University of Alberta

EFFECTS OF CHRONIC HYPERGLYCEMIA ON GENE EXPRESSION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

BY

DAN YOU ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **EFFECT OF CHRONIC HYPERGLYCEMIA ON GENE EXPRESSION OF SKELETAL MUSCLE IN STREPTOZOTOCIN (STZ)-DIABETIC RATS** submitted by **DAN YOU** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in experimental medicine.

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ABSTRACT

Hyperglycemia, inherent to diabetes, can impair insulin secretion and insulin action, a phenomenon called glucose toxicity. Studies have suggested a gene regulatory mechanism for glucose toxicity in beta-cells and this may confound the search for primary genetic defects in NIDDM. We used mRNA differential display technique and a STZ (streptozotocin)-induced diabetic rat model to investigate whether glucose toxicity-induced insulin resistance in skeletal muscle is associated with any alteration of gene expression. Three groups of rats, STZ-induced diabetic, STZ-diabetic, insulin treated and controls were studied. Forty differentially expressed cDNAs were identified in differential display. Twenty-two of these 40 bands were cloned, sequenced and verified by Northern analysis. Three fragments, G24, G25, and C16, showed differential expression. G24 is 57% upregulated, while G25 (63%) and C16 (60%) are significantly downregulated in both STZ-treated groups relative to the control group, independent of glucose and insulin levels. G24 is 95% homologous to rat mitochondrial 16S rRNA, G25 is 93% homologous to rat mRNA of ATP synthase subunit, and C16 is 93% homologous with rat muscle tropomyosin mRNA. Our results suggest that STZ can modify gene expression in skeletal muscle independent of glucose and insulin levels. The effect of STZ on ATP synthase may have a physiological significance by decreased activation of hexokinase in skeletal muscle. Therefore, caution must be used when employing the STZ-diabetic model to evaluate pathogenic mechanisms of insulin resistance.

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ABBREVIATIONS

A	Adenosine
ATP	Adenosine triphosphate
amp	Ampicillin
β-ME	Mercaptoethanol
C	Cytidine
cDNA	Complementary deoxyribonucleic acid
cpm	Counts per minute
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
G	Guanosine
IPTG	Isopropyl-β-D-Thiogalactopyranoside
hnRNA	Heterologous nuclear RNA
LB	Luria broth
mRNA	Messenger ribonucleic acid
min	Minute

μg	Microgram
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
T	Thymidine
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris (hydroxymethy) aminomethane
U	Units
V	Volts
Xgal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

I. FACTORS DETERMINING GLUCOSE HOMEOSTASIS

A. Glucose Homeostasis

In normal subjects the blood glucose concentration is tightly controlled in a narrow range despite intermittent feeding behavior and long periods without food intake [1]. This glucose homeostasis is controlled by a very efficient hormonal regulatory system, consisting of a closed feedback loop between the circulating glucose level and pancreatic islet hormones [2]. The fluctuation of plasma glucose has a direct effect on the pancreas to modulate insulin and glucagon secretion. In the postprandial state, the glucose level is high and insulin secretion is increased from the beta cells while alpha cell glucagon secretion is reduced. When hypoglycemia prevails, glucagon secretion is enhanced while insulin release is diminished. Glucagon stimulates hepatic glucose production while insulin inhibits glucose release from the liver and stimulates glucose utilization in the peripheral insulin- responsive tissues, including fat and skeletal muscles. Any change of the components in this loop such as hormone levels, or plasma glucose concentration, or glucose utilization, will result in other modulations to maintain the balance between glucose appearance (entry into blood) and glucose utilization (disappearance from the blood).

There are three critical points in the regulatory system which will determine glucose homeostasis: (i) the islet alpha- and beta-cell sensitivity to glucose and their secretion

capacity, particularly insulin secretion; (ii) the rate of hepatic glucose production and (iii) the efficiency of glucose uptake by peripheral tissue. The last two points are mainly regulated by insulin action. Thus, insulin secretion in response to glucose, and insulin action in the target tissues, will largely determine glucose homeostasis [2].

B. Insulin Secretion

Insulin is secreted from clumped endocrine cells in the pancreas known as the islets of Langerhans. Four distinct types of cells are found within the islets. The insulin-secreting beta cells are the most abundant, and constitute about 70-90% of the islet endocrine cell population. Alpha cells which secrete glucagon make up the majority of the remaining cells. The remaining few percent of islet cells include delta cells which secrete somatostatin, and the F cells which secrete pancreatic polypeptide [3,4].

Insulin is synthesized in beta cells by the peptide synthetic mechanisms which begin with transcription of the insulin gene to hnRNA, which is stimulated by increased blood glucose level through an unknown mechanism [5]. It has been suggested that some nuclear protein, in response to glucose, can act on the promoter-enhancer region of the insulin gene and initiate insulin gene transcription [6]. hnRNA is processed to mRNA through splicing and polyadenylation in the nucleus. mRNA then diffuses into the cytoplasm where it binds to ribosomes for translation into protein. In beta cells, insulin mRNA is translated into a precursor polypeptide, proinsulin, which is transported to the Golgi apparatus. The Golgi apparatus concentrates and packages the proinsulin into

secretory granules in which converting enzymes cleave proinsulin to yield insulin and C-peptide [7]. The mature membrane-bound granules contain equimolar amounts of mature insulin and C-peptide. The mature granules move toward the plasma membrane, and release the contents into the extracellular space by a process known as exocytosis [8]. It is now known that cytoplasmic free calcium (Ca^{++}) concentration is of paramount importance in this process, and that Ca^{++} concentration is dependent on glucose metabolism in the beta cells [3].

Insulin secretion in response to postprandial glucose elevation depends on metabolism of glucose in the beta-cells. Glucose enters the beta-cells via a high Km glucose transporter (GLUT2) and is then phosphorylated by glucokinase which works as a glucose sensor and is the rate limiting enzyme for glucose metabolism. Subsequently glycolysis and mitochondrial oxidative phosphorylation lead to an elevation of ATP level and the closure of ATP-dependent K^+ channels. This results in depolarization of the membrane which is followed by the opening of Ca^{++} channels. The resultant influx of Ca^{++} activates enzyme phospholipase C, which generates inositol triphosphate (IP_3) and diacylglycerol (DAG), which both mobilize further Ca^{++} from intracellular stores in the endoplasmic reticulum. The increased intracellular Ca^{++} triggers insulin secretion [3,9].

Under normal conditions, insulin release in response to intravenous glucose load is characterized by a two-phase release; there is a rapid and transient burst of insulin secretion immediately after glucose administration, called the acute or first phase of

insulin response that subsides within about 10 minutes. This acute phase insulin secretion is released from previously stored insulin granules that are adjacent to the beta cell membrane [3,10] and the use of inhibitors to insulin synthesis *in vitro* does not prevent this first phase insulin response [10]. Clinically the acute insulin response is considered as an important parameter of beta cell function. In contrast, the second phase of insulin release is characterized by gradual secretion which progressively increases for four hours after glucose exposure [3]. This phase is believed to result from the release of newly synthesized insulin granules [10]. This biphasic insulin secretion in response to a glucose load has a significant physiological role in restoring postprandial euglycemia.

Once insulin is secreted and released into blood stream, it reaches the first target tissue the liver. The liver is an important site of insulin action, but it also degrades and clears 40-60% of the insulin [7,11]. Therefore, the insulin concentrations beyond the liver are much lower. Insulin has two other main target tissues, muscle and fat, which depend on insulin to promote glucose metabolism.

C. Insulin Action

Insulin is a very important hormone which regulates carbohydrate, fat and protein metabolism and also has an influence on the secretion of other hormones and mineral metabolism. In terms of glucose homeostasis, insulin is regarded as the major hormone regulating blood glucose concentration [12]. Some tissues such as brain, renal medullar and erythrocytes, are entirely dependent on glucose as a source of energy and glucose

utilization in these tissues is not insulin-dependent. Liver, peripheral muscle and adipose tissue are the major tissues in which insulin regulates glucose production and utilization, so the term " insulin sensitivity" is used to define the ability of insulin to enhance glucose disappearance in peripheral tissues and to suppress glucose production in the liver [12].

