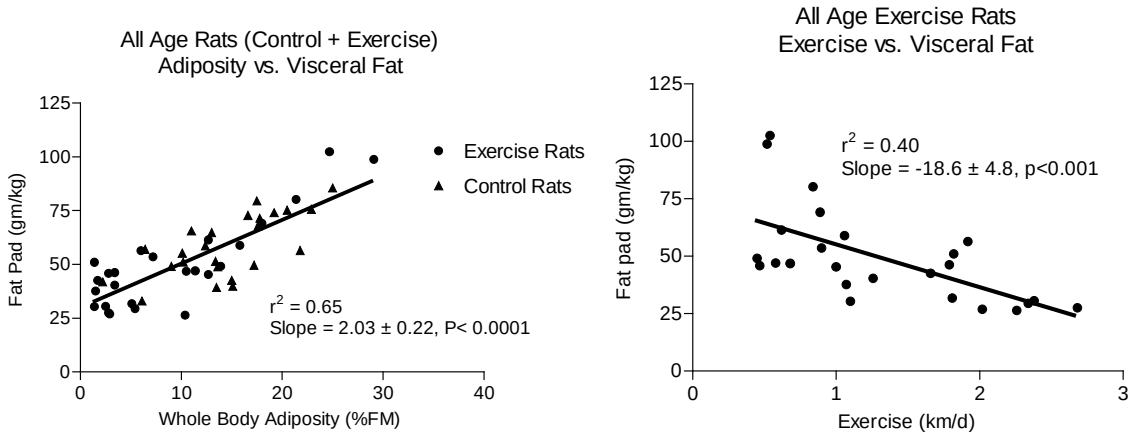


Fig. 15: Fat pad vs. %FM and fat pad vs. exercise (protocol 1): The whole body adiposity was measured as percent total body fat mass (%FM) and determined by bioelectrical impedance analysis. The visceral fat mass was expressed as total fat pad (gm kg-1) collected from the perinephric, epididymal, and perienteric regions. The linear relationships between whole body adiposity and visceral fat mass are shown for the control and exercise rats of pooled age groups. The negative correlation between exercise and visceral fat content is also shown for the combined age groups. (Note: Left panel: Exercise group: $r^2 = 0.73$, slope = 2.24 ± 0.28, $p < 0.0001$; Control group: $r^2 = 0.45$, slope = 1.74 ± 0.41, $p < 0.001$). [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

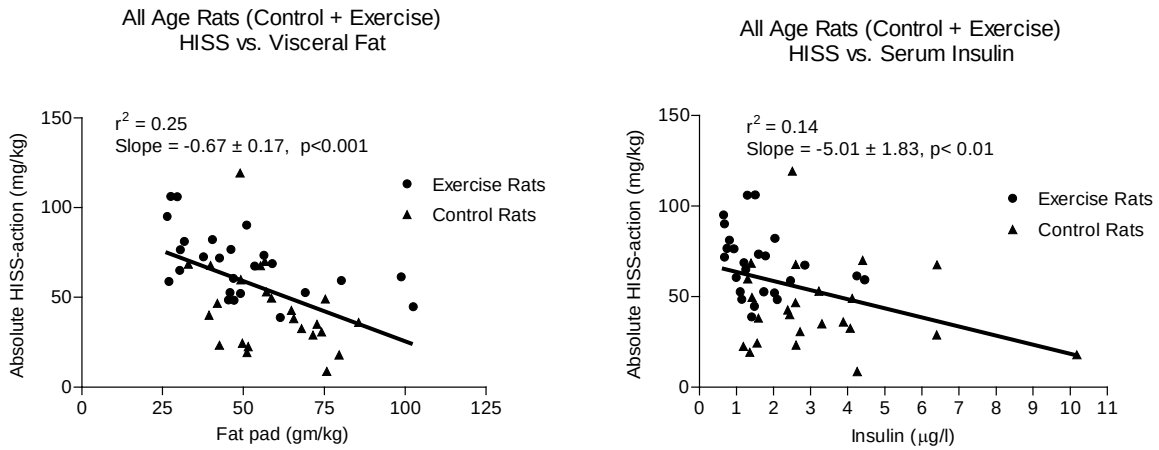


Fig. 16: HISS vs. fat pad and HISS vs. serum insulin concentration (protocol 1): The negative associations between HISS-action and visceral fat mass/serum insulin are shown for the control and exercise rats of pooled age groups. As HISS action decreased, fat mass and postprandial serum insulin concentration increased. (Note: Left panel: Exercise group: $r^2 = 0.29$, slope = -0.47 ± 0.15, $p < 0.01$; Control group: $r^2 = 0.16$, slope = -0.66 ± 0.32, $p = 0.052$. Right panel: Exercise group: $r^2 = 0.08$, slope = -5.11 ± 3.64, p = NS; Control group: $r^2 = 0.02$, slope = -1.72 ± 2.40, p = NS). [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

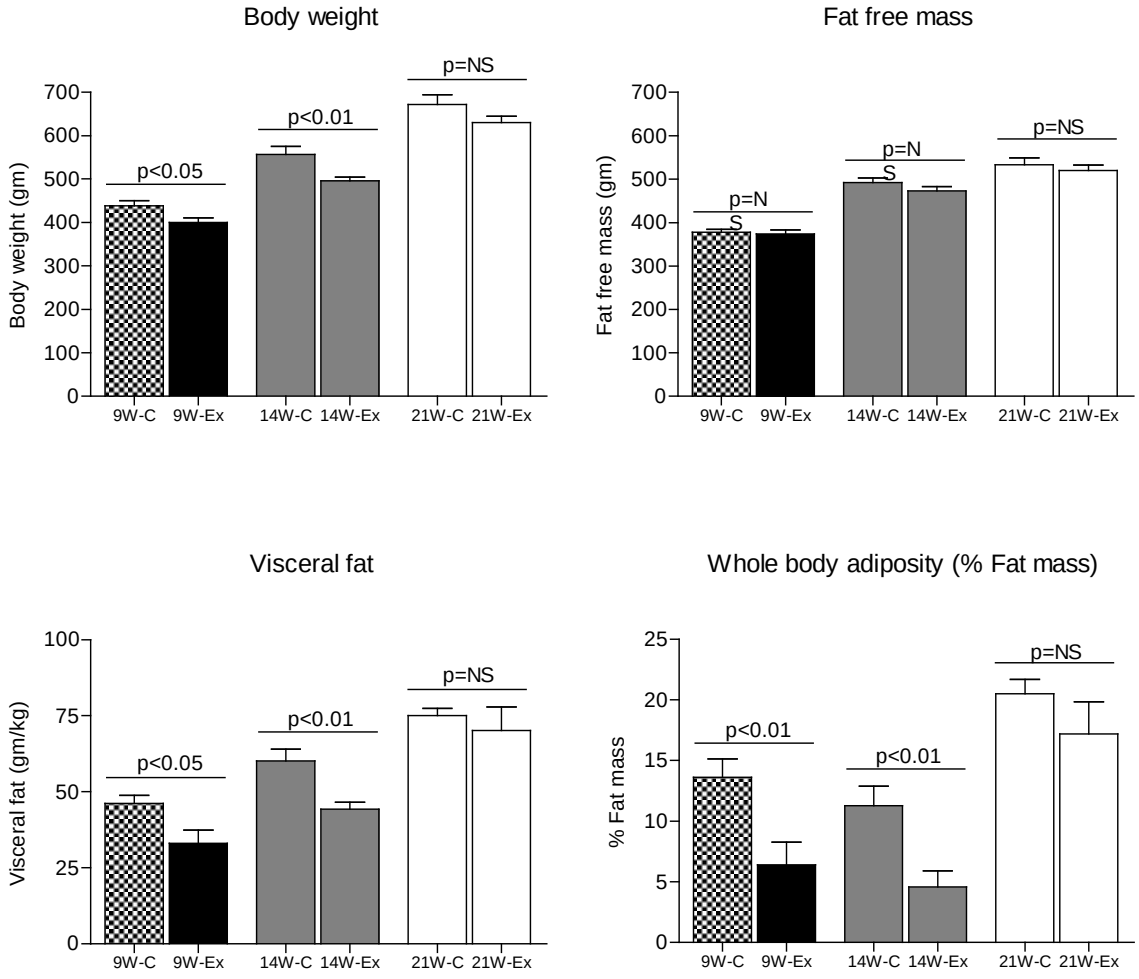


Fig. 17: Body composition (protocol 1): Body weight is increased with age and decreased by exercise. Alteration in whole body adiposity and visceral fat contributes to the progressive weight gain in aging (or weight loss with exercise). The lean body mass (fat free mass) is increased with age, as HISS decreases. The fat free mass is not changed with exercise, and increased muscle-mass with age does not compensate for the decrease in HISS action. [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

4.1.4 Blood Glucose, Serum Insulin and Hepatic Glutathione

Postprandial blood glucose and serum insulin levels were determined by taking samples from the arterial side of the AV-shunt, 30 min after surgery and approximately 2 hours after feeding. A statistically significant ($p<0.01$ and $p<0.05$ respectively) decrease in postprandial glycemia was found in 9 and 14-week rats with exercise, compared to the age-matched controls. Postprandial serum insulin concentration was also decreased significantly ($p<0.05$, $p<0.05$, and $p<0.01$ respectively) with exercise in 9, 14, and 21-week age groups. The homeostatic model assessment for insulin resistance (HOMA - IR) was done using the blood glucose level and serum insulin concentration. HOMA-IR value {(glucose level X insulin level)/405} was decreased by exercise in all age groups. Hepatic glutathione level was not significantly affected by age or exercise (Table 4).

TABLE 4: The postprandial blood glucose, serum insulin, and hepatic glutathione concentrations in the age-matched control and exercise groups. [*Reprinted from: Chowdhury KK, Legare DJ, Lautt WW (2011), Insulin sensitization by voluntary exercise in aging rats is mediated through hepatic insulin sensitizing substance (HISS); Exp Gerontol., 46(1):73-80, © 2010 with permission from Elsevier*]

	9W groups		**14W groups**		**21W groups**	
	Control (N=9)	Exercise (N=7)	Control (N=9)	Exercise (N=10)	Control (N=6)	Exercise (N=8)
Postprandial Blood Glucose (mg/dl)	114.8 ± 2.4	103.8 ± 2.5	114.3 ± 3.8	103.1 ± 1.7	108.6 ± 3.3	104.1 ± 2.6
Exercise-induced Decrease in Postprandial Blood Glucose	11.0 ± 3.5 (p<0.01)		11.2 ± 4.0 (p<0.05)		4.5 ± 4.1 (p=NS)	
Serum Insulin (µg/l)	2.81 ± 0.55	1.26 ± 0.23	3.02 ± 0.93	1.31 ± 0.17	4.24 ± 0.49	2.40 ± 0.47
Exercise-induced Decrease in Serum Insulin	1.54 ± 0.65 (p<0.05)		1.71 ± 0.90 (p<0.05)		1.84 ± 0.69 (p<0.01)	
HOMA-IR ($mg.dl^{-1}$-$\mu g.l^{-1}$)	0.80 ± 0.16	0.33 ± 0.07	0.87 ± 0.28	0.34 ± 0.04	1.14 ± 0.14	0.62 ± 0.12
Exercise-induced Decrease in HOMA-IR	0.47 ± 0.19 (p<0.05)		0.53 ± 0.27 (p<0.05)		0.52 ± 0.19 (p<0.01)	
Hepatic Glutathione (µmol/g)	6.03 ± 0.17	5.87 ± 0.10	6.57 ± 0.14	6.22 ± 0.26	6.38 ± 0.09	6.07 ± 0.26
Exercise-induced Decrease in Hepatic Glutathione	0.16 ± 0.21 (p=NS)		0.35 ± 0.31 (p=NS)		0.31 ± 0.31 (p=NS)	

4.2 Protocol 2: Impact of voluntary exercise in diet-induced insulin resistant rats

4.2.1 Exercise Performance

The day-to-day exercise, for each individual rat over the 7-day training session, was recorded and demonstrated a gradual increase in distance run per day in all groups. The average distance run in Hf-9 (n=6), Sc-14 (n=7), and Sc-21 (n=7) was 1.7 ± 0.28, 1.4 ± 0.37 and 0.60 ± 0.07 km/day respectively (Fig. 18).

4.2.2 Insulin/HISS Dynamic Action

The total glucose uptake in non-exercised rats of the Hf-9, Sc-14, and Sc-21 groups was 75.9 ± 4.9, 68.0 ± 3.5, and 70.9 ± 4.4 $mg \cdot kg^{-1}$ respectively. If we compare these values with that of the age-matched healthy controls (140.1 ± 11.0, 101.9 ± 6.3, and 89.3 ± 4.1 $mg \cdot kg^{-1}$ respectively), the insulin sensitivity is found to have declined significantly with the high-fat diet (HF) and sucrose supplement (SS) in the respective treatment groups. The RIST index of the exercised groups was 119.6 ± 13.1, 108.8 ± 6.0, and 86.5 ± 7.0 $mg \cdot kg^{-1}$ respectively. It indicates that voluntary exercise caused at least partial reversal of the response to insulin (combined HISS-action and HISS independent insulin-action) in the insulin resistant groups (Fig. 19).

The HISS-independent glucose uptake was mostly unaltered by diet and exercise in the respective groups. The HISS-independent glucose uptake in Hf-9, Sc-14, and Sc-21 non-exercised groups was 63.7 ± 3.9, 55.8 ± 3.3, and 64.6 ± 4.3 $mg \cdot kg^{-1}$ respectively. In exercised rats the HISS-independent glucose uptake was 65.7 ± 7.2, 56.7 ± 3.8, and 56.4

± 3.1 mg·kg^{-1} respectively. HISS-independent glucose uptake representing the direct action of insulin did not change significantly with exercise in any group (Fig. 19).