After an overnight fast, plasma insulin declines to basal levels of 70-105 pM which permits hepatic glucose production by glycogenolysis and/or gluconeogenesis to proceed [13,14]. Liver glucose production in the fasting state is the only source for glucose which is consumed by brain and blood cells, tissues which depend on a constant supply of glucose [15,16]. The critical role of insulin in this state is to alter the liver glucose production. Insulin has a paracine effect on alpha cells, regulating glucagon release. In turn, glucagon directly stimulates hepatic glucose production [17].

After glucose ingestion, the ensuing hyperglycemia and hyperinsulinemia work together to regulate glycemia by suppressing endogenous hepatic glucose production and stimulating glucose uptake by the splanchnic bed (liver and gut) and peripheral skeletal muscle and fat tissue [18]. One third of the ingested glucose will be absorbed by the liver and used for glycogen formation and triglyceride synthesis. One third of ingested glucose is used by muscle and fat along insulin-dependent pathways and the rest is used by the brain and other noninsulin-dependent tissues to meet their consistent ongoing obligate glucose needs [18]. The degree of postprandial glycemia is thus simultaneously

dependent on these three processes: splanchnic glucose uptake, suppression of endogenous hepatic glucose production and peripheral glucose utilization [18].

It has been suggested that the liver is more sensitive than the peripheral tissues to minor changes in glucose and/or insulin concentration. The concentration of insulin required to achieve half-maximal suppression of glucose production was shown to be (210-350 pM), much less than that required to achieve half-maximal stimulation of peripheral glucose utilization (840 pM) [19,21]. Although hyperglycemia augments suppression of hepatic glucose production, it is not the critical factor; insulin plays the major role in suppressing this endogenous glucose production [23]. Experimental evidence suggests that hyperglycemia alone can increase splanchnic glucose uptake in the presence of basal insulin level by mass action and a change of insulin level during this state will increase glycogen and triglyceride synthesis. The observation that splanchnic glucose uptake is six times higher after oral than intravenous glucose administration raised the speculation that gut peptides secreted by the gastrointestinal system (gastric inhibitory polypeptide) may be responsible for the enhancement of splanchnic glucose uptake [22,24]. Now evidence suggests that the arterial- portal glucose gradient may play an important role in the regulation of splanchnic glucose uptake based on studies performed in dogs, in which net splanchnic glucose uptake was twofold higher after intraportal rather than peripheral glucose administration while hepatic glucose load, insulin, glucagon and arterial glucose concentration remained identical under the experimental conditions [25].

Although hyperglycemia can increase peripheral glucose uptake after a meal by mass-action, insulin is necessary for the maintenance of a normal rate of glucose storage and oxidation [26]. Therefore, insulin plays a key role in the maintenance of normal glucose tolerance in the postprandial state by the suppression of endogenous glucose production and stimulation of peripheral glucose utilization [27].

All the physiologic actions of insulin are mediated by cellular mechanisms. The binding of the hormone to specific high-affinity cell surface receptors is the initial event [28,29]. An insulin receptor consists of two α-subunits and two β-subunits linked by disulfide bonds to form an $\alpha_2 \beta_2$-heterotetramer. The α-subunits contain the insulin binding domain, whereas the β-subunits contain an insulin-stimulated tyrosine kinase. The autophosphorylation of the β-subunits of the receptor after insulin binding is the first step in the signal transduction pathway [30]. The signal transduction network has not been fully elucidated for any of the actions of insulin. Recently, since the discovery of insulin receptor substrate-1 (IRS-1), rapid advances have been made in understanding the downstream components of the insulin-signaling pathway. The first evidence proving the existence of IRS-1 came from the experiments using antibodies against phosphotyrosine. Within seconds after cells were stimulated with insulin, a high molecular weight tyrosine phosphorylated protein (Mr 160,000-185,000) was detected by antibodies on the SDS-gel [37]. Studies in the cells containing point mutation of the insulin receptor at tyrosine 972 showed that no phosphorylation of IRS was detected in these receptors which still maintain normal insulin binding, autophosphorylation and kinase activity. The lack

phosphorylation of IRS in these mutated insulin receptors parallels an inability of these receptors to mediate insulin-stimulated glycogen and DNA synthesis, suggesting that IRS is critical to insulin action [38]. The sequence of this protein showed a unique cytosolic protein with no homology to any known protein and it was termed IRS-1 for insulin receptor substrate-1. Later additional evidence demonstrated the functional significance of this protein in the insulin signaling pathway. IRS-1 phosphorylated by insulin receptors acts as a docking protein which binds and activates several cytosolic signaling components, resulting in a modulation of some of the downstream effects of insulin [217]. It has been demonstrated that a number of proteins containing SH2 domains such as phosphatidylinositol 3-kinase (PI3-kinase), the subunit of p21 ras-GTP activating protein (GAP), phospholipase C, and the adaptor molecule GRB-2, are associated with IRS-1 and mediate insulin's metabolic and growth effects [218,220].

Two models exist for the downstream signal transduction pathway after IRS-1. One model is a phosphorylation cascade [31,33]. In this case, receptor tyrosine kinase activates one or more serine kinases which phosphorylate other kinases or substrates. These events lead to the activation of insulin effector enzymes. In the second model which involves the mediator hypothesis [34,36], insulin activates other signaling molecules such as phospholipase C and phosphatidylinositol 3-kinase, leading to release of phosphoinositolglycan (PI-glycan) and phosphoinositides, which act as mediators of mechanisms that regulate insulin effector enzymes.

The signaling pathway for insulin-regulated glycogen synthase activation and GLUT4 is currently better understood than the pathways of other insulin effector components. Glycogen synthase is activated by a phosphorylation cascade involving several kinases which are associated with IRS [221]. The translocation and activation of GLUT4 by insulin has been suggested to be mediated through an IRS-1 activated PI3-kinase, the latter generating phosphoinositides which in turn serve as mediators to modulate the downstream components of insulin action on GLUT4 translocation and activation [222]. Although evidence suggests this is the predominant pathway for activation of glycogen synthase and translocation of GLUT4, actually the signaling network for insulin action is complicated by cross talk between different kinases and mediators. Thus we cannot yet isolate a single component in these complicated pathways.

In summary, insulin plays a key role in the maintenance of normal glucose homeostasis by suppressing endogenous hepatic glucose production and stimulating glucose utilization by its target tissues during a meal. In the fasting state, levels of insulin will fall to basal levels which are important for allowing endogenous glucose production to proceed and maintaining glucose levels during a fasting state.

II. DEFECTS IN DIABETES

A. Concepts of Diabetes

Diabetes is characterized by hyperglycemia during both fasting and postprandial periods. It is caused by both defects in insulin secretion and insulin action which normally determine glucose homeostasis under these conditions.

After insulin was purified and used as a therapeutic agent, Himsworth first recognized that diabetes could be differentiated into "insulin sensitive" and "insulin insensitive" types based on the blood glucose response to administered insulin [39]. The development of the radioimmunoassay (RIA) for insulin by Yalow and Berson and their landmark studies by oral glucose tolerance test (OGTT) gave definitive proof for two types of human diabetes [40,41] and lead to the current classification of diabetes. Since then, as more techniques have been devised to estimate *in vivo* insulin secretion and insulin action, more evidence has accumulated regarding the pathophysiologic aspects of this disease. It is now well known that the two types of diabetes are distinctive diseases, with different pathogenic mechanisms and defects.

B. Defects in Type I Diabetes (Insulin-dependent Diabetes Mellitus or IDDM)

In IDDM, insulin is not synthesized or secreted in sufficient quantities to maintain blood glucose concentration at a normal level [42]. In addition, the insulin that is produced, does not have the same effects on target cells, a condition known as insulin resistance

[43,44]. This loss of sensitivity to insulin is caused by both insulin deficiency and hyperglycemia [45,47].

C. Defects in Type II Diabetes (Noninsulin-dependent Diabetes Mellitus or NIDDM)

In NIDDM, as in IDDM, both insulin secretion and insulin action are impaired, however, insulin secretion is much less affected. It remains unclear which abnormality precedes the development of NIDDM, insulin resistance or deficiency of insulin secretion. Insulin resistance or insulin deficiency alone does not generally produce clinical diabetes in experimental models [48,49], so both insulin resistance and diminished insulin secretion are required for overt glucose intolerance to occur in NIDDM [50].

In contrast to IDDM, insulin resistance is the predominant defect in NIDDM. Insulin resistance is manifested as decreased glucose uptake in peripheral tissues, and continued glucose production, in the presence of hyperinsulinemia [51,52]. As mentioned earlier, the primary action of insulin on the liver is to reduce net glucose release and the liver is believed to be more sensitive to the increments of plasma insulin concentration than peripheral tissues. In NIDDM subjects, the dose-response curve for suppression of hepatic glucose production by insulin is shifted to the right, indicating hepatic insulin resistance [53]. In NIDDM, the liver continues or even increases the release of glucose in the face of hyperglycemia and relative hyperinsulinemia [54]. Net hepatic glucose release appears resistant to exogenously infused insulin in NIDDM subjects, further supporting the conclusion that hepatic insulin resistance is present [86]. In peripheral

tissues, a reduction of more than 50% of the normal glucose disposal rate in type II diabetic patients has been demonstrated. Dose-response curve analysis suggests that this defect is due to both a reduction of insulin receptor binding and a postbinding (intracellular) defect, as indicated by the right shift of the dose-response curve and marked decrease in the maximal rate of glucose disposal [56].

Fasting plasma insulin levels have been reported as low, normal or elevated in NIDDM in the presence of insulin resistance [57]. However, subjects with NIDDM, matched for body weight and glycemia, show deficiency of both basal insulin secretion [58] and glucose-stimulated insulin response marked by the absence of first phase insulin release [59,60]. The second phase insulin response can be either normal or exaggerated, but compared to weight-matched normal subjects with a matched plasma glucose level, NIDDM patients have impaired second-phase insulin secretion [60]. Abnormal beta cell function in NIDDM is also seen as a reduced ability of glucose to potentiate the insulin secretory response to other non-glucose secretogogues. The dose-response relationship between glucose and insulin release to intravenous arginine, between normal subjects and individuals with NIDDM, shows a maximal response less than 25% of the control value in NIDDM. This indicates an impaired beta cell secretory capacity, while the plasma glucose level for half-maximal potentiation, reflecting beta cell sensitivity to glucose, was identical between the two groups [58].

In addition, islet alpha cell glucagon secretion is also altered in NIDDM. Glucagon secretion, both at the basal state and in the glucose-stimulated state, is inappropriately elevated in NIDDM [58]. Since islet blood flow is known to proceed from beta- to alpha- and delta- cells, the primary defect in the beta cell causing impaired insulin secretion to glucose may be the underlying cause of the derangement in glucagon secretion through a paracrine mechanism [61,62].

III. PATHOGENESIS OF NIDDM

A. Two Categories of Pathogenic Mechanisms

Diabetes is among the most common of all metabolic disorders affecting up to 11% of the population, type II diabetes representing 90 % of the affected population [63]. For decades, many research efforts have focused on searching for pathogenic factors involved in defects in insulin secretion and insulin action through characterization of major elements of insulin secretion and insulin action. Defects in insulin secretion and insulin action in NIDDM are the results of two different pathogenic mechanisms [65]. Primary genetic factors play a key role in the development of NIDDM, and they initiate the clinical onset of NIDDM and metabolic derangements such as hyperglycemia, alterations of insulin levels and elevated free fatty acids. These metabolic factors can then cause secondary pathogenic mechanisms which will aggravate the primary defects in NIDDM.

B. Primary Pathogenic Factors

Evidence from epidemiological studies strongly suggest that genetic factors play a predominant role in the development of this disease. When comparing the prevalence of the glucose intolerance among specific relatives of NIDDM individuals to that found among similar relatives of a control group, there is significantly greater prevalence rate among the close relatives of diabetic (10-30%) than among similar relatives of nondiabetic individuals (1-6%) [66]. Twin studies further confirmed the importance of genetic factors in the development of diabetes with NIDDM. When glucose tolerance tests were performed in monozygotic (identical) twins and dizygotic (fraternal) twins, the frequency of concordance for diabetes in monozygotic (45-96%) is significantly higher than for dizygotic twins (3-37%) [67]. The evidence derived from these familial aggregation and twin studies indicates the importance of genetic factors in the etiology of NIDDM.

A great deal of effort has been made to explore the " primary" lesion in NIDDM. Mutations of the glucokinase gene have been identified as being responsible for one form of NIDDM-maturity-onset diabetes (MODY) [68]. Mutations of mitochondrial subunits of the respiratory chain complexes are associated with another form of genetically mediated diabetes [69]. Mutations of the insulin gene affecting its expression and biologic action have also been reported in some families diagnosed with type II diabetes [70,71]. Point mutations of the insulin receptor gene and altered expression of two

isoforms of the receptor through alternative mRNA splicing are the underlying basis for insulin resistance in some NIDDM patients [72]. Altered activities of the insulin receptor kinase and glycogen synthase have been shown to be an early event in some pre-diabetic high-risk populations [73,74], but there is no data available about whether these alterations are due to gene mutation or a consequence of altered regulatory mechanisms. Considerable progress has been made in the search for primary defects in NIDDM, but the genetic heterogeneity of this disease is a significant obstacle to success. The mutations identified to date account for only a minority of diabetes cases. Furthermore, secondary pathogenic mechanisms obscure the fundamental defects by altering insulin secretion and insulin action via changes in gene expression.

C. Hyperglycemia is the Major Secondary Pathogenic Factor

C. 1. Glucose Toxicity

Hyperglycemia is inevitably present in NIDDM and has been considered as a major secondary pathogenic factor for the defects in NIDDM. The concept of " glucose toxicity" refers to the effect of hyperglycemia on beta cell function and insulin action [50]. Chronic hyperglycemia decreases insulin secretion and insulin action by a toxic effect and plays a central role in the pathogenesis of the beta cell defect and insulin resistance in diabetes. This concept was proposed based on observations from both human and animal models of diabetes.

In human diabetic subjects, tight metabolic control, independent of the method by which it is achieved (diet, insulin, sulfonylureas), leads to improvement in beta cell function and insulin sensitivity [75,77]. These observations support the conclusion that hyperglycemia exerts a deleterious effect on beta cell function and insulin action. Studies in animal diabetic models provide a more rigorous test of this glucose toxicity hypothesis, because the application of one agent, phloridzin, an inhibitor of renal tubular glucose transport, leads to normalization of plasma glucose without a change in plasma insulin levels. Futhermore, the doses used in these studies, phloridzin has no effect on gut or muscle glucose transport. Studies have shown that hyperglycemia alone can impair both insulin secretion and insulin action [78,85].

C. 2. Glucose Toxicity on Beta-cells

The effects of chronic hyperglycemia on insulin secretion was evaluated in an animal model of NIDDM which was developed by 90% partially pancreatectomy [78]. Three groups of animals were studied; group 1 received a sham operation, group 2 received partial pancreatectomy, and group 3 was partially pancreatectomized and received phloridzin to normalize plasma glucose. To examine the effects of glycemia on insulin secretion, a hyperglycemic clamp was performed. There is a typical biphasic pattern of insulin secretion in the control rats, while in partially pancreatectomized rats the first phase was lost and the second phase was markedly impaired. In the phloridzin-treated group, both phases of insulin secretion were significantly enhanced compared with the hyperglycemic group but less than normal. In fact, the insulin response was restored to

normal in this phloridzin-treated group when considered per gram of residual pancreatic tissues. But the plasma insulin response to arginine, a nonglucose insulin secretagogue, was increased 3- fold in the hyperglycemic group, but returned to normal when the hyperglycemia was corrected with phloridzin. These results clearly demonstrated that chronic hyperglycemia in rats produced an impaired beta cell response to glucose, but not to the other secretogogues, suggesting that glucose toxicity, at the level of the beta cells, causes a defect in glucose sensing rather than a defect in insulin secretion per se. When the mechanism of this reversible impairment of beta cell function by hyperglycemia was examined, it appeared that the decrease in insulin secretion was not due to changes in intracellular glucose metabolism or insulin synthesis [79,81], but coupling of glucose metabolism to the secretory process was desensitized. Phosphoinositol metabolism has been shown to play a role in this hyperglycemia-induced deficiency of insulin secretion [50,82,83].