The HISS-dependent glucose uptake was significantly increased by exercise in all study groups, and thus accounted for the major improvements in whole body insulin sensitivity. The HISS-dependent glucose uptake was considerably decreased by diet in Hf-9, Sc-14, and Sc-21 groups to 12.3 ± 3.1, 12.3 ± 2.3, and 6.4 ± 1.2 mg·kg^{-1} respectively. In the exercised groups the HISS-dependent glucose uptake was 53.8 ± 7.9, 52.1 ± 6.4, 30.0 ± 6.1 mg·kg^{-1}, and represents an exercise-induced improvement in the HISS-action by 41.6 ± 7.4 ($p<0.0001$), 39.9 ± 6.2 ($p<0.0001$), and 23.7 ± 6.2 ($p<0.01$) mg·kg^{-1} in the respective groups. Although the oldest rats ran less, the extent of improvement, when expressed as HISS-action per unit exercise (km·day^{-1}), was highest in this group (Fig. 19). The whole body insulin sensitivity was improved in proportion to the amount of performed exercise, and a linear relationship exists between the HISS-dependent glucose uptake and the running distance (Fig. 20).

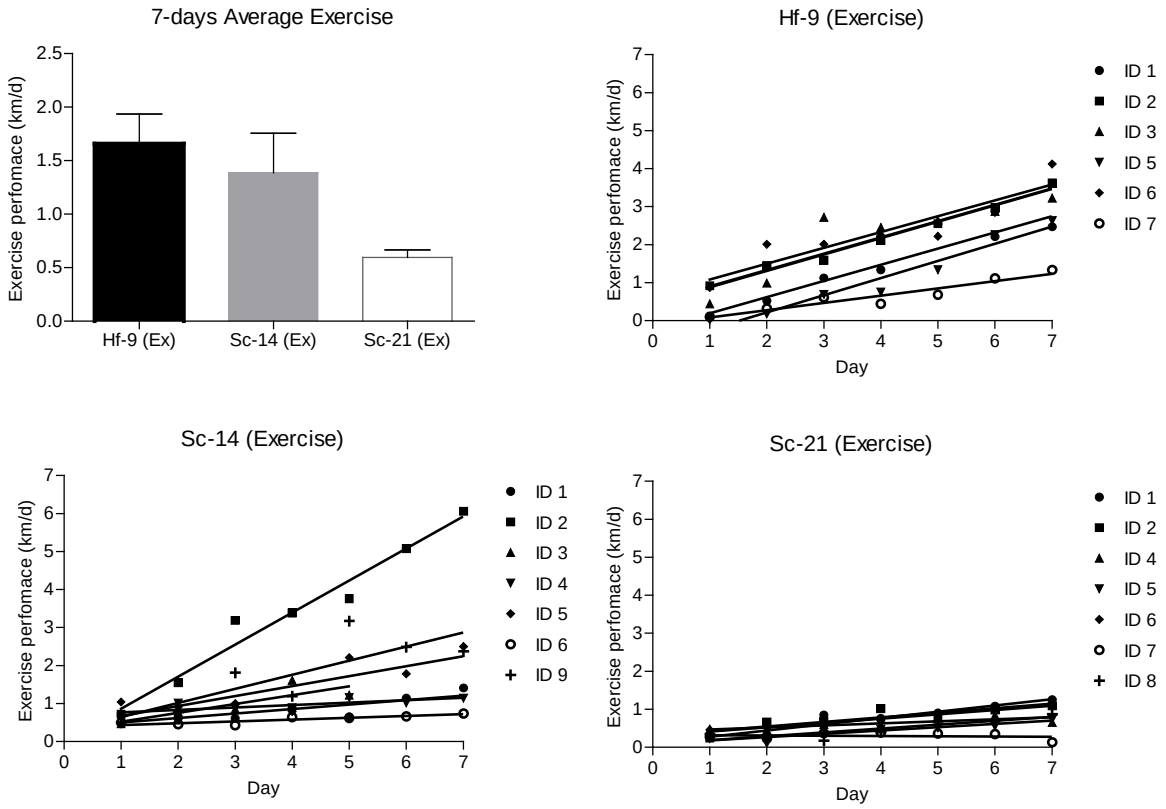


Fig. 18: Exercise performance (protocol 2): The average level of voluntary exercise was reduced with age. The day-to-day running distance for each individual rat was recorded and indicated a gradual increase in exercise performance over the 7-day period. Note that a few rats were lost during the surgery and therefore were eliminated from the study.

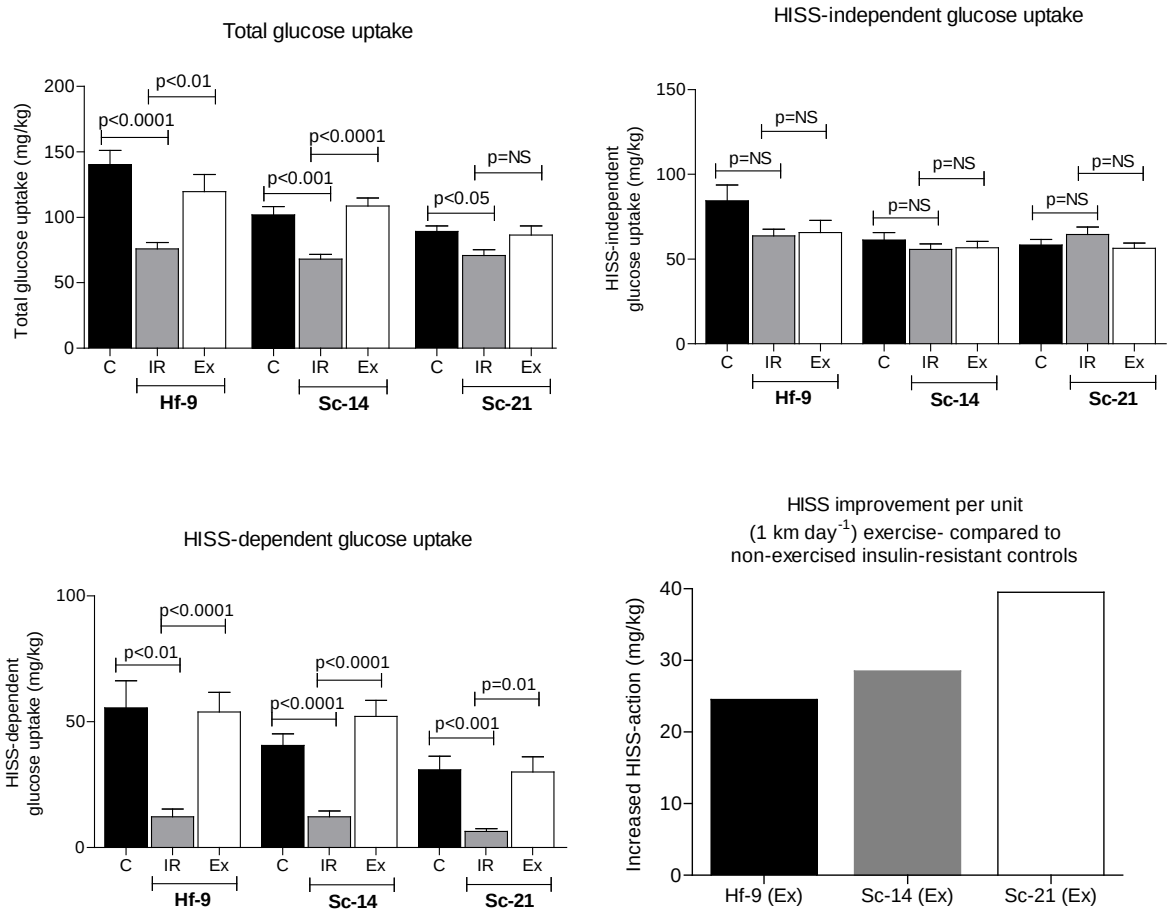


Fig. 19: Insulin sensitivity (protocol 2): The total glucose uptake in insulin resistant rats (IR) was compared to that of the exercised rats (Ex) and age-matched healthy controls (C). The postprandial insulin sensitivity was decreased significantly by dietary insult and reversed by exercise. The development of insulin resistance was caused primarily due to the blockade of HISS-action; conversely exercise reversed the insulin resistance by restoring the HISS-pathway. The HISS-independent glucose uptake (or direct insulin action) was not affected by diet or exercise. Although the older rats ran less, the extent of improvement in HISS-action per unit exercise (1 km·day^{-1}) was higher in these groups.

4.2.3 Body Composition

There was a corresponding increase in fat pad content with the increase in whole body adiposity; a linear relationship (r^2 = 0.75) was obtained when total fat pad was plotted against the percent fat mass (Fig. 21). The extent of decrease in adiposity or fat content by exercise depends on the amount of voluntary running, and an inverse relationship (r^2 = 0.35) exists between the fat pad mass and the running distance (Fig. 21). There was also an inverse relationship between HISS-action and fat pad mass and/or serum insulin concentration (Fig. 22).

Voluntary exercise caused a tendency to decrease body weight in rats of all groups. The body weight of non-exercise vs. exercise in Hf-9, Sc-14, and Sc-21 groups was 425.6 ± 11.2 vs. 397.5 ± 10.4, 570.9 ± 18.8 vs. 516.0 ± 29.4, and 702.3 ± 44.0 vs. 657.1 ± 18.6 gm respectively. The whole body adiposity (% fat mass) was decreased significantly by exercise in most of the study groups. The combined mass of perinephric, epididymal and perienteric fat pads tended to decrease with exercise in all groups, but the changes were not statistically significant. The lean body mass was determined by subtracting total body fat (calculated from % fat mass) from body weight. The muscle-mass was not altered by exercise in the insulin resistant groups (Fig. 23).

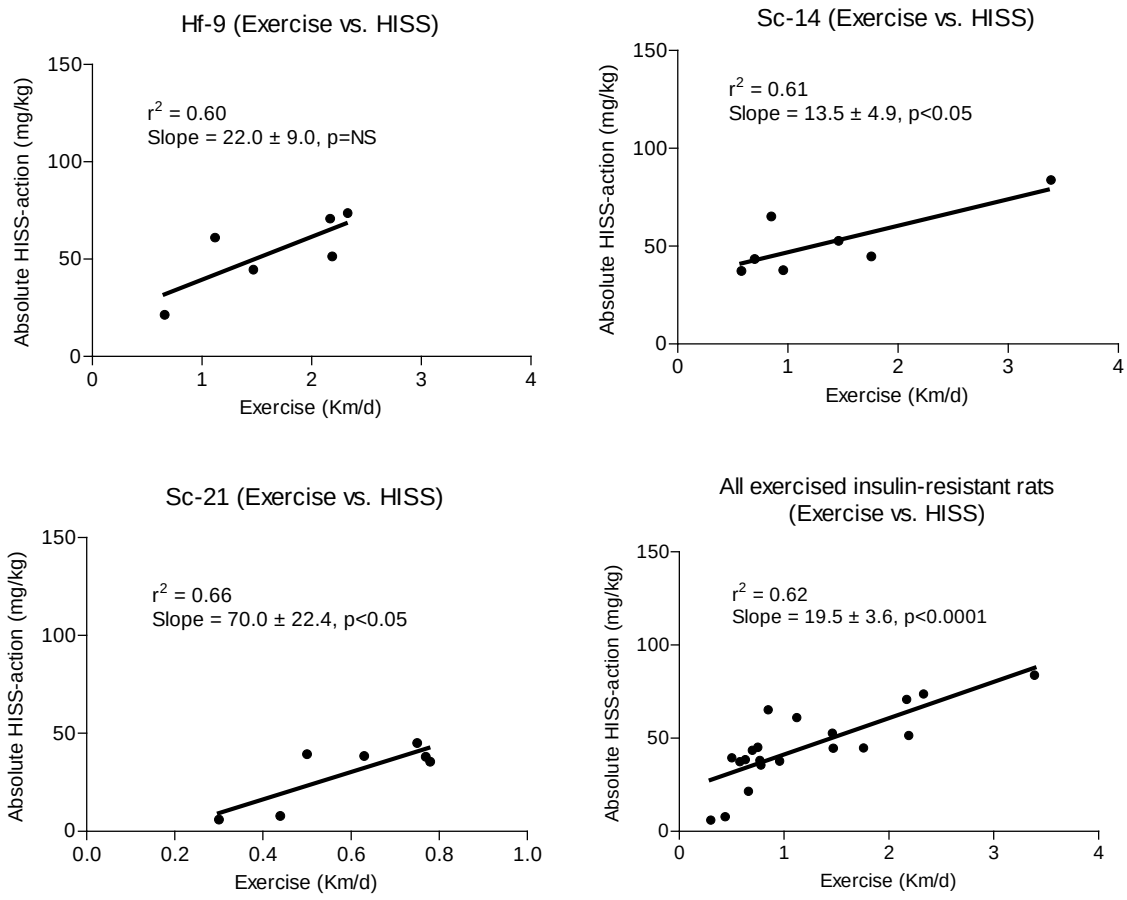


Fig. 20: HISS vs. exercise (protocol 2): To determine the impact of exercise on the improvement in HISS-dependent glucose uptake, correlation analysis was done between the running distance and the HISS-action for individual and pooled study groups. The positive correlation between exercise and HISS-action signifies a performance dependent enhancement in insulin sensitization in exercised rats. Note that the exercise-axis is on a different scale for the oldest rats which exercised the least.