C. 3. Glucose Toxicity on Insulin Action

Unger and Grundy [84] first proposed the role of hyperglycemia in the impairment of insulin-mediated glucose disposal in both type I and type II diabetic subjects. This was confirmed in an experiment using the previously described animal model of NIDDM [85]. Euglycemic insulin-clamp studies were performed to assess the effect of chronic hyperglycemia and euglycemia on insulin sensitivity. Results showed that plasma glucose levels were inversely correlated with the degree of insulin-mediated glucose uptake and phloridzin treatment completely restore insulin sensitivity to normal. Under

euglycemic hyperinsulinemic conditions, it was demonstrated that most (> 80-90%) of the infused glucose load is disposed by peripheral tissues, primarily muscle [16,86]. Therefore, these results suggest that the muscle is the major organ responsible for the development of insulin resistance after exposure to chronic hyperglycemia [85,88]. Consistent with this, basal hepatic glucose production (measured with [3-^{3}H] glucose) and its suppression during insulin infusion in clamp experiment were not altered by hyperglycemia [85].

Though many studies have been performed to investigate the mechanisms underlying glucose toxicity on insulin resistance, it remains unclear. Since Traxinger and Marshall [89,90] first observed that overactivity of the hexosamine biosynthetic pathway mediates glucose-induced insulin resistance in rat adipocytes, more evidence has suggested that this pathway might be involved in the insulin resistance caused by this hyperglycemia. Basically, this hypothesis proposed that a small fraction (2-3%) of glucose is metabolized to a hexosamine product (glucosamine-6-P) in the presence of glutamine. The hexosamine products decrease the activity of the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) activity, the rate-limiting step in this pathway, and desensitize the glucose transport system in the presence of insulin. When hyperglycemia prevails, it increases the rate of glucose uptake in a concentration-dependent manner, so does the hexosamine pathway which will sense the rate of glucose entry and desensitize glucose transport activity to normalize the flux of glucose [89,90]. *In vivo* infusion of glucosamine products of this pathway for 8 hours in rats gave results consistent with this

hypothesis that increased flux via the hexosamine pathway is involved in glucose-induced insulin resistance by desensitizing glucose transporters in skeletal muscle [91].

The hexosamine pathway hypothesis provided an explanation for the observation *in vitro* that the distribution and intrinsic activity of the glucose transporter was decreased in skeletal muscle and is the major defect induced by hyperglycemia [92,94]. However, the observation by Haring [95] suggested that hyperglycemia can also inhibit tyrosine kinase activity of the insulin receptor via activation of certain protein kinase C isoforms which translocate up to plasma membrane and form a complex with the insulin receptor and modulate tyrosine kinase activity through phosphorylation of the receptor beta subunit at serine residues.

C. 4. Genetic Regulatory Mechanism of Glucose Toxicity

More investigation into the mechanism of glucose toxicity must be performed. This issue may be particularly important in human NIDDM subjects, who may be exposed to abnormally high glucose concentrations for years, even before they are diagnosed. It is possible that chronic hyperglycemia may cause irreversible and increasingly deleterious damage to beta cell function and insulin action through a variety of cellular mechanisms. Studies by Robertson et al [96,97] suggested this possibility. They used *in vitro* insulin-secreting cell lines HIT-T15 and BTC-6 to study the chronic effect of hyperglycemia (25 weeks) on beta cells. They observed that after chronic exposure to a supraphysiological glucose concentration (11.1mM) these cell lines lost insulin responsivity to glucose and

had greatly diminished level of insulin content and insulin mRNA, whereas the cells grown under a low glucose concentration (0.8mM) retain insulin mRNA, insulin content and insulin responsivity to glucose. No differences were found in cell population doubling time, or GLUT2 levels between cells grown under different glucose concentrations and no mutations were found in the insulin gene in cells grown under high glucose. This strongly suggested a potential gene regulatory mechanism of hyperglycemic toxicity on beta cells. This mechanism is likely mediated through glucose-response transcription factors binding to cis-elements in the promoter-enhancer region of the insulin gene [96,97]. Glycosylation of an insulin transcription factors or their gene, which could prevent the transcription factor interacting with their regulatory elements of the insulin gene is another possible mechanism [98]. Consistent with this observation, Eizirik et al [99] observed diminished insulin content for human islets cultured in high glucose concentration for 7 days and this change was partially reversed by subsequent culture in lower glucose concentration. Orland et al [100] observed a decrease in both insulin content and mRNA levels in db/db mice after several weeks of hyperglycemia. Based on these observations, a new definition for glucose toxicity was proposed which implies irreversible cellular damage caused by chronic exposure to hyperglycemia, while glucose desensitization more properly fits in the category of reversible cellular damage because of the relatively shorter exposure to hyperglycemia [101].

This gene regulatory mechanism of glucose toxicity in beta cells raises the possibility that glucose may have a similar gene regulatory mechanism on insulin action in skeletal muscle, the major tissue responsible for insulin resistance induced by hyperglycemia. This new concept of glucose toxicity underlines the importance of further investigation into the mechanism of glucose toxicity, with respect to insulin action. The main goal of this project is to investigate a potential gene regulatory mechanism of glucose toxicity in skeletal muscle, which could lead to insulin resistance.

IV. MODELS USED FOR STUDY OF GLUCOSE TOXICITY

A. *In Vitro* Cell Culture

In vitro cell culture studies have provided considerable evidence about the possible mechanisms of glucose toxicity. These studies have the advantage of assessing the effects of hyperglycemia on beta cell function without a change in insulin level. The most commonly used cell lines for studies of insulin action are 3T3L1 cell lines from adipose tissue [102]. However, adipose tissue is not the major tissue targeted by hyperglycemia. Unfortunately, an appropriate muscle cell line is not currently available. L6 and L8 myocyte cell lines are from muscle, but they contain more GLUT1 rather than GLUT4 [94,103]. GLUT4 is the major glucose transporter isoform in skeletal muscle and is responsible for insulin-stimulated glucose uptake *in vivo*. Therefore, a better way to investigate the effect of glucose toxicity on insulin action is to use an *in vivo* diabetic animal model.

B. *In Vivo* Pancreatectomy Model

As described earlier, 90% pancreatectomized animals have been used for this purpose, in combination with phloridzin, an inhibitor of the renal tubular glucose transporter. This model has provided valuable evidence about glucose toxicity [78,85].

In another animal model used for this aim by Weir et al [104], a 60% pancreatectomy was performed in rats, and plasma glucose levels in these animals which were similar to controls. These pancreatectomized animals were divided into two groups; one was given tap water and their normal rat chow, while the another group received 10% sucrose instead of tap water. The mean nonfasting plasma glucose concentration was elevated in sucrose-fed rats compared with other two groups. Using this animal model, insulin secretion and action were evaluated and compared and it was found that the elevation of plasma glucose concentration can lead to impairment of insulin secretion in this model.

Pancreatectomized rats are a good model for the study of the effects and mechanism of glucose toxicity, but application of this model has been limited due to the difficulty of the surgical procedure and traumatic complications from the surgery.

C. Streptozotocin-induced Diabetic Model

A streptozotocin (STZ)-induced diabetic model is an alternative approach for studying glucose toxicity. STZ is a naturally occurring antibiotic and has diabetogenic effects [105,106]. The structure of this chemical is a methyl-nitrosourea side chain linked to the

C2 position of a D-glucose molecule [107,108]. The diabetogenic action of STZ was found to be dependent on the glucose moiety of this toxin, which directs this chemical to the beta cell specifically [109]. The nitrosourea moiety will alkylate the DNA of the beta cell, especially the mitochondrial DNA [110,112]. Damage to mitochondrial DNA leads to dysfunction of the mitochondria and subsequently affects glucose oxidation, and ATP synthesis, major functions of mitochondria which are the crucial steps in insulin synthesis and secretion [113,114]. So administration of STZ generates a insulinopenic, hyperglycemic diabetic state.