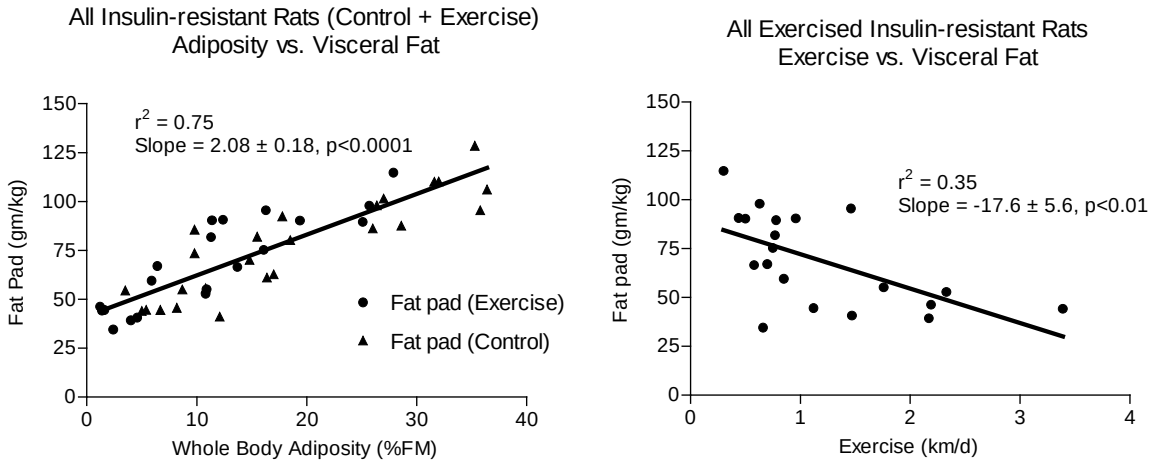


Fig. 21: Fat pad vs. %FM and fat pad vs. exercise (protocol 2): The increase in measured fat pad compared to the increase in whole body adiposity (%FM) demonstrates the existence of a strong positive correlation. The 7-day voluntary exercise reduced adiposity. The fat pad mass was reduced in proportion to the amount of exercise performed. (Left panel: Exercise group: $r^2 = 0.77$, slope = 2.52 ± 0.32, $p<0.0001$; Control group: $r^2 = 0.78$, slope = 2.06 ± 0.23, $p<0.0001$).

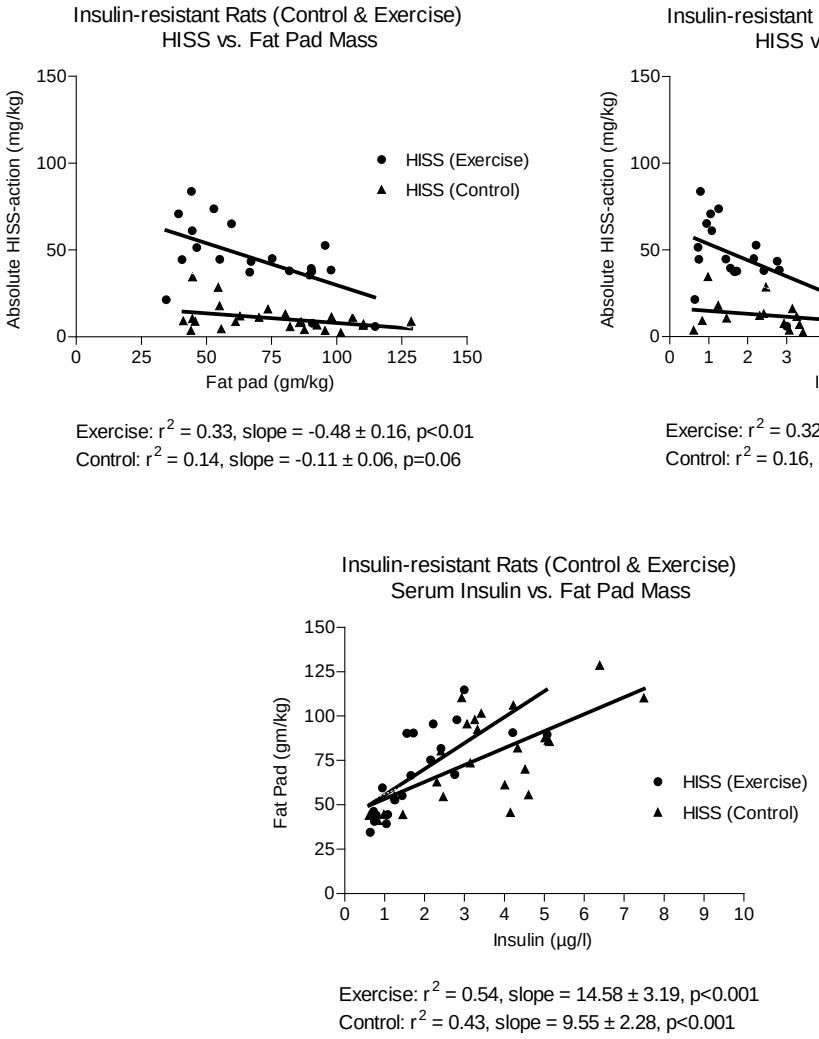


Fig. 22: HISS vs. fat pad, HISS vs. serum insulin concentration, and fat pad vs. serum insulin concentration (protocol 2): The inverse relationship between HISS-action and postprandial serum insulin concentration or fat content, and the positive correlation between insulin level and fat pad mass are consistent with the pathologies being secondary to the impaired HISS-action. In exercised insulin-resistant groups, the HISS-pathway was recovered and a higher HISS-action was correlated with lower adiposity and serum insulin level with steeper regression lines. In non-exercised insulin-resistant control groups, the HISS-pathway was almost blocked, leading to progressive adiposity and hyperinsulinemia over the range of a very low HISS-action, providing flat regression lines.

4.2.4 Postprandial Blood Glucose, Serum Insulin and Hepatic Glutathione

The metabolic status of the rats was determined by assessing postprandial parameters including postprandial glycemia and serum insulin concentration. The blood glucose level tended to decrease with exercise in all groups, and was statistically significant in the Sc-14 group. The serum insulin concentration demonstrated statistically significant decreases in the exercised rats, signifying that the postprandial insulinemia was improved by voluntary running. The hepatic glutathione level was decreased significantly by exercise in the Hf-9 group, but remained unchanged in others (Table 5).

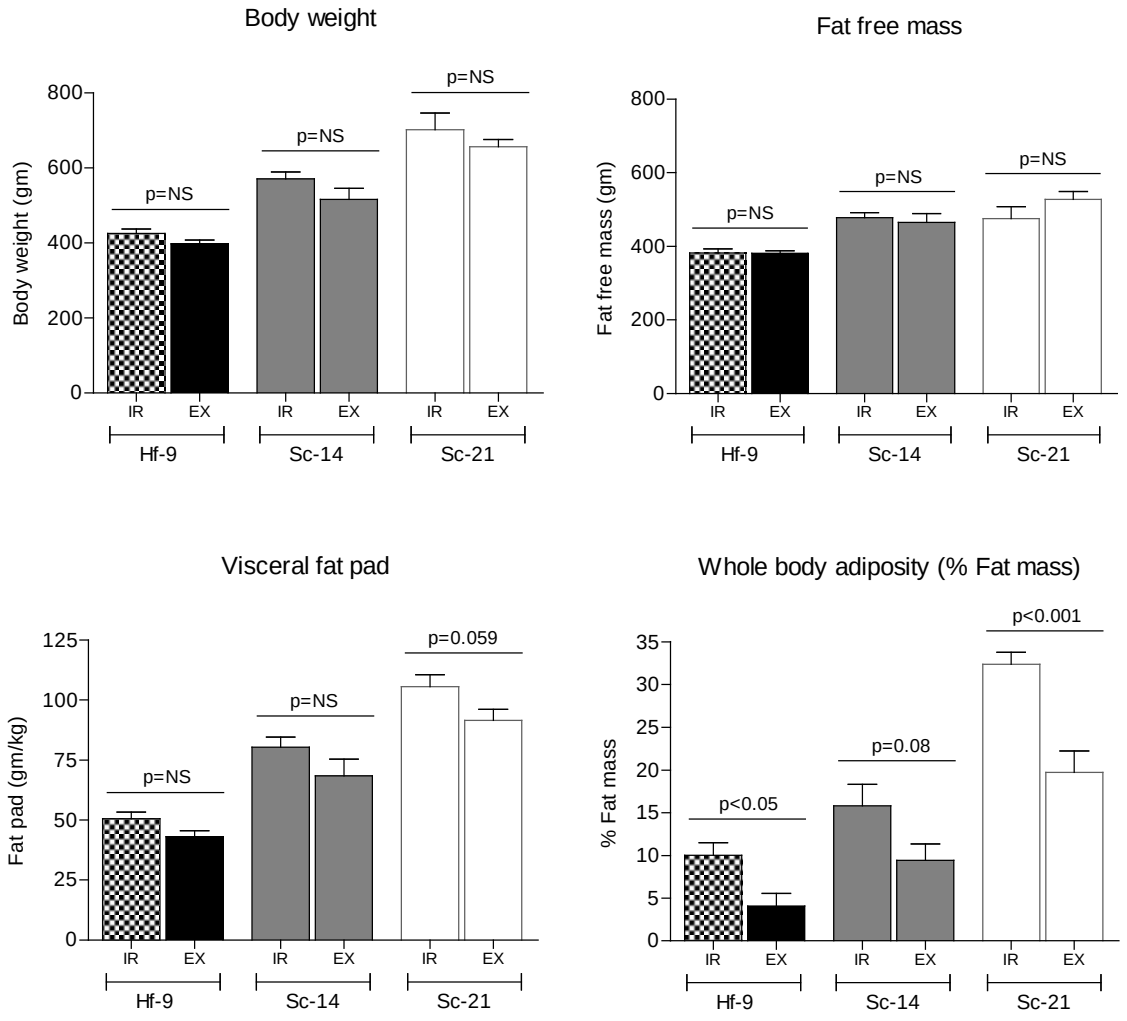


Fig. 23: Body composition (protocol 2): The 7-day voluntary exercise reduced body weight and adiposity/fat pad mass in all groups. The lean body weight or fat free mass was not changed significantly by exercise, signifying that the increase in muscle mass is not the primary mechanism by which voluntary exercise causes insulin sensitization in diet-induced insulin resistant rats.

TABLE 5: The postprandial blood glucose, serum insulin, and hepatic glutathione concentration in the control and exercised rats of the insulin-resistant groups. The metabolic parameters were adversely affected by high fat diet or 35% sucrose supplement, and led to the development of HISS-dependent insulin resistance. Voluntary exercise reversed the diet-induced HISS-dependent insulin resistance and improved the associated conditions.

	Hf-9 groups		**Sc-14 groups**		**Sc-21 groups**	
	Control (N=9)	Exercise (N=6)	Control (N=9)	Exercise (N=7)	Control (N=7)	Exercise (N=7)
Postprandial Blood Glucose (mg/dl)	114.8 ± 2.8	106.8 ± 2.6	111.3 ± 2.4	103.6 ± 2.6	109.0 ± 5.4	103.8 ± 5.1
Exercise-induced Decrease in Postprandial Blood Glucose	8.1 ± 4.1 (p=0.07)		7.7 ± 3.6 (p<0.05)		5.2 ± 7.4 (p=NS)	
Serum Insulin (µg/l)	2.25 ± 0.53	0.92 ± 0.10	3.74 ± 0.35	1.65 ± 0.26	4.65 ± 0.66	3.03 ± 0.46
Exercise-induced Decrease in Serum Insulin	1.33 ± 0.66 (p<0.05)		2.10 ± 0.46 (p<0.001)		1.62 ± 0.81 (p<0.05)	
Hepatic Glutathione (µmol/g)	5.30 ± 0.26	4.12 ± 0.41	6.39 ± 0.24	5.65 ± 0.27	6.06 ± 0.17	6.17 ± 0.27
Exercise-induced Change in Hepatic Glutathione	(-)1.18 ± 0.46 (p<0.05)		(-)0.74 ± 0.36 (p=NS)		(+)0.11 ± 0.32 (p=NS)	

4.3 Protocol 3: Impact of SAMEC and voluntary exercise in normal and insulin resistant rats

4.3.1 Exercise Performance

The day-to-day exercise, for each individual rat over the 7-day training session, demonstrated a gradual increase in exercise performance in all study groups. The average exercise in H-NE (n=10), H-SE (n=8), IR-NE (n=7), and IR-SE (n=8) was 1.27 ± 0.16, 1.10 ± 0.19, 1.39 ± 0.37, and 0.98 ± 0.15 km/day respectively (Fig. 24). There were no statistically significant differences in mean voluntary runs by the study groups.

4.3.2 Insulin/HISS Dynamics

4.3.2.1 Healthy Rats:

The total glucose uptake in control vs. exercise of the no-SAMEC (H-NC vs. H-NE) and SAMEC-supplemented (H-SC vs. H-SE) groups was 101.9 ± 6.3 vs. 142.3 ± 6.8 and 102.0 ± 6.5 vs. 139.7 ± 10.7 mg/kg respectively. There was a statistically significant increase in whole body insulin sensitivity stimulated by exercise in rats with/without SAMEC-supplement. SAMEC supplementation during the 7 days of exercise did not attenuate/augment the exercise benefits on insulin sensitivity (Fig. 25).

The HISS-dependent glucose uptake in H-NC vs. H-NE and H-SC vs. H-SE was 40.6 ± 4.6 vs. 68.2 ± 4.5 and 33.6 ± 4.7 vs. 54.0 ± 5.8 mg/kg respectively. The 7-day voluntary exercise caused statistically significant improvement in the HISS-dependent glucose uptake. SAMEC supplementation for 7 days did not influence the HISS-dependent glucose uptake in rats with/without exercise (Fig. 25).