Although no evidence is currently available which demonstrates a direct toxic effect of STZ on skeletal muscle, it has been shown that this molecule has toxic effects on liver, kidney, lymph tissue, intestine and other tissues [115,116]. The D-glucose structure of this toxin that directs it to the beta cells may also direct STZ to other glucose utilizing tissue. So DNA damage by STZ cannot be ruled out when using this model to study the gene regulatory mechanism of glucose toxicity on insulin action on skeletal muscle.

V. METHODS USED FOR ANALYZING GENE EXPRESSION IN DIABETES

A. Candidate Gene Approach

Attempts to study alterations in gene expression that might occur in diabetic patients have been largely based on a "candidate-gene" approach. Candidate genes are screened for a difference between diabetic and control subjects and then a larger population is studied for the linkage between mutations of this candidate gene and glucose intolerance within

pedigrees segregating for diabetes [117]. This approach has been successful in identifying a glucokinase gene mutation in some families with maturity-onset diabetes of youth (MODY) [68,118]. However, this approach has not been widely successful due to the genetic heterogeneity of NIDDM [68,69,70,71,72], and since this method only permits screening of known genes on an individual basis.

B. Subtractive Hybridization

Subtractive hybridization is another approach which can identify changes in gene expression in one population compared to another. This method avoids the limitations imposed by availability of probes for candidate genes and is performed without preconceptions about probable differences in gene expression [119]. Briefly, a cDNA library is made from cells of interest and hybridized with RNA from the another cell population as control, then all cDNA/RNA hybrids are removed leaving only unhybridized cDNAs that represent unique mRNAs of the cells of interest. Although this technique is very powerful, it is technically difficult to construct the cDNA library and a large amount of high quality RNA is required. Hybridization is necessary to select the differentially expressed gene. Hybridization involves many steps and is not only time consuming, but genes of interest might be lost during the procedure [120].

C. mRNA Differential Display

Differential display is a new methd for screening unknown differential expression [121]. This method combines both the power of polymerase chain reaction (PCR) and the high

resolution of denaturing polyacrylamide gel electrophoresis for separation of amplified cDNA products. PCR is a powerful technique developed within the last decade which allows rapid amplification of nucleic acids *in vitro* using a thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase) [123]. One cycle of PCR represents three sequential steps: denaturation, annealing and extension. Initially, the two strands of template DNA are denatured by high temperature after which the temperature is dropped to allow short synthetic oligonucleotides (called primers) to anneal to the complementary sequence of the template. In the presence of substrate dNTPs, the extension step will occur in the 5′ to 3′ direction from one primer to the other at 72°C. Nucleic acid sequences defined by two primers will be synthesized in one cycle and repeated cycling results in the exponential amplification of the target sequence.

Differential display is based on the assumption that every cell expresses approximately 15,000 genes which can be reverse transcribed to cDNA and amplified by the polymerase chain reaction (PCR) [121,130]. Total RNA is purified from cells of interest and reverse transcribed to produce DNA copies of the messenger RNA (cDNA) using four 3′ primers: $T_{12}MA$, $T_{12}MC$, $T_{12}MG$ and $T_{12}MT$ (M is degenerate mixture of dA, dC and dG). These primers take advantage of the polyadenylated [poly(A)] tail present on eukaryotic mRNAs to anchor the primer at the 3′ end of mRNA, while the two additional bases give the primers specificity and subdivide the cDNA into four groups, since each primer permits initiation of reverse transcription of only this subpopulation. Then each subpopulation of cDNA is amplified by PCR using the same 3′ primer and an arbitrary

decamer as the 5′ primer. The 5′ primers are arbitrary 10-mer oligonucleotides that hybridize randomly with complementary sequences located at varying distances from the 3′ end of each cDNA strand. Because the arbitrary 5′ primer will only anneal to a small proportion of the total cDNA pool, the pair of primers selected will provide amplification of a subpopulation of only ~150 of the available cDNA species. By the use of different sets of primers, (26 arbitrary 5′ primers along with four degenerate oligo(dT) 3′ primers to give 104 reactions per sample) 90% of all mRNA expressed in a given cell type will be evaluated. Radio-labeled PCR products are visualized by separation on a 6% w/v denaturing polyacrylamide gel followed by autoradiography. Through side by side comparison on the gel of cDNA bands between different groups, differential expression can be detected by visual inspection. This method is much more sensitive, quicker and easier than the subtractive hybridization technique. It has not only been applied to screen qualitative (on/off) gene expression in cancer [121,124,125], it also has been used to investigate the quantitative (up/down) gene expression under different physiologic and pathologic conditions [126,128].

VI. OBJECTIVES OF THIS STUDY

The main aim of this project is to investigate the secondary pathogenic mechanisms of insulin resistance in skeletal muscle of diabetic animals. We created a chemically induced diabetic animal model by administration of STZ. After 5 weeks of diabetes, we screened for differential expression of genes in skeletal muscles from these animals using

the differential display technique to determine whether altered gene expression could cause secondary insulin resistance in these diabetic animals.

MATERIALS AND METHODS

ANIMAL CARE AND MONITORING:

Male Spague-Dawley rats, purchased from Biologic Science Animal Services (U of A) at 8 - 10 weeks of age, were housed in individual cages, and maintained on 12h light, 12h dark cycle. Animals had free access to food and water. After 3 days acclimatization, basal body weight was measured, and plasma glucose and insulin levels were monitored from the tail vein. Blood was collected from the tail vein of nonfasted conscious animals into heparinized microhematocrit tubes between 10:00 - 14:00 h. These samples were immediately centrifuged and the plasma was stored at -20°C until later analysis. The whole blood glucose level was measured by One-Touch II blood glucose meter (Lifescan Canada Ltd, Johnson & Johnson company Burnaby, B. C.)

ANIMAL PREPARATION:

Experimental rats were randomly subdivided into 3 groups. (1) Streptozotocin (STZ) - induced diabetic rats (n = 16), (2) STZ - induced diabetic rats treated with insulin to maintain euglycemia (n = 14), (3) Normal rats to serve as a control group (n =10). Animals were made diabetic with a tail injection of STZ (55mg/kg). STZ was dissolved in 0.1 M acetate buffer pH4.0. Control animals received a sham injection of vehicle. All animals were monitored for their weight, plasma glucose and insulin daily for 3 days to verify the development of hyperglycemia. The group of animals on insulin treatment

received Ultralente (Connaught Novo Nordisk) insulin 1 U/100g subcutaneously twice a day to normalize blood glucose levels, and their whole blood glucose levels were monitored daily by glucose meter. The other two groups of animals had blood glucose levels monitored twice a week by glucose meter. All the animals had their plasma glucose, insulin and body weight determined weekly. Five weeks later, a hind leg muscle biopsy was performed. Animals were fasted and anaesthetized i.p. with sodium pentobarbitol (60mg/kg, 30mg/kg in the diabetic animals) together with atropine (0.1mg). Hind muscle(soleus plantaris, gastrocnemius, and vastus lateralis) samples were removed and snap frozen in liquid nitrogen and kept at -70°C until analysis. Animals were euthanized without recovering from the anaesthetic.

CHEMICALS:

Agarose, acrylamide, bis-acrylamide, random hexamer labeling kit, TRIzol™ RNA extraction solution, Superscript reverse transcriptase, DNase I, restriction enzymes and T7 polymerase were purchased from GIBCO BRL (Burlington, ON). Taq polymerase was prepared as described [213]. [^{32}P] dATP were obtained from Amersham. The TA cloning kit were purchased from Invitrogen. Poly A isolation spin columns were from New England Biolabs. Kodak BioMax™ film was used for autoradiography. A phosphorimager (FUJI Photo Film, Tokyo, Japan) was used to analyze the autoradiographs of Northern blots. Geneclean™ was purchased from Bio 101 Inc (La Jolla CA 92038-2284).

STRAIN, VECTORS, and MEDIA.

A. E.coli strain DH5α was used for transformation. The genotype of these cells is: F^-, endA1, hsdR17(r_K^-, m_K^+), supE44, thi-1, λ-, recA1, gyrA96, Δ(argF-Laczya) U169, ϕ80LacZΔM15. The ϕ80LacZΔM15 marker allows α complementation of the β-galactosidase gene from pUC. Blue/white color selection was used to screen for colonies with insert containing plasmids.