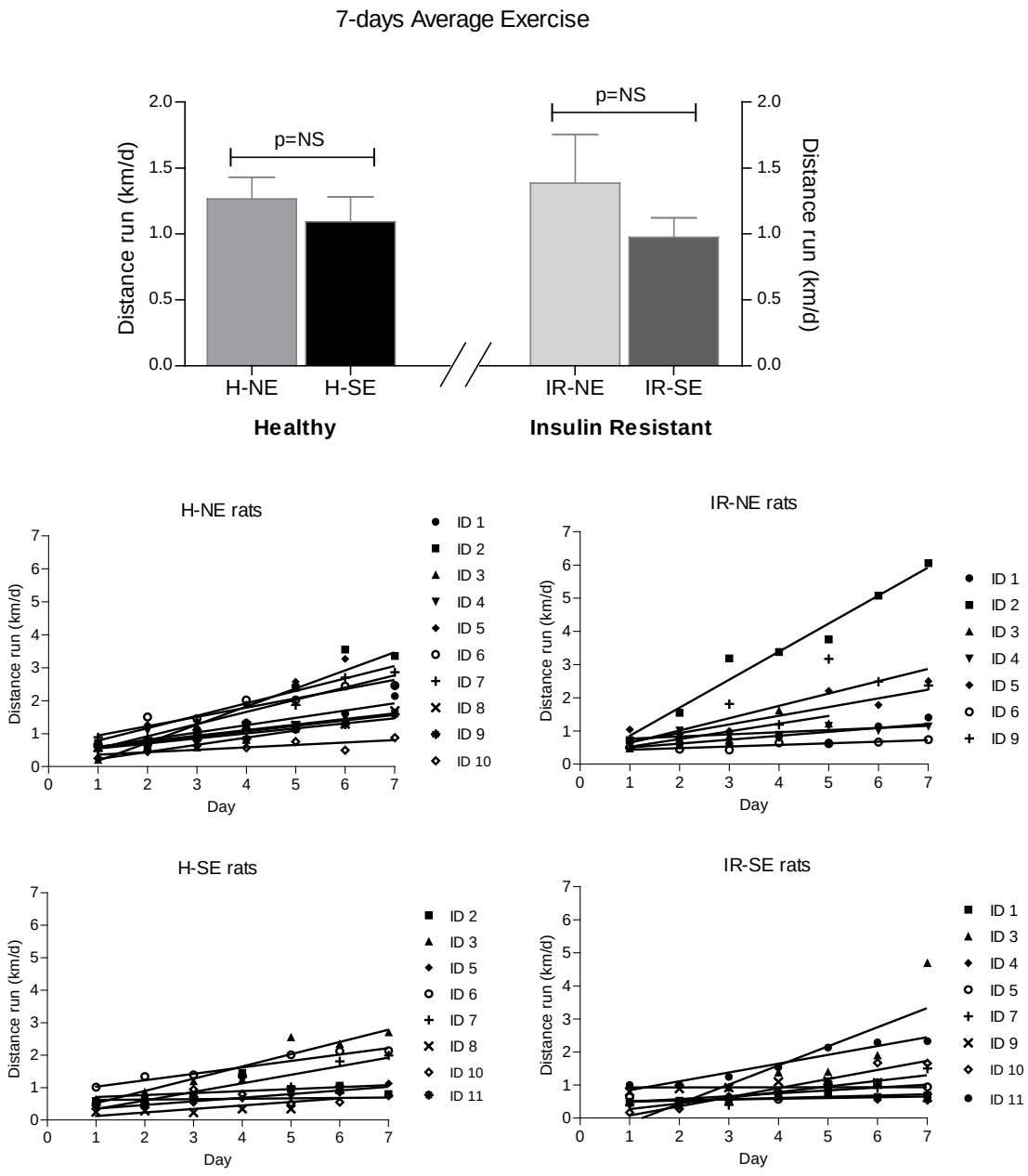


Fig. 24: Exercise performance (protocol 3): The day-to-day running distance for each individual rat was recorded and indicated a gradual increase in exercise performance over the 7-day period. Abbreviations: H-NE: healthy rats on normal (no-SAMEC) diet with exercise, H-SE: healthy rats on SAMEC-supplemented diet with exercise, IR-NE: insulin-resistant rats on normal (no-SAMEC) diet with exercise, IR-SE: insulin-resistant rats on SAMEC-supplemented diet with exercise.

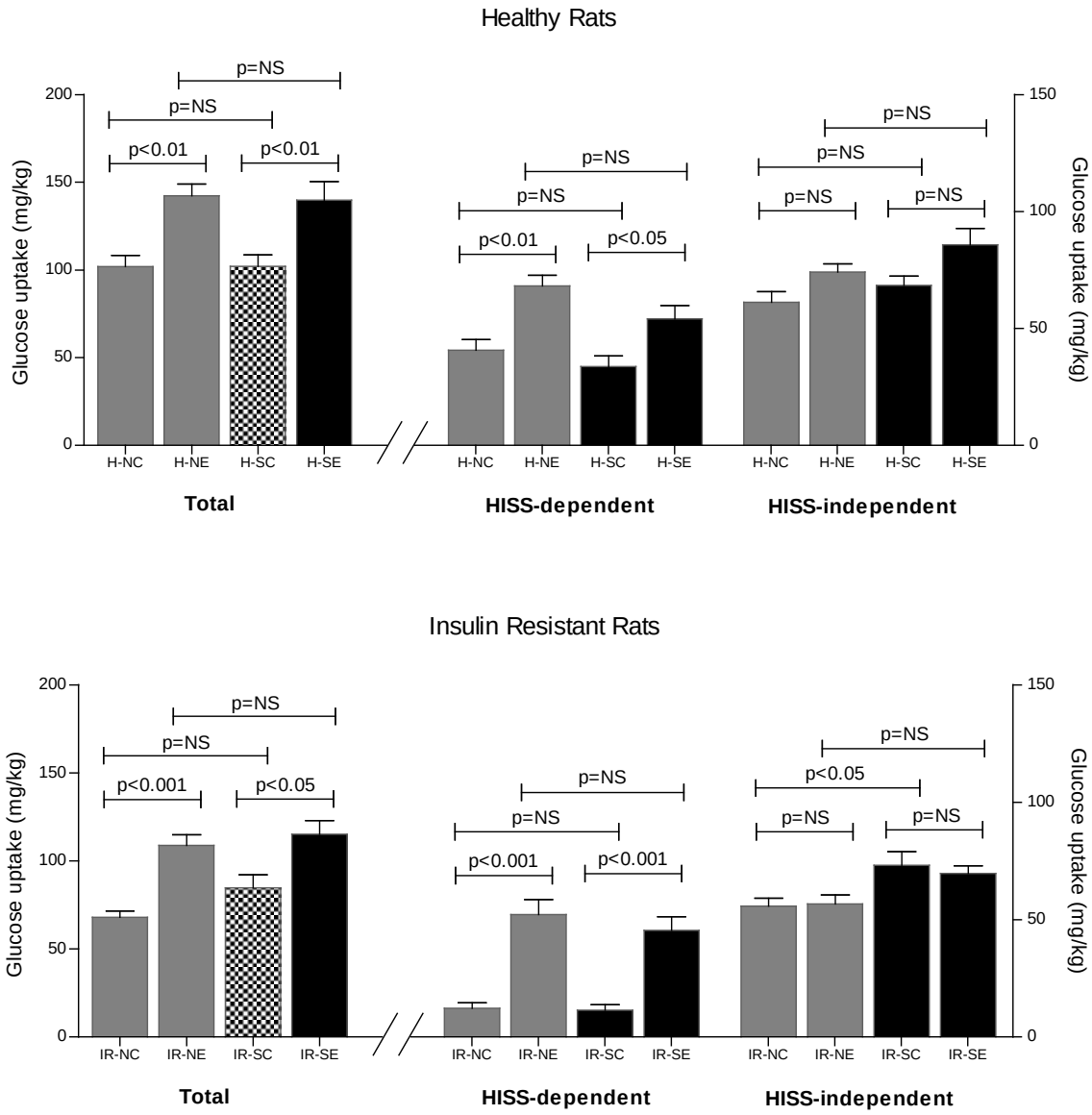


Fig. 25: Insulin sensitivity (protocol 3): The total, HISS-dependent and HISS-independent glucose uptake in various subgroups of the healthy and insulin resistant rats. Abbreviations: H-NC: healthy rats on normal (no-SAMEC) diet without exercise, H-NE: healthy rats on normal (no-SAMEC) diet with exercise, H-SC: healthy rats on SAMEC-supplemented diet without exercise, H-SE: healthy rats on SAMEC-supplemented diet with exercise, IR-NC: insulin-resistant rats on normal (no-SAMEC) diet without exercise, IR-NE: insulin-resistant rats on normal (no-SAMEC) diet with exercise, IR-SC: insulin-resistant rats on SAMEC-supplemented diet without exercise, IR-SE: insulin-resistant rats on SAMEC-supplemented diet with exercise.

The HISS-independent glucose uptake was mostly unaltered by exercise ± SAMEC in the respective study groups. The HISS-independent glucose uptake in H-NC vs. H-NE and H-SC vs. H-SE was 61.3 ± 4.4 vs. 74.1 ± 3.6 and 68.4 ± 4.0 vs. 85.6 ± 7.1 mg/kg respectively (Fig. 24).

4.3.2.2 Insulin Resistant Rats:

The rat models of insulin resistance (IR-NC, IR-NE, IR-SC and IR-SE) were made by chronic exposure to 35% sucrose solution for 9 weeks. The total glucose uptake in IR-NC vs. IR-NE and IR-SC vs. IR-SE was 68.0 ± 3.5 vs. 108.8 ± 6.0 and 84.6 ± 7.5 vs. 115.1 ± 7.7 mg/kg respectively. Compared to the age-matched healthy controls (H-NC, RIST: 101.9 ± 6.3 mg/kg), the whole body insulin sensitivity was reduced significantly in sucrose-fed rats indicating the development of insulin resistance. Voluntary exercise reversed the sucrose-induced insulin resistance and SAMEC did not interfere with the increase in insulin sensitization by exercise (Fig. 25).

The HISS-dependent glucose uptake was impaired in sucrose-induced insulin resistance and restored by the 7-day voluntary exercise. The HISS-dependent glucose uptake in IR-NC vs. IR-NE and IR-SC vs. IR-SE was 12.3 ± 2.3 vs. 52.1 ± 6.4 and 11.4 ± 2.4 vs. 45.4 ± 5.8 mg/kg respectively. Exercise improved insulin sensitivity in sucrose-fed rats through reversal of the HISS-dependent glucose uptake, and SAMEC supplementation for 7 days during the training session did not affect these benefits. SAMEC alone did not reverse HISS-dependent insulin resistance in the non-exercise groups (Fig. 25).

The HISS-independent glucose uptake was not affected by exercise ± SAMEC. In IR-NC vs. IR-NE and IR-SC vs. IR-SE, the HISS-independent glucose uptake was 55.8 ± 3.3 vs. 56.7 ± 3.8 and 73.1 ± 5.8 vs. 69.7 ± 3.3 mg/kg respectively (Fig. 25).

To analyze the effect of SAMEC on the performance-dependent enhancement in HISS-action, we plotted the HISS-dependent glucose uptake against the exercise performance for pooled groups (healthy + insulin resistant) with/without SAMEC. The slope of the regression lines between no-SAMEC (14.9 ± 4.8) and SAMEC (15.2 ± 8.5) groups does not vary significantly, indicating that SAMEC does not interfere with the pattern of interaction between exercise and the HISS-pathway (Fig. 26).

4.3.3 Body Composition

The body weight tended to be less with exercise in all healthy and insulin-resistant groups, and was statistically significant in the H-NE and H-SE groups. The body weight (in gm) in the respective groups was H-NC (556.9 ± 18.0) vs. H-NE (496.1 ± 8.2), H-SC (550.7 ± 15.1) vs. H-SE (491.0 ± 16.3), IR-NC (570.9 ± 18.8) vs. IR-NE (516.0 ± 29.4) and IR-SC (601.9 ± 21.7) vs. IR-SE (558.8 ± 18.8). There was no statistically significant difference of weight reducing benefits of exercise between the SAMEC and no-SAMEC groups (Fig. 27).

The whole body adiposity and fat pad mass were decreased by exercise in all study groups, and SAMEC did not affect these benefits of voluntary exercise. The fat pad mass (in gm/kg) was H-NC (60.1 ± 4.0) vs. H-NE (44.3 ± 2.3), H-SC (58.7 ± 4.6) vs. H-SE (45.1 ± 4.0), IR-NC (80.4 ± 4.3) vs. IR-NE (68.4 ± 7.0) and IR-SC (86.8 ± 10.0) vs. IR-SE (75.4 ± 4.4) (Fig. 27).

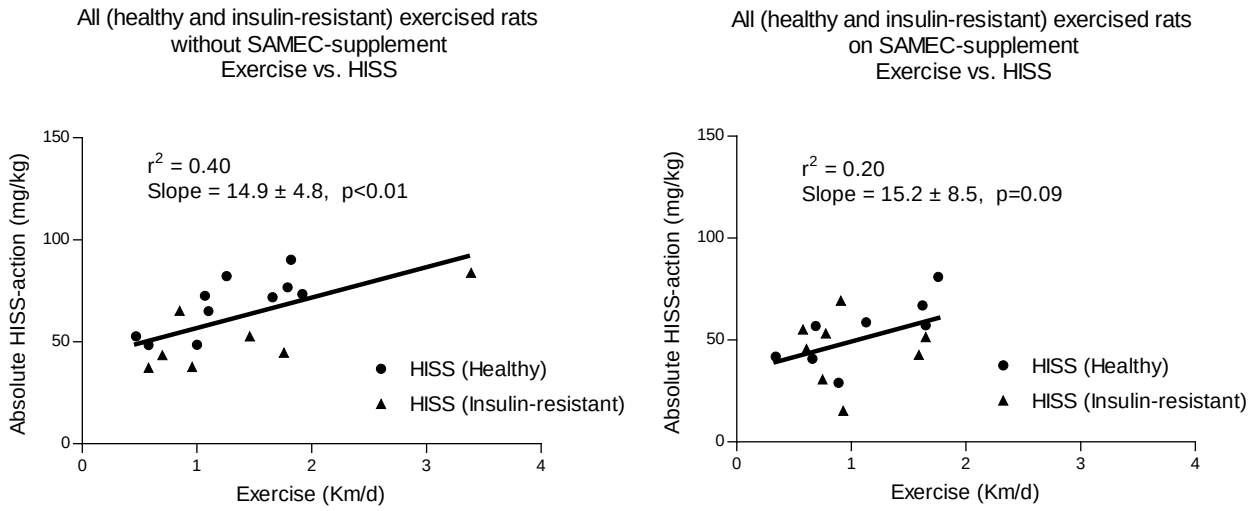


Fig. 26: HISS vs. exercise (protocol 3): To test the influence of SAMEC on the pattern of interaction of exercise with the HISS-pathway, the HISS-action was plotted against the running distance for the pooled rat groups with/without SAMEC. The slope of the regression lines does not differ significantly between the SAMEC and no-SAMEC groups, indicating that SAMEC does not interfere with the performance dependent enhancement of the HISS-pathway.

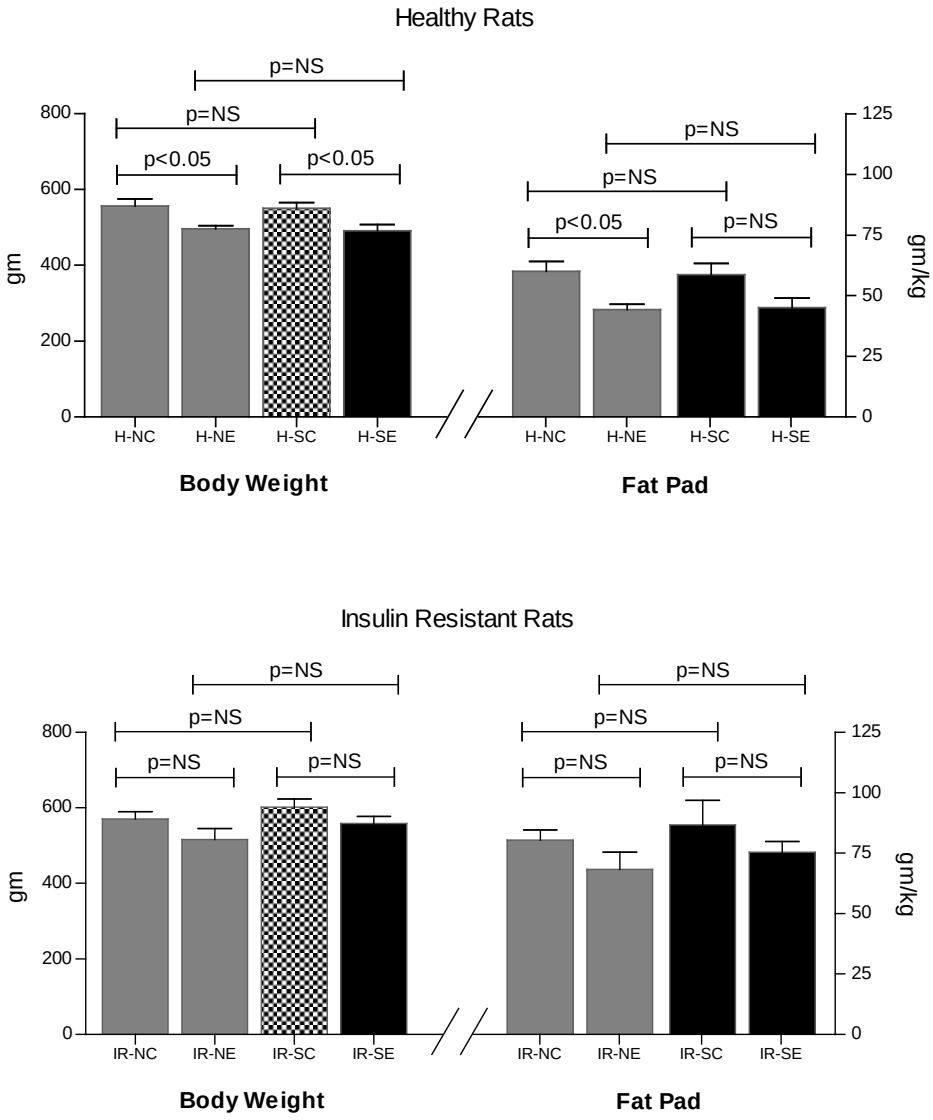


Fig. 27: Body composition (protocol 3): SAMEC supplementation during the exercise period did not influence the benefits of exercise on body compositions. Abbreviations: H-NC: healthy rats on normal (no-SAMEC) diet without exercise, H-NE: healthy rats on normal (no-SAMEC) diet with exercise, H-SC: healthy rats on SAMEC-supplemented diet without exercise, H-SE: healthy rats on SAMEC-supplemented diet with exercise, IR-NC: insulin-resistant rats on normal (no-SAMEC) diet without exercise, IR-NE: insulin-resistant rats on normal (no-SAMEC) diet with exercise, IR-SC: insulin-resistant rats on SAMEC-supplemented diet without exercise, IR-SE: insulin-resistant rats on SAMEC-supplemented diet with exercise.

4.3.4 Postprandial Blood Glucose, Serum Insulin, and Hepatic GSH

The metabolic status of the rats was determined by assessing postprandial parameters including the dynamic actions of HISS and insulin, postprandial glycemia and serum insulin concentration. The blood glucose level was decreased by exercise in most of the study groups, and was statistically significant in the H-NE and IR-NE groups. The serum insulin concentration was decreased significantly in the exercised rats of all study groups, signifying that the postprandial insulinemia is improved by voluntary running. The extent of decrease in serum insulin concentration between the SAMEC and no-SAMEC groups was not significantly different while considering the group variability of distance ran. The hepatic GSH level remained unchanged with exercise alone, but decreased in groups on SAMEC with/without exercise (Table 6 and 7).

TABLE 6: The metabolic profiles in different subgroups of healthy rats with/without SAMEC. Values are mean ± SE. Statistical significance was considered at $p<0.05$. Postprandial blood glucose and serum insulin concentration were improved by exercise in most of the study groups. The application of multivariate analysis of variance (MANOVA) to assess the differential impact of exercise in presence/absence of SAMEC indicates that, there is no significant difference between the SAMEC and no-SAMEC groups in obtained metabolic benefits considering the variability in voluntary run by rats in these groups.

	H-NC (N=9)	**H-NE** (N=10)	**H-SC** (N=10)	**H-SE** (N=8)
Postprandial Blood Glucose (mg/dl)	114.3 ± 3.8	103.1 ± 1.7	115.1 ± 3.3	115.3 ± 3.4
Exercise-induced Decrease in Postprandial Blood Glucose	11.2 ± 4.0 ($p<0.05$)		-0.2 ± 4.8 (p=NS)	
Serum Insulin (µg/l)	3.02 ± 0.93	1.31 ± 0.17	3.39 ± 0.53	2.22 ± 0.08
Exercise-induced Decrease in Serum Insulin	1.71 ± 0.90 ($p<0.05$)		1.17 ± 0.60 ($p <0.05$)	
Hepatic GSH (µmol/g)	6.57 ± 0.14	6.22 ± 0.26	4.63 ± 0.17	5.02 ± 0.27
Exercise-induced Decrease in hepatic GSH	0.35 ± 0.31 (p=NS)		(-) 0.39 ± 0.30 (p=NS)	

TABLE 7: The metabolic profiles in different subgroups of prediabetic rats with/without SAMEC. Values are mean ± SE. Statistical significance was considered at $p<0.05$. Postprandial blood glucose and serum insulin concentration were improved by exercise in most of the study groups. The application of multivariate analysis of variance (MANOVA) to assess the differential impact of exercise in presence/absence of SAMEC indicates that, there is no significant difference between the SAMEC and no-SAMEC groups in obtained metabolic benefits considering the variability in voluntary run by rats in these groups.

	IR-NC (N=9)	**IR-NE** (N=7)	**IR-SC** (N=7)	**IR-SE** (N=8)
Postprandial Blood Glucose (mg/dl)	111.3 ± 2.4	103.6 ± 2.6	110.7 ± 3.7	107.3 ± 2.2
Exercise-induced Decrease in Postprandial Blood Glucose	7.7 ± 3.6 ($p<0.05$)		3.4 ± 4.1 (p=NS)	
Serum Insulin (µg/l)	3.74 ± 0.35	1.65 ± 0.26	3.40 ± 0.60	2.32 ± 0.12
Exercise-induced Decrease in Serum Insulin	2.10 ± 0.46 ($p<0.001$)		1.08 ± 0.58 ($p<0.05$)	
Hepatic GSH (µmol/g)	6.39 ± 0.24	5.65 ± 0.27	4.44 ± 0.12	3.68 ± 0.46
Exercise-induced Decrease in hepatic GSH	0.74 ± 0.36 (p=NS)		0.76 ± 0.50 (p=NS)	

4.4 Protocol 4: The HISS-Pathway and Insulin/AMPK Signaling

4.4.1 Degree of Activation of Insulin Receptor-β (INR-β) and Insulin Receptor Substrate-1 (IRS-1)

The possible interaction of HISS with the insulin signaling pathway was tested by targeting the INR-β and its substrate IRS-1. There was no statistically significant difference in the cellular content of INR-β and IRS-1 between HISS-positive and HISS-negative in the soleus and EDL muscles. The phosphorylated form of INR-β and IRS-1 was not different between the respective samples. The degree of activation of the receptor/substrate was calculated from the ratio of the phosphorylated to the total form, which was not different between the HISS-positive and HISS-negative samples. It indicates that HISS works in skeletal muscle through a mechanism that is independent of the insulin signaling pathway (Fig. 28 and 29).

Similar to testing the insulin signaling pathway, we targeted the AMPK pathway in our HISS-positive and HISS-negative samples. There was no significant difference in the total, phosphorylated and phosphorylated/total AMPK between the samples. This indicates that HISS does not involve AMPK pathway for its action in skeletal muscle (Fig. 30).