B. Media: LB plates (to 950ml water add 10g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, adjust to PH 7.4, add 15g agar and water to 1 litter, autoclave to sterilize.) were used to support growth of the transformed cells. The plates also contained 100 mg/ml ampicillin, to select for plasmid transformed cells.

OLIGONUCLEOTIDES:

Synthetic oligonucleotides were designed for use in mRNA differential display as described in [129]. The four 3′ primers were T_{12}MA, T_{12}MC. T_{12}MT and T_{12}MG (M is a degenerate mixture of dA, dC and dG). Twenty-six 5′ primers with arbitrary sequences were selected from a list of random sequences considering the following characteristics: (i) presence of 50% G+C and A+T, and (ii) absence of uninterrupted self-complementarity of more than two nucleotides, (iii) thirteen primers have a GATC sequence at the 5' end in common (Tab.1). M13 reverse primer was used for DNA sequencing.

Oligonucleotides were synthesized on the 391 DNA Synthesizer (Applied Biosystem Inc. PCR Mate^{TM}) and purified by reverse phase chromatography on Sep-Pac C18 cartridges as described by Atkinson and Smith (1984). Purified oligonucleotides were suspended in dH_2O at a final concentration of 20mM and stored at -20°C until used.

DIFFERENTIAL DISPLAY

A. RNA Isolation:

Total RNA was extracted from frozen muscle tissue using TRIzol Reagent (GIBCO. BRL), a mono-phasic solution of phenol and guanidine isothiocyanate. The frozen samples were homogenized in TRIzol Reagent using a tissue homogenizer (Polytron), followed by incubation at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1ml of TRIzol Reagent was added, the sample was mixed vigorously by hand for 15 seconds and incubated at room temperature for 3 minute, followed by centrifugation at 17,000rpm for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into 3 phases and the upper colorless aqueous phase containing RNA was transferred to a fresh tube and precipitated with an equal volume of isopropanol followed by centrifugation. The integrity of RNA was assessed by ethidium bromide fluorescent staining of 18S and 28S ribosomal RNA on denaturing agarose gel.

Table 1. List of all 5′ primers used for a complete analysis of expressed RNA by DDPCR.

No.	Sequence (5′ to 3′)
1.	T A C A A C G A G G
2.	T G G A T T G G T C
3.	C T T T C T A C C C
4.	T T T T G G C T C C
5.	G G A A C C A A T C
6.	A A A C T C C G T C
7.	T C G A T A C A G G
8.	T G G T A A A G G G
9.	T C G G T C A T A G
10.	G G T A C T A A G G
11.	T A C C T A A G C G
12.	C T G C T T G A T G
13.	G T T T T C G C A G
14.	G A T C A A G T C C
15.	G A T C C A G T A C
16.	G A T C A C G T A C
17.	G A T C T G A C A C
18.	G A T C T C A G A C
19.	G A T C A T A G C C
20.	G A T C A A T C G C
21.	G A T C T A A C C G
22.	G A T C G C A T T G
23.	G A T C T G A C T G
24.	G A T C A T G G T C
25.	G A T C A T A G C G
26.	G A T C T A A G G C

B: Removal of DNA contamination from RNA

50 ug of total RNA was treated with 7 U of RNase free DNase I (BRL) in 10mM Tris.Cl, pH 8.3, 50 mM KCl, 1.5 mM$MgCl_2$ and in the presence of 1.5 unit of human placental RNase inhibitor (BRL). After extraction with phenol/chloroform (3:1), the supernatant was ethanol precipitated in the presence of 0.3 M NaOAC pH5.2, and RNA was redissolved in diethyl pyrocarbonate (DEPC)-treated H_2O.

C: Reverse Transcription of RNA

Four reverse transcriptions were performed for each RNA sample using 0.2 μg DNA-free total RNA in 1× reverse transcription buffer, 10mM DTT, 20uM each of dGTP, dATP, dTTP and dCTP, and 1 μM of either $T_{12}MG$, $T_{12}MA$, $T_{12}MT$ or $T_{12}MC$ oligonucleotide where M is three fold degenerate for G, A, and C. The primer and RNA were denatured at 65 °C for 5 min and quickly chilled on ice to allow the primer to anneal to the RNA. 200 units of reverse transcriptase (Gibco, BRL) were added and the reaction mixture was incubated at 37 °C for 1 hour. The reaction was stopped by incubation at 95 °C for 5 minutes. Alternatively, a total cDNA pool was generated in one reaction using oligodT as the primer then divided into 4 aliquots.

D: Amplification of cDNA by PCR

Each subpopulation of first strand cDNA generated was then amplified by polymerase chain reaction using the same 3′ primer and a arbitrary decamer as 5′ primer. PCR was performed in a reaction mixture containing 0.1 vol. of RT reaction mixture, 1× PCR

buffer, 2 μM each of dGTP, dATP. and dCTP, 1 μCi [α- ^{32}P] dCTP, 2.5 μM of the respectiveT_{12}MX oligonucleotide, 0.5 μM of 26 different specific arbitrary 10-mer oligonucleotides (see table 1) and 2.5 U of Taq DNA polymerase. The PCR reactions were incubated at 94 °C for 45s, 40°C for 90s, 72°C for 30s for forty cycles, followed by an additional extension period at 72°C for 5 min.

E: Denaturing polyacrylamide gels

DNA fragments from the PCR reaction were electrophoresed on 6% w/v polyacrylamide gels at 60 Watts until the bromophenol dye front reached the bottom of the gel. Gels were prepared by adding 8.3 M urea and the appropriate volume of 38:2 acrylamide: bisacrylamide stock solution to the running buffer, 1 × TBE (50mMTris base, 50mM boric acid, 1mM EDTA). Polymerization was initiated by addition of 0.066% (w/v) ammonium persulphate and 0.024% (w/v) TEMED. 5 μl formamide loading buffer was added to 5 μl PCR reaction mixture on microtiter plates, heated to 95°C for 5 min prior to loading on the gel. After electrophoresis, gels were dried onto 3 mm paper using a gel drier (Model 583 Bio-rad) and exposed to Kodak X-ray film at room temperature. The autoradiogram and dried gel were oriented with a fluorescent maker.

Samples from five different animals from each experimental group were run in parallel on the gel, and bands which showed a difference in all 5 samples between groups were identified.

BAND RECOVERY AND REAMPLIFICATION OF cDNA

Differentially displayed cDNA were located by needle punch after orienting the dried gel and autoradiograph, then excised along with 3 mm backing paper from the dried gel. The dried gel slice was incubated in 100 ul dH_2O at room temperature for 10 min to rehydrate the polyacrylamide gel slice, then the cDNA was extracted by boiling the gel slice for 15 minute in a tightly capped microcentrifuge tube, followed by centrifugation to remove the filter paper and urea. cDNA was recovered from the supernatant by ethanol precipitation in the presence of 0.3 M NaOAC, 3 ul of 0.25% linear polyacrylamide as a carrier, and redissolved in 10 ul dH_2O. 5 µl of the eluted cDNA was reamplified by PCR in a 40 µl reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentration was 20 µM instead of 2 µM and no isotope was added. Amplified DNA was visualized on 1 % w/v agarose gels and PCR samples were stored at -20°C for subsequent cloning and probe generation.

DNA CLONING

A. TA cloning system

PCR products which appeared as a single band (no non-specific amplification) following agarose gel electrophoresis, were directly cloned into linearized PCR 2.1 vector which has single 3′ deoxythymidine (T) residues. DNA products generated by PCR with Taq polymerase usually have a single deoxyadenosine (A) added to the 3′ end in a nontemplate-dependent manner. PCR products were quantified by agarose gel

electrophoresis and concentration estimated against a known DNA ladder. The ligation reaction was roughly adjusted to a 1:1 molar ratio of vector: PCR products using 25 ng (1μl) vector. Ligation reactions were performed in 10 μl reaction volumes in the presence of 1× ligation buffer and 4 U T_4 DNA ligase at 14°C for overnight. The volume of PCR sample used in the ligation reaction was less than 2 μl because the salts in the sample would inhibit T_4 DNA ligase.

B: Blunt end cloning into Bluescript II K/S vector:

(I) Purification of DNA fragments to be cloned:

PCR reactions in which there appeared to be multiple bands or primer dimers, were cloned in a blunt-end fashion. DNA fragments were fractionated by 1% agarose gel electrophoresis, the most prominent band was cut from the agarose gel using a sterile scalpel and DNA was recovered using the Geneclean protocol as described by the manufacturer. Gel purification can remove some of the 3'A-overhangs necessary for TA cloning, so it is more efficient to clone the purified PCR product into a pBS vector by blunt-end cloning.

(II) Blunt-ending PCR products and pBS vetor:

Klenow fragment of E.coli DNA pol I was used to complete the conversion of PCR fragments ends to blunt-ends and a kinase reaction was performed to add a phosphate group to the 5' ends. First, 25 μl reaction volumes containing 10ul PCR product (0.5 pmoles molecules), 1× kinase buffer (60 mMTris-HCl, 10 mM$MgCl_2$, pH7.8), 15 mM

2-ME, 0.33 uM ATP, and 10 units of T_4 kinase, were incubated at 37 °C for 1 hour. The enzyme was heat inactivated at 85°C for 15 min, then cooled to room temperature. Fill-in reactions were performed at room temperature for 30 min by adding dNTP (final concentration 0.25 mM) and 6 units of Klenow. Final reaction mixtures were used directly for blunt end cloning.

The vector Bluescript IISK$^+$ (pBS) was cut with EcoRV and dephosphorylated with calf intestine alkaline phosphatase to minimize the recirculization of the vector and increase the efficiency of blunt end ligation. The linearized vector was then recovered by phenol/chloroform extraction and ethanol precipitation.

(III) Ligation of DNA fragments into pBS:

Conditions for the ligation reaction were the same as TA cloning except the concentration of the vector ends and insert ends were twofold higher than with TA cloning, and the time for ligation was 24 hour.

C. Transformation of recombination DNA into E. coli competent cells

A 2 μl aliquot of the ligation mixture was diluted 10 times in TE (Tris 10 mM, EDTA 0.1mM) pH7.4, and combined with 100 μl of competent E.coli DH 5α cells in a 1.5 ml eppendorf tube. The cells were incubated on ice for 30min, then subjected to heat shock at 42°C for 45 sec. 400 μl of LB liquid medium was added to the tube and the cells were grown at 37° with gently shaking for 1 hour. Transformed cells were plated on

LB/Amp/IPTG/X-gal agar plates and incubated at 37°C overnight. White colonies were selected for subsequent DNA isolation.

D. Isolation of plasmid DNA (miniprep method)

Plasmid DNA was isolated using the procedure of Lee and Rasheed [212]. In short, individual colonies were grown at 37°C with vigorous shaking in LB medium overnight, centrifuged, resuspended in buffer, lysed with SDS and sodium hydroxide, then contaminating proteins were removed by "salting out" with ammonium acetate and the plasmid DNA was precipitated with isopropanol.

DNA SEQUENCING AND RESTRICTION DIGESTION

The chain termination method (Sanger et al.1977) was used for DNA sequence analysis as modified for double stranded templates [214,215]. Double stranded DNA was prepared for sequencing by boiling 10 μg DNA in 0.2M NaOH for 2 min, followed by ethanol precipitation. This step destroys contaminating RNA and denatures the double stranded template. The pellet was resuspended in 7 μl dH_2O. To the DNA solution, 1 ul of 2 μM M13 reverse primer and 2 μl of 5 × sequencing buffer were added and the sequencing primer was annealed to the DNA by denaturing the mixture at 65°C for 2 min, then the mixture was cooled slowly to 35°C. A labeling reaction was performed for 3 min by adding 1× labeling mix, diluted T_7 polymerase (1:8), 1 μl α^{35}S-dATP, and 0.1 M DTT to the annealed DNA mixture. 2.5 μl termination mix (ie. ddG, ddA, ddT, ddC) was added to the 3.5 μl of the labeling reaction, mixed and incubated at 37°C for 5 min.

The reaction was stopped by adding 4 µl loading buffer dye. After boiling for 4 min to denature the dideoxynucleotide-terminated strands, 2.5 µl of each mixture was loaded onto a denaturing 6 % w/v polyacrylamide sequencing gel.

NORTHERN ANALYSIS

The poly (A) RNA was purified using a polyA spin mRNA Isolation kit according to the manufacturers protocol. 5 µg of polyA RNA was fractionated by denaturing 1 % w/v formaldehyde agarose gel electrophoresis and transferred to Hybond^{TM}- N^+ nylon membrane (Amersham Inc. U.K.) by downward capillary action (Northern Blotting). The blot was UV-cross linked to immobilize the RNA on the membrane (Stratagene UV-Stratalinker^{TM} 1800). Hybridization was performed using 7 % w/v sodium dodecyl sulfate solution (0.5 M $NaPO_4$, 7% SDS, 2 mM EDTA, 1 mg NaPPi) described by Gilbert [216]. Membranes were placed in Seal-a-Meal bags, heat sealed and prehybridized at 60 °C with 25 ml of 7 % SDS solution. After a 4 hour prehybridization, the buffer was discarded and replaced with fresh hybridization buffer (as above) containing > 10^8 cpm/µg of probe. ^{32}P-dCTP labeled probe was generated from cDNA clones using a random primer labeling system following the protocol described by the manufacturer. Hybridization was carried out in a 60°C water bath overnight.

Following hybridization, blots were removed from the bag and rinsed for 5 min in 2×SSC/0.1% w/v SDS at room temperature, the same solution at 60 °C for 15 min, 1× SSC/0.1% w/v SDS at 60 °C 15 min twice, then 0.5×SSC/0.1% w/v SDS twice.

mRNA expression was quantified by a Phosphorimager and loading differences were normalized using γ- actin as a control cDNA probe. The blots were stripped by pouring boiled 2% w/v SDS solution onto the membrane, cooled to room temperature, checked for residual probe on the phosphorimager, and then re-probed as above using a γ- actin probe.

RESULTS

ANIMAL MODEL

The plasma glucose levels were similar in all animals at the start of the study. After streptozotocin treatment, the glucose levels rose promptly. The group treated with insulin had a successful restoration of normal glycemia (Fig.1A), but this control required supraphysiological amounts of insulin (Fig.1B). Following STZ administration, the animals lost weight and the group left hyperglycemia showed no weight gain during the five week recovery period. The group treated with insulin recovered their weight and were identical to the control by 4 weeks post STZ (Fig.1C).

DIFFERENTIAL DISPLAY, SUBCLONING, SEQUENCING AND NORTHERN ANALYSIS

The expression of genes within the skeletal muscle from STZ-diabetic, diabetic with insulin treatment, and control rats was compared using mRNA differential display. A total of 26 arbitrary 10-mer primers in combination with all four $T_{12}MN$ primers were used to screen mRNA species (104 reactions per sample). As described, each differential display lane yields ~150 discrete bands allowing evaluation of over 15,000 RNA species, representing 80-90% of the estimated 10,000 ~ 20,000 cellular mRNAs [121,130]. Separate experiments using the same primer pair produced equivalent band pattern while patterns were markedly different between different primer pairs. Side-by-side comparisons were made on 5 RNA samples from each group. An RNA sample without

reverse transcription was amplified by PCR and loaded on the same differential display gel as a negative control. No bands were seen on this control which indicated there was no DNA contamination in the DNase I treated RNA samples used for reverse transcription and PCR reaction. As exemplified by Fig.2A, on differential display gels, 40 cDNA bands appeared differentially expressed when samples from three groups were compared. 30 of them showed increased expression in the STZ-diabetic group, 8 of them showed decreased expression in the STZ-diabetic group, and 2 of them showed increased expression in the STZ-induced, insulin-treated, diabetic group. Consistent difference shown in all 5 samples in one group compared with the other group was considered necessary to minimize the false positive results generated from individual sample differences.

Of 40 differential expressed bands were excised, 22 of them were successfully recovered. and re-amplified in a single 40-cycle PCR amplification. The rest of them appeared as a smear on 1% agarose gel after re-amplification. In the 22 successful re-amplified samples, 12 had a single PCR product by 1% agarose gel electrophoresis (G2, G5, G7, G35, G24, G25, T32, T34, A24, A2, A4, A5), and were directly cloned into PCR2.3 vector. PCR products shown multiple bands on 1% agarose gel, including $C8^{(1)}$, $C8^{(2)}$, C9, C10, C15, C16, C30, C31, A30, T33, were excised from the gel, purified by Geneclean and blunt-end cloned into pBluesript S K II vector.