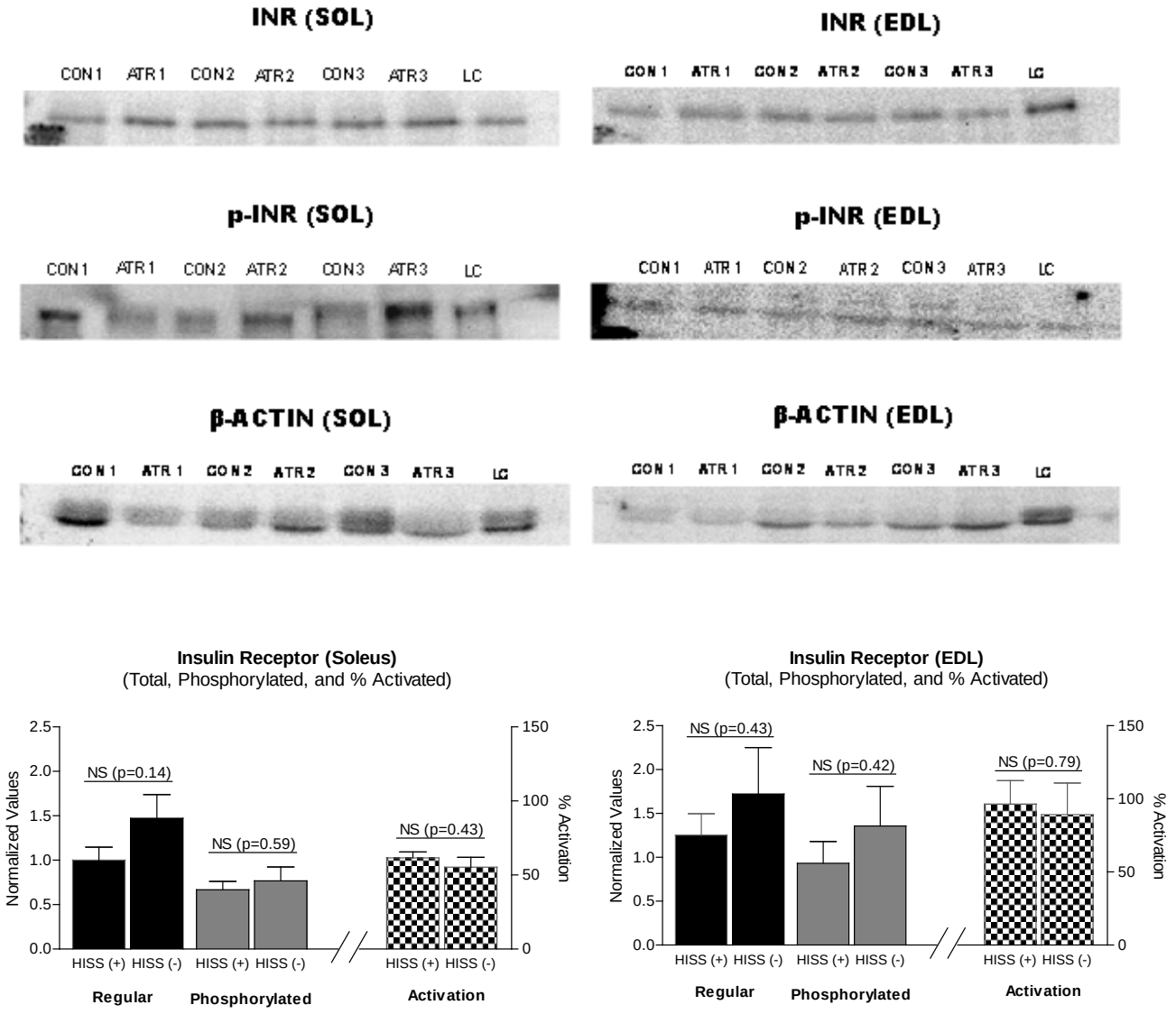
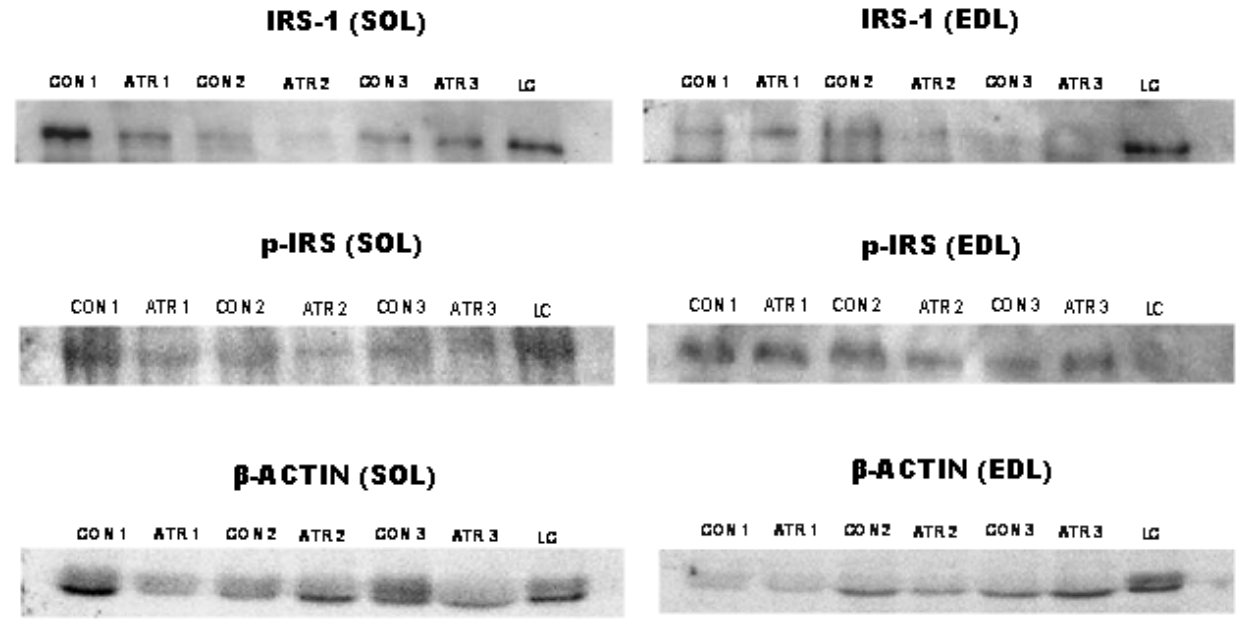
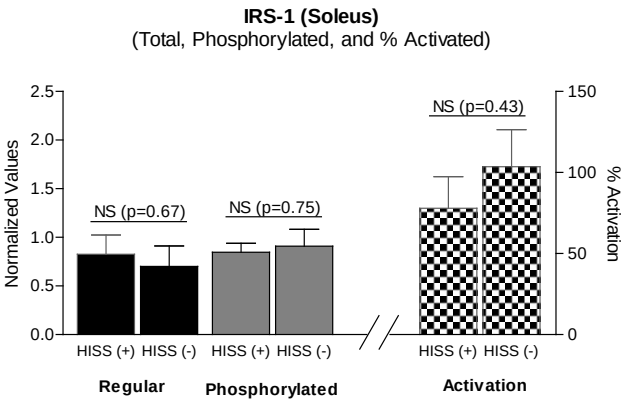


Fig. 28: Western blots of INR-β and p-INR-β (protocol 4): The total and phosphorylated form of insulin receptors in HISS-positive (CON, N=6) and HISS–negative (ATR, N=6) samples were estimated by Western blots, and each value was normalized by using an internal loading-control (LC) and β-actin. The degree of activation of the receptor was determined by calculating the percent ratio of the phosphorylated to the total insulin receptor. It indicates that there is no statistically significant difference of the insulin receptor content, receptor phosphorylation and its activation between the HISS-positive and –negative rats.





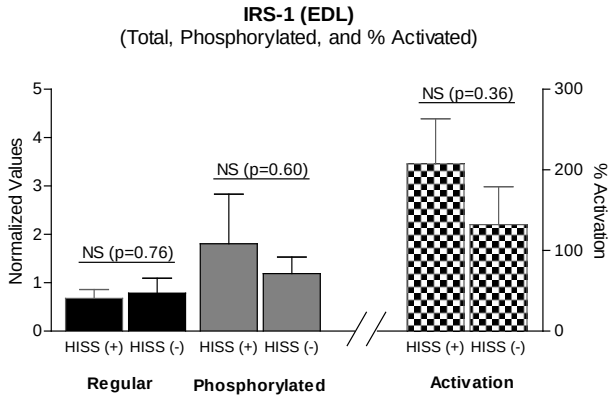


Fig. 29: Western blot of IRS-1 and p-IRS-1 (protocol 4): IRS-1 is an upstream substrate of the insulin signaling cascade. The degree of activation of IRS-1, calculated from the ratio of the phosphorylated to the total substrate, was not significantly different between the HISS-positive and HISS–negative rats.

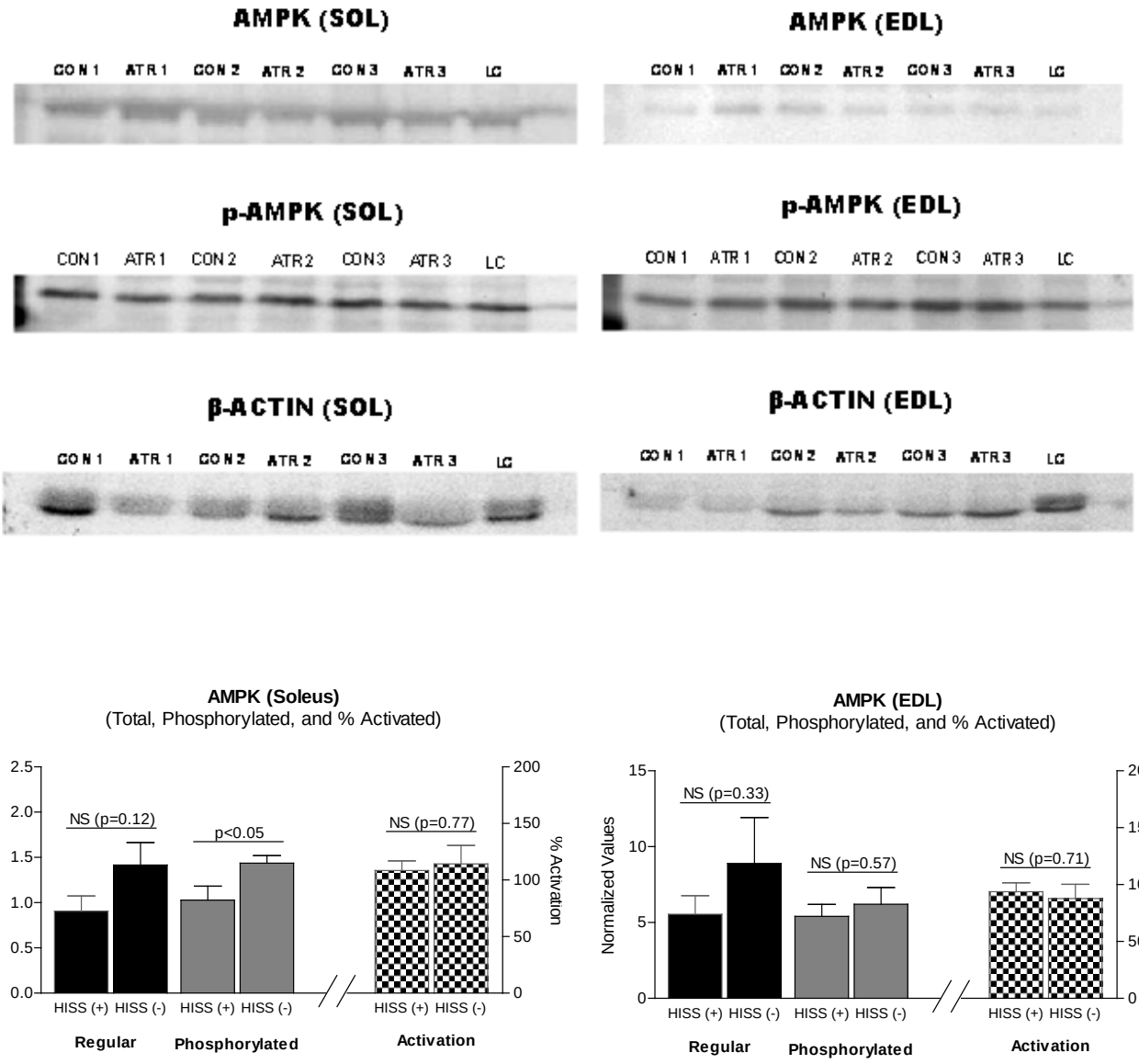


Fig. 30: Western blots of AMPK and p-AMPK (protocol 4): The AMPK content was estimated in its total and phosphorylated forms, and its activation was determined from the ratio of the phosphorylated to the total substrate in HISS-positive and HISS-negative samples. There was no differential activation of the AMPK-pathway between the HISS-positive and –negative rats.

4.5 Protocol 5: Glycogen Synthesis Kinetics in Presence/Absence of HISS

The rate of glycogen synthesis was estimated in terms of the measurement of total glycogen, G6P, and pGSK3:GSK3 in soleus and EDL samples of HISS-positive and HISS-negative rats. Note that the tissues were collected only 10 min after the administration of a bolus insulin (100 U/kg), and same samples were utilized in the signaling studies for INR-β and AMPK (protocol 4). There was a clear tendency of increase in the glycogen content, G6P, and pGSK3:GSK3 in soleus (but not in EDL) muscle of HISS-positive samples. The presence of HISS caused a differential activation of the downstream pathways resulting in increased rate of glycogen synthesis in skeletal muscle. However, this finding is reconfirmed in an extended protocol (not a part of the thesis), where we have shown that mimicking the feeding signals in fasted rats increases glycogen content significantly in soleus (but not in EDL) and left ventricle (but not in right ventricle) muscles. HISS does not affect glycogen content in the liver and gut (see details in Discussion).

TABLE 8: The measurement of glycogen, G6P and pGSK3/GSK3 in soleus and EDL muscles of HISS-positive and HISS-negative rats

	Soleus		**EDL**	
	HISS-positive	HISS-negative	HISS-positive	HISS-negative
Glycogen (%)	0.31 ± 0.02	0.23 ± 0.04	0.09 ± 0.04	0.18 ± 0.04
	p=0.08		p=NS	
G6P (nmol/mg)	7.54 ± 1.29	5.26 ± 0.68	9.91 ± 0.68	8.81 ± 0.68
	p=0.07		p=NS	
pGSK3:GSK3 (unit/ng)	0.79 ± 0.14	0.42 ± 0.18	0.46 ± 0.05	0.41 ± 0.04
	p=0.08		p=NS	

SECTION V

DISCUSSION

5. DISCUSSION

The major findings of our exercise studies follow: 1. Voluntary exercise for 7 days is able to potentiate MIS, and prevent the progression to the AMIS syndrome in healthy aging rats. 2. The 7-day voluntary exercise reverses the diet-induced AMIS through restoration of the MIS process. 3. Administration of antioxidant cocktail, SAMEC, during one week of voluntary running does not augment or attenuate the benefits of exercise on the whole body response to insulin.

The significant findings of our signaling studies include: 1. The action of HISS in skeletal muscle is independent of the insulin- and AMPK-signaling pathways. 2. The HISS action in skeletal muscle results in an increase in the cellular kinetics of glucose uptake and storage.

5.1 Technical Considerations

The exercised rats received 7-day voluntary running-wheel exercise, and the insulin and HISS action was measured at least 24 hours after the last bout of exercise. This interval was adopted to minimize the acute effects of exercise and to avoid the complications of testing immediately after the last session. The studies relate to the chronic effects of aging and/or dietary imbalance, and the sustained effects of exercise. The beneficial metabolic effects of exercise persisted after 24 hours, though the onset and duration of exercise effect on AMIS was not examined. A more prolonged exercise is expected to provide more metabolic benefits, since each rat showed a gradual increase in

day-to-day running distance over the 7-day session and the degree of improvement correlated with the distance run.

The rapid insulin sensitivity test (RIST), which is a transient euglycemic clamp in response to a bolus of insulin, was used to measure the whole body glucose uptake in response to insulin. In fed state the response to insulin has two components, the HISS-dependent and HISS-independent glucose uptake. The HISS-dependent glucose uptake results through the insulin-induced release of HISS from the liver and its action in peripheral tissues. The HISS-independent glucose uptake represents the direct action of insulin in the periphery. If HISS release is blocked, the remaining response is attributed entirely to the direct action of insulin. The RIST was done before and after atropine infusion that blocks the hepatic parasympathetic feeding signal, and therefore blocks HISS-release. Atropine produces an effect similar to the blockade of hepatic nitric oxide synthase or hepatic denervation. Atropine is a useful tool in this regard, as it is capable of eliminating the HISS response in fed animals and has no effect on direct insulin actions (Lautt et. al., 2001). The RIST can be repeated up to four times with a low coefficient of variance in the same rat over 6-8 hour test period (Lautt et. al., 1998).

Atropine is a non-selective competitive antagonist of the muscarinic acetylcholine receptors, and therefore might block acetylcholine action in organs other that the liver. However, the reduction in insulin sensitivity with atropine is similar to that produced by surgical denervation of hepatic parasympathetic nerves. Atropine does not further affect the insulin sensitivity in fasted rats and that in rats with hepatic denervation. Atropine

reduces insulin sensitivity without changes in plasma concentration of glucagon and insulin (Xie and Lautt, 1995a; Lautt et. al., 2001). Therefore, we assume that atropine induces postprandial insulin resistance primarily through blockade of the hepatic parasympathetic nerves, and the off-target effects of atropine influencing insulin sensitivity in minimal.

A fast-feed protocol of 12-2 h was adopted in current protocols (protocol 1-5) to quantitate MIS, and the action of HISS. In order to measure the whole body glucose uptake in fed state, it is necessary to ensure that a stable metabolic condition is established. The animals were fasted overnight and re-fed 2 hours prior to testing MIS at a stable postprandial state. Typically a stable glucose and insulin response after the meal are established after 2 hours, with the primary metabolic dysfunction being able to be demonstrated over the first 60-90 minutes (Lautt et al. 2011). Although the utilized animal models of AMIS demonstrated a clear decrease in the postprandial glucose uptake response to insulin, the 2 hr postprandial blood glucose and plasma insulin level was not elevated significantly in the prediabetic rats. Nevertheless, exercise caused a significant decrease in plasma insulin concentration and sensitization of insulin response in all study groups. The AMIS syndrome proposes a progressive dysfunction initially demonstrated by early postprandial hyperglycemia and hyperinsulinemia over the first 90 minutes. Adiposity increases as the earliest chronic symptom of AMIS. Only later in the development of the AMIS syndrome does 2 hour postprandial hyperinsulinemia and hyperglycemia occur. The very early effect on adiposity is confirmed in current studies.

Unlike muscle and adipose tissue insulin resistance, which remain relatively stable throughout the course of the disease (Weyer et. al., 1999), the rise of hepatic glucose production (HGP) occurs late in the natural history of diabetes, but appears to worsen progressively, and to become refractory to treatment (Monnier et. al., 2007). We assume that abnormal HGP and impairment of the hypothalamic control on HGP are not associated with the postprandial insulin resistance in early prediabetic animal models of AMIS. We measure insulin sensitivity at the 2h postprandial state, when a stable metabolic condition is established. Denervation of the hepatic parasympathetic nerves decreases glucose uptake in response to insulin in skeletal muscle, heart and kidney, but not in the liver (Fernandes et. al., 2011). Therefore, the role of HGP on extrahepatic postprandial insulin sensitivity in our healthy and AMIS animal models would be minimal.

To study the effect of exercise in diet-induced AMIS, the protocol 2 utilized three established models of AMIS that employs two different diet types, high-fat diet and 35% sucrose supplement. The high fat diet was used for 4 weeks, and 35% sucrose solution was used for 9 or 16 weeks. What we wanted to test is that exercise would be capable of reversing AMIS in the 3-diet/age models that were previously shown to have a reduced postprandial insulin response, secondary to the absence of HISS-action. The sucrose diet was given for 9 weeks when full blockade of HISS release would have existed for at least 7 weeks (Ribeiro et. al., 2005). By increasing the duration of the sucrose insult it was expected that signs and symptoms of the AMIS syndrome would show a predictable progression of pathologies.

In protocol 2 and 3, during the final week prior to experimentation the rats were returned to the normal diet and were allocated to the exercise/no-exercise subgroups. Removal of the dietary insult was done in order to avoid acute effects of the diet and to carry out the testing in an established and stable disease state. It allowed us to overcome the complex inference of intervention diets with exercise ± SAMEC. Both the high-fat diet and sucrose supplementation cause an absence of HISS-action (Afonso et. al., 2010; Ribeiro et. al., 2005) that does not recover spontaneously for at least a week following withdrawal of the dietary insult, and returning the rats to the normal diet and water (pilot study). This finding is reconfirmed in the present study, demonstrating that the HISS-dependent glucose uptake is significantly low in non-exercised rats after 1 week of withdrawal of the intervention diets.

SAMEC was used, as a balanced antioxidant cocktail in the protocol 3, to study the interaction of antioxidant with exercise. Free radicals with various chemical properties are widely spread throughout different tissues and cellular components, and the chemical property of an individual antioxidant can only allow it to scavenge the free radicals located in a specific cellular compartment, e.g. lipid or aqueous phase. The combination of antioxidants designed to protect the mitochondria (S-adenosylmethionine), the lipid phase (vitamin E), and the aqueous phase (vitamin C) provides a synergistic antioxidant effect, which can prevent the development of HISS-dependent insulin resistance in thioacetamide- (Ming et al. 2006) and age/sucrose- (Ming et al. 2009) induced animal models. The unbalanced combination of vitamin C and E, or SAM alone was ineffective (Ming et al. 2006).

In cell signaling studies, the hindlimb muscles (soleus and EDL) were collected after 10 min of bolus insulin (100 U/kg) administration through the jugular vein. This time point was selected from the observation that the glucose infusion rate is maximal between 10 and 15 min of the RIST, when the action of HISS is maximal. Note that insulin-stimulated tyrosine phosphorylation in hind limb is seen at the earliest time point of 30 sec, and is maximally activated at 2 min of insulin administration. Tyrosine phosphorylation decreases slowly over time, but the residual insulin action remains evident upto 15 min (Giorgino et. al., 1992). The experiments designed to study cellular signaling (protocol 4) and glycogen synthesis kinetics (protocol 5) utilized same hindlimb samples that were collected only 10 min after the administration of insulin. Though 10 min interval is not adequate to fully activate the downstream pathways, we found a clear tendency of increase in glycogen content and the rate of glycogen synthesis in the HISS-positive samples (details in section 5.2.6).

5.2 Major Findings and Discussion

5.2.1 Voluntary exercise provides sustained metabolic benefits in aging- and diet-induced models of AMIS

The absence of HISS release is compensated in type 2 diabetes by elevated insulin secretion, which leads to numerous metabolic abnormalities. It raises the possibility that an intervention which potentiates the HISS-pathway will slow/reverse the progression to AMIS, prediabetes and diabetes. Voluntary exercise was used as an intervention to modulate the HISS-pathway in aging and diet-induced models of AMIS. In our studies, exercise restored the HISS-dependent glucose uptake in age- and diet-associated prediabetic rats, and the benefits were seen 24 hours after the last bout of exercise. These

benefits might be obtained through the adaptive effects of exercise that resulted in augmentation of HISS-action. The metabolic improvements were also seen as beneficial changes in other parameters like adiposity and body composition.

5.2.2 HISS, the primary pathway attenuated by aging or dietary stress, and improved by exercise

Aging and intervention diets significantly decreased the response to insulin due to attenuation of the HISS pathway. Only the HISS-dependent glucose uptake was impaired, while the direct insulin action remained mostly unaffected. The 7-day voluntary exercise reversed the aging- and diet-induced AMIS, and the improved insulin response was achieved through restoration of HISS-action. There was no significant impact of exercise on the direct insulin action. The exercise-induced metabolic benefits correlated directly with the enhancement in HISS-dependent glucose uptake. The benefits might be attained through augmentation of the feeding signals (hepatic parasympathetic nerve activity and glutathione concentration) required for HISS release. Hepatic glutathione level was not changed by exercise in most of the study groups. Therefore, it is possible that the increased HISS-action was mediated by the nerve feeding signals. Muscle can work as a secretory organ to release paracrine and endocrine factors that regulate local and peripheral glucose uptake. Exercise stimulates the expression of these factors, and therefore increases whole body insulin sensitivity (Pedersen and Febbraio, 2012). We assume that the exercise-induced secretion of myokines might stimulate HISS release from the liver, causing an increase in the postprandial response to insulin.

5.2.2.1 Does an enhanced muscle glycogen synthesis during the post-exercise period influence the HISS-pathway?

Muscle glycogen is the primary fuel source during prolonged moderate-to-high intensity exercise (Romijn et. al., 1993). After an intense bout of exercise, muscle glycogen content may be depleted to less than half of the initial level (Davie et. al., 1999; Lacombe et. al., 2001; Pratt et. al., 2007), while there is an increase in whole body and skeletal muscle insulin sensitivity, and a concurrent increase in insulin-stimulated glycogen synthase activity in muscle (Christ-Roberts and Mandarino, 2004; Conlee et. al., 1978; Nielsen et. al. 2001). The magnitude of post-exercise increase in insulin sensitivity and glycogen synthesis is inversely related to the muscle glycogen content, suggesting that muscle glycogen itself may regulate the enhanced insulin action on glucose metabolism after exercise (Ivy and Kuo, 1998; Nielsen et. al. 2001).

Therefore, it is important to scrutinize whether the metabolic benefits of voluntary running are obtained primarily because of the improvements in HISS-pathway, or of the local mechanisms of skeletal muscle providing an added contribution and totaling the benefits. Here, we discuss the process and mechanisms involved in skeletal muscle glycogen replenishment during the post-exercise period, the durability of these effects, and their contribution to the HISS-mediated glucose uptake in muscle.

Skeletal muscle glycogen synthesis after exercise is dependent on several factors, including initial muscle glycogen content, carbohydrate availability, glucose transport in muscle, and the activity of glycogen synthase (Christ-Roberts and Mandarino, 2004; Conlee et. al., 1978). Muscle glycogen synthesis in the post-exercise recovery period has

such a high metabolic priority that intramuscular triglycerides are utilized at an increased rate to supply lipid fuel for oxidative muscle metabolism (Kiens and Richter, 1998). Glycogen recovery in muscle after exercise occurs in 2 phases; the initial period is characterized by a rapid synthesis of glycogen that lasts about 30-60 minutes. This process is insulin independent and involves myogenic/contractile mechanisms, causing increased translocation of glucose transporters (Glut-4) to the membrane surface and increased glucose uptake into the muscle. The second phase occurs at a slower rate involving insulin dependent mechanisms, and it lasts for several hours (Jentjens and Jeukendrup, 2003). Depending on the extent of glycogen depletion and provided that sufficient carbohydrate is consumed, the complete restoration of muscle glycogen can occur within 24 hours (Casey et. al., 1995; Keizer et. al., 1987; Kochan et. al., 1979).

Our protocols assigned an interval of at least 24 hours between the last bout of exercise and the measurement of insulin sensitivity in rats. This approach allowed us to minimize the exercise-mediated acute metabolic effects associated with the glycogen replenishment mechanisms in the muscle. Exercise was able to manipulate only the HISS-dependent glucose uptake that was impaired with age and intervention diets. The direct action of insulin remained unaffected with exercise. These findings suggest that the local glucoregulatory mechanisms through glycogen recovery in muscle do not have significant contributions 24 hours after the last exercise session; rather the enhancement/restoration of the HISS-pathway was the primary mechanism by which exercise offered persistent metabolic benefits.

Moreover, the protocol for dynamic testing of insulin sensitivity allows us to repeat RIST for consecutive 4 times with a low coefficient of variance, and after a stabilization period (of approximately 30 min) between each test, as assessed by samples taken at 5-min intervals until three stable basal glucose levels are determined (Lautt et. al., 1998). Also, mimicking the feeding signals with Benac in 24 hr fasted condition, when the muscle glycogen content significantly depletes, can restore the whole body glucose uptake to the level of fed state. These observations bring about the assumption that the glucose uptake capacity of skeletal muscle, under the action of insulin in non-contractile state, is very high; and the intramascular glycogen content is not the primary regulator of the whole body response to insulin. Therefore, the dynamic control of glucose uptake by muscle at normal condition is regulated primarily by the action of insulin and HISS, irrespective of the degree of changes in intramuscular glycogen content.

5.2.3 Exercise-induced metabolic benefits are secondary to the improvement in HISS-action

Inability of the release of HISS causes impaired glucose utilization and storage in peripheral tissues. The chronic shift in nutrient storage from muscle glycogen to fat, as a consequence of impaired HISS-action, results in a progressive and predictable series of metabolic, cardiac and vascular dysfunctions (Lautt et. al., 2010; Ming and Lautt, 2011; Ming et. al., 2009; Ming et. al. 2011). The 7-day voluntary exercise increased postprandial insulin response and tended to decrease postprandial blood glucose in all prediabetic groups. The plasma insulin concentration was significantly decreased by exercise, indicating that voluntary running had insulin sparing effect. The whole body

adiposity and fat pad mass decreased with exercise. The inverse relationship between HISS-action and serum insulin or fat content (and the positive correlation between insulin concentration and fat mass) suggests that the impact of exercise on various metabolic parameters was attained primarily through augmentation of the HISS-dependent glucose uptake.

5.2.3.1 Is adiposity the cause or a consequence of insulin resistance? How the exercise impacts correlate?

There is a strong relationship between obesity and the incidence of diabetes (Whitlock et. al., 2009). Fat accumulates mainly in subcutaneous adipocytes, but deposition is also found in ectopic sites such as abdominal/visceral area, liver, muscle, heart, and pancreas (Gastaldelli and Basta, 2010). Visceral fat represents approximately 10-15% of total body fat (Gastaldelli and Basta, 2010), and there is a strong correlation not only between visceral fat and whole body adiposity (Ming et. al., 2009) but also between visceral fat and ectopic fat deposition of various organs like liver (Gastaldelli et. al., 2007; Kotronen and Yki-Jarvinen, 2008), heart (Icobellis et. al., 2003; Sironi et. al., 2004) and muscle (Gastaldelli and Basta, 2010). There are two opposite schools of thought that provide explanation of the possible links between insulin resistance and obesity. Firstly, it is believed that obesity causes an increase in visceral fat mass that secretes free fatty acids and inflammatory cytokines into the circulation, leading to peripheral insulin resistance (Hajer et. al., 2008; Galic et. al., 2010; Hotamisligil et. al., 1993; Uysal et. al., 1997). Conversely, it is also suggested that the failure of postprandial dynamic HISS action to sequester glucose causes hyperglycemia and hyperinsulinemia, leading to a shift in the nutrient storage from glycogen to fat and resulting in progressive

adiposity (Lautt et. al., 2010). Because of the nature of simultaneous progression of compromised insulin sensitivity and increased adiposity, it is hard to accurately predict which pathology initiates first, and subsequently leads to other metabolic abnormalities.

The HISS-hypothesis proposes that the failure to develop MIS following a meal results due to the impairment of HISS release from the liver. When HISS is absent, hyperglycemia and compensatory hyperinsulinemia develops. Insulin acts on fat cells and liver to progressively lead to increased adiposity. There is a strong inverse correlation between the extent of HISS-action and plasma insulin concentration, and body adiposity (measured by bioimpedance estimation) or visceral fat mass (combined mass of perinephric, epididymal, and perienteric fat pads). Insulin resistance caused through impairment of the HISS-pathway is fully developed after only 2 weeks of dietary stress with voluntary consumption of 35% sucrose solution. The sucrose-fed rats show a higher weight/fat gain in relation to the length of sucrose exposure for 6 and 9 weeks, while there is no additional increase in HISS-dependent insulin resistance in these groups (Ribeiro et. al., 2005). These findings are consistent with the HISS-hypothesis suggesting that adiposity is a secondary consequence of the impairment of HISS-pathway. By this paradigm, the 7-day voluntary exercise improves the HISS-pathway, and therefore decreases the whole body adiposity.

Although intramuscular fat makes up only a fraction (approximately 1-2%) of the total fat stores, it is metabolically active and plays a significant role in energy homeostasis of skeletal muscle (Shaw et. al., 2010). It has been suggested that muscle fat

accumulation in insulin resistance is due to an increase in fatty acid uptake (Bonen et. al., 2004; Glatz et. al., 2010; Holloway et. al., 2009a; Luiken et. al., 2001), and not due to a decrease in the intrinsic capacity of fat utilization in muscle (Holloway et. al., 2009a; Holloway et. al., 2009b; Holloway et. al., 2007; Mogensen et. al., 2007; Turner et. al., 2007). However, we have demonstrated that provision of the dual feeding signals for MIS by a single acute intraportal administration of bethanechol plus N-acetylcysteine (Benac) can successfully reverse postprandial insulin resistance in the sucrose-fed diabetic models. This finding shows that accumulation of intramuscular fat is not the primary etiology behind the development of insulin resistance. After HISS release is restored by Benac, the response to insulin in the skeletal muscle of insulin-resistant rats is restored. The effect of Benac allows us to disprove the hypothesis that accumulation of fat in muscle results in skeletal muscle insulin resistance. Therefore, we propose that impairment of the HISS-pathway causes an inability of the muscle to sequester postprandial glucose and storage as glycogen, and subsequently results in accumulation of carbohydrate as fat in various tissues including skeletal muscle. At this advanced stage of the AMIS syndrome, true in vivo resistance to insulin action does develop, but only after whole body adiposity has become significantly elevated.

5.2.4 Increased muscle mass does not contribute to the primary metabolic benefits of exercise

Skeletal muscle accounts for >85% of glucose utilization (DeFronzo et. al., 1985; Kuk et. al., 2008). While it could be possible that an increase in skeletal muscle mass might cause the insulin sensitization response to exercise, the metabolic characteristic of the skeletal muscle is a more important determinant than the muscle mass for glucose

metabolism (Kuk et. al., 2008). The muscle mass increased with age, but was unaffected by 1-week of voluntary exercise. The increased muscle mass with age does not compensate for decreased whole body HISS-action, and exercise benefits in aging are obtained primarily through enhancement/restoration of the HISS-pathway (Chowdhury et. al., 2011). The improved metabolic dynamics of the muscle through HISS, but not an increased muscle-mass, is the primary mechanism by which 7-day voluntary exercise provides insulin sensitization in age- and diet-associated insulin resistance.

5.2.5 SAMEC does not affect the insulin sensitization benefits of exercise in healthy and prediabetic rats

Oxidative stress and reactive oxygen species (ROS) are implicated in the pathogenesis of systemic inflammation, endothelial dysfunction, and insulin resistance (Meydani and Azzi, 2009). The natural defense mechanism of the body possesses the ability to balance the ROS level; however increased ROS generation or reduction in the antioxidant defenses may result in elevated oxidative stress and development of type 2 diabetes (Ruhe and McDonald, 2001, Barbagallo et. al., 1999; Montonen et. al., 2004; Meigs et. al., 2007; Head, 2009).

There are disagreements between the ideas of whether the increased mitochondrial ROS generation by exercise is favorably or adversely linked to the benefits of exercise on insulin sensitivity. Reduced mitochondrial metabolism has been functionally connected with type 2 diabetes, and exercise stimulates mitochondrial metabolism, which might provide benefits in insulin resistance (Simoneau and Kelley, 1997). It is suggested that oxidative stress may mediate some health promoting effects

(McClung et. al., 2004; Goldstein et. al., 2005; Schulz et. al., 2007; Birringer et. al., 2007; Gomez-Cabrera et. al., 2008), and increased mitochondrial ROS generation is required for the insulin sensitizing benefits of physical exercise (Ristow et. al., 2009). Therefore, it could be possible that suppression of the exercise-induced ROS generation by supplemental antioxidants might attenuate the metabolic benefits of physical exercise. This suggestion was supported by a recent study (Ristow et. al., 2009) demonstrating that vitamin E plus vitamin C attenuated the insulin sensitizing effects of exercise. It raises a serious concern for those who attempt to live a healthy lifestyle through the use of exercise and antioxidant supplements. Our study (protocol 3) was based on the hypothesis that the balanced antioxidant cocktails, SAMEC, would not affect the benefits of exercise on insulin sensitivity in health and prediabetes.

The postprandial insulin sensitivity was increased by exercise, primarily through enhancement of the HISS-dependent glucose uptake, which remained unaffected by SAMEC supplement. Direct insulin action was unaltered by exercise ± SAMEC. While chronic supplementation of SAMEC is a strong preventative against age- and sucrose diet-induced AMIS syndrome, it was not capable after one week to restore HISS action in the established sucrose-treated AMIS model. SAMEC supplementation for 7 days does not either harm or add benefit to the positive effects of exercise on insulin sensitivity in rats. Moreover, more recent studies (Lavie and Milani 2011; Yfanti et al. 2011) have not supported the finding of Ristow et al. (2009), as the use of combined vitamin E and C have not been confirmed to impair the effect of exercise on insulin sensitivity in healthy volunteers. While SAMEC is a demonstrated preventive against the progressive loss of

HISS action, this short-term supplementation was not capable of reversing the established disease state.

Hepatic GSH level was decreased in SAMEC supplemented groups with/without exercise. Hepatic GSH determinations made from a single fed or fasted state provides minimal information about the hepatic GSH feeding signal. Hepatic GSH levels are highly variable depending on the duration since last meal and the acute or chronic exposure to free radical stress. Hepatic GSH increases by 47% after a meal (Guarino et. al. 2003). After a 24 hour fast, GSH levels are largely independent of the feeding signal, and the remaining GSH serves a myriad of other functions including serving as a first line mitochondrial free radical scavenger. An acute free radical stress, such as alcohol or thioacetamide, can rapidly deplete GSH, but the levels return to, or above, the pre-stress level within hours. In present study, exercise alone did not change the hepatic GSH, while the total hepatic GSH was decreased by SAMEC ± exercise. To make a conclusive interpretation of the impact of exercise and antioxidants on GSH related to MIS, we require measurements made in fasted versus fed state in otherwise identical situations. The hepatic GSH levels in the current study, therefore, do not provide any conclusive explanation about the non-interaction of SAMEC with the benefits of exercise on insulin sensitivity.

5.2.6 HISS increases glucose uptake and the rate of glycogen synthesis in skeletal muscle through insulin- and AMPK-independent pathways

Insulin and AMPK are the major regulators of cellular energy homeostasis. Though there is a significant advancement in our knowledge about insulin and AMPK

signaling pathways, it is not fully clear whether several alternate or complementary mechanisms exist, and how they are regulated under different cellular energy states, including the glucose-rich postprandial condition. The augmented whole body glucose uptake during the postprandial state, though, can be explained by the HISS-hypothesis, it is not known which signaling pathways are involved with the HISS action in peripheral tissues. A recent study of Macedo's team, using radioactive tracer (2-deoxyglucose), has shown that HISS controls glucose uptake in skeletal muscle, heart, and kidney but not in liver and adipose tissue (Fernandes et. al., 2011).

The present study (protocol 4) measured the degree of activation of insulin- and AMPK-pathways, in presence and absence of HISS, through targeting the upstream receptor/substrate. We have demonstrated that HISS does not involve the insulin and/or AMPK signaling to cause augmented glucose uptake in skeletal muscle. This finding convinces us to speculate that HISS might share a common downstream signaling, secondary to the activation of an insulin/AMPK-independent pathway, leading to an increased rate of glycogen synthesis and storage.

Glycogen synthase (GS) is often described as the rate limiting enzyme in the synthesis of glycogen (Farrace and Rossetti, 1992; Villar-Palasi, 1991; Ivy, 1991; Shulman et. al., 1995). However, the GS activity depends on two regulatory mechanisms; positive flux control by G6P through hexokinase, and inverse functional control by GSK3 (see section 1.6.2) (Shulman et. al., 1995). Therefore, to estimate the rate of glycogen synthesis in HISS-positive and HISS-negative samples we measured the total glycogen,

G6P, and the ratio of pGSK3 (inactive form) to GSK3 (active form). The results indicate that there is a clear tendency of increase in the glycogen synthesis rate in soleus muscle (but not in EDL) by HISS only 10 min after a bolus administration of insulin. However, in a recently completed study (not a part of this thesis project) we mimicked the two feeding signals (for HISS release) in 24 hour fasted rats with BENAC, and measured glycogen content in various tissues collected at the end of the RIST (approx. 35-40 min after the bolus insulin administration). HISS increased glycogen content significantly in soleus (but not in EDL) and left ventricle (but not in right ventricle) muscle. There was also a clear tendency of increase in glycogen content in less metabolically active muscles including kidneys and spleen. However, HISS did not affect glycogen content in the liver and gut. Further studies are required to provide conclusive answers about the complementary signaling pathways and their net effect on glycogen synthesis, due to the action of HISS in skeletal muscle (details in section 6).

SECTION VI

SUMMARY

6. SUMMARY

This doctoral thesis is based on the HISS-hypothesis and the AMIS syndrome hypothesis that bring about a paradigm shift in our knowledge regarding prediabetes, obesity, and full stage diabetes. The sensitization of the whole body response to insulin by a meal (i.e. meal-induced insulin sensitization, MIS) contributes to the dynamic regulation of glucose utilization during the postprandial state. This glucoregulatory mechanism is mediated through the insulin-induced release of hepatic insulin sensitizing substance (HISS) that enhances glucose uptake in skeletal muscle, heart, and kidney (Fernandes et. al., 2011). If HISS is not released, it causes the absence of meal-induced insulin sensitization (AMIS), chronically leading to the precipitation of a cluster of metabolic, vascular, and cardiac dysfunctions, which we refer to as components of the AMIS syndrome.

Taking into account the mechanistic significance of MIS in health, and of AMIS in progression to type 2 diabetes, we wanted to examine the effects of a non-pharmacological intervention (i.e. exercise, with/without antioxidants) on the aforementioned phenomena. We have shown that the metabolic benefits of exercise in health and in prediabetes are conferred through potentiation or restoration of the MIS process. However, the sensitization of postprandial insulin response by exercise remains unaffected by the supplementation of antioxidant cocktail, SAMEC.

My thesis project also included the cell signaling studies associated with MIS, which indicates the existence of alternate signaling pathway(s) to the insulin or AMPK pathway. However, the downstream signaling mechanisms controlling the rate of glycogen synthesis were up-regulated by HISS, independent of the activation of the insulin/AMPK-signaling at the proximal end. We assume that HISS activates an insulin-independent upstream pathway that shares the common downstream regulators of glycogen synthesis in the cell. In a recent study (not a part of this thesis project), we have shown that HISS increases glycogen content in hindlimbs and the heart, but not in the liver and gut. There is also a consideration to test the association of NO signaling pathway, that increases glucose uptake through a mechanism distinct from the insulin and contraction pathways (Higaki et. al., 2001), with the action of HISS in skeletal muscle.

A limitation of these studies is the lack of chemical identification of HISS, which is an impediment to the general acceptance of this novel hypothesis of the development of insulin resistance and progression to type 2 diabetes. The endocrine nature of HISS is confirmed by blocking its action through hepatic denervation, and restoring the action through intraportal (but not systemic) administration of acetylcholine (Xie and Lautt, 1996; Moore et. al., 2002) or nitric oxide donors (Sadri and Lautt, 1999). Moreover, the metabolic and cardiovascular abnormalities of AMIS can be prevented or reversed by using pharmacological agents that are designed to target the MIS feeding signals. Even without HISS identification, the HISS-hypothesis provided us a clear insight to better understand the dynamic metabolic control or nutrient partitioning after a meal, and its impairment in pathologies. Indeed, the HISS-pathway remains an attractive target to treat

various metabolic abnormalities with pharmacological and non-pharmacological interventions.

---- THE END ----

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