DNA sequence analysis revealed flanking sites complementary to the 5′ and 3′ PCR primers in most of the clones, although some clones showed sequence complementary to only one of the two primers used in the PCR reaction. Part of the sequence may have been lost during the cloning step. The sequences of the 22 clones were used to search the Genbank database for homologous sequences. 4 clones $C8^{(1)}$, $C8^{(2)}$, C9, C30 were 95% homologous to rat MLC2 gene for muscle myosin light chain. Fragments corresponding closely to previously reported sequences were summarized in Table 2.

Every clone containing an insert with the expected size upon restriction digestion was then used as a probe for Northern analysis. All cloned differential display fragments were 200-300 bp in size. Northern blot analysis was performed to verify the differential expression on 3 samples per group using ^{32} P-labeled DNA from subcloned inserts as probes. 5 μg of poly (A) RNA was loaded per lane. Of 22 fragments cloned, equal expression was observed in 12 clones (54%), differential expression was noted from all three animals in 3 clones (14%), differential expression was observed from two of the three animals tested in 3 clones (14%). The rest of the clones were undetectable on northern analysis, presumably because the cloned fragment was a spurious sequence generated by the low specificity PCR or because of very low mRNA levels.

The first differentially expressed cDNA detected on both differential display and northern analysis was G25 (Fig.2). This mRNA was expressed at a lower level in 5 animals from both STZ-diabetic and STZ-diabetic, insulin-treated groups. On northern analysis,

average signal intensity for three animals per group showed a 63% decrease in these two groups compared with the control when quantitated by phosphorimager. The cDNA sequence of G25 was shown in Fig.3 and is 93% homologous with the sequence of rat ATP synthase subunit mRNA from Genbank.

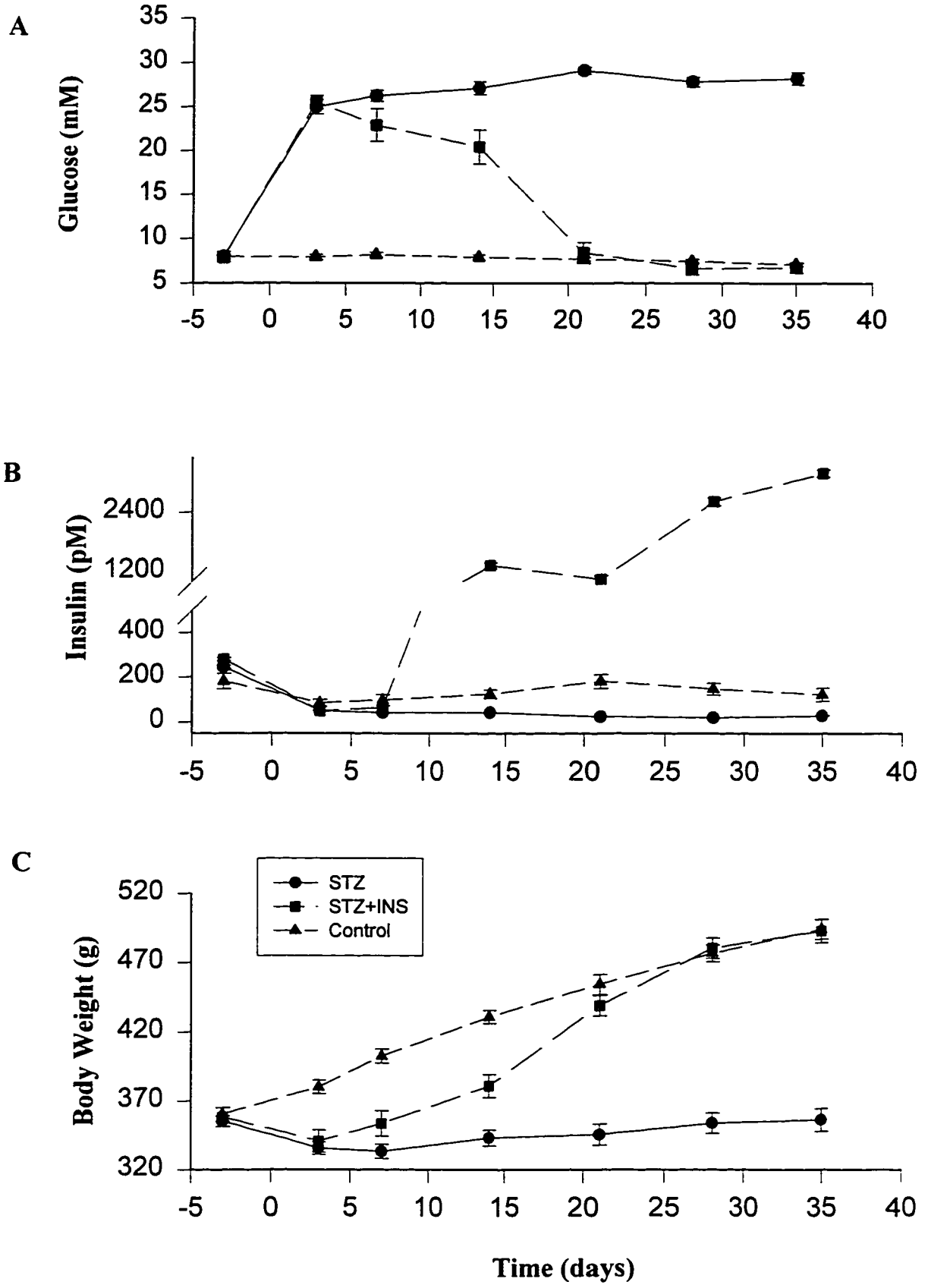
Clone G24 showed an increased level in STZ-diabetic and STZ-diabetic, insulin- treated groups on both differential display and northern analysis (Fig.4). The signal was 57% higher compared with the control group by quantitation on the phosphorimager. The cDNA sequence of G24, shown in Fig. 5, has 95% homology with rat mitochondrial 16s ribosomal RNA.

The third clone C16 was decreased in both STZ-diabetic and STZ-diabetic, insulin-treated groups by differential display, confirmed by northern analysis in all three animals tested. The signal was 60% decreased compared to the control group as quantitated by northern analysis (Fig. 6). The cDNA sequence of C16 shown in Fig.7 has 90% homology with rat striated muscle alpha tropomyosin mRNA.

The sequence of four clones $C8^{(1)}$, $C8^{(2)}$, C9, C30, showed 93% homology with rat muscle myosin light chain. They are all from the same differential display gel and were amplified by the same set of primers. All four clones showed increased signal on differential display, while northern analysis showed decreased signal, so the northern analysis image was not shown. The quantitation results are summarized in Table 2.

Using clone A5, G5, G7, on northern analysis, two of the three animals tested show decreased (A5, G5) or increased (G7) expression, consistent with the differential display signal. The signal on northern analysis was very weak which could account for inaccurate quantitation. Although a figure is not shown, the quantitation result from Northern analysis is summarized in Table 2.

Fig. 1A, B, C. Animal plasma glucose (A), insulin (B) and body weight (C) profile during 5 weeks period of the study. Plasma glucose, insulin and body weight in control (n=10), and STZ-diabetic (n=16) and STZ-diabetic with insulin treatment rats (n=14). STZ was administered during week 0, and insulin treatment started at 3 day post STZ. Data points are the mean ± SE.


A
Glucose (mM)
35
30
25
20
15
10
5
-5
0
5
10
15
20
25
30
35
40
B
Insulin (pM)
2400
1200
400
200
0
C
Body Weight (g)
520
470
420
370
320
STZ
STZ+INS
Control
Time (days)

* differential expression was confirmed by northern analysis in all 3 rats tested.
◊ differential expression was confirmed by northern analysis in 2 of 3 rats tested.
differential expression was detected in northern analysis which is in opposite direction with DD-PCR.
----------: represent the same clone as $C8^{(1)}$

Table 2. Summary of DDPCR, northern analysis and sequence results for 22 cDNAs

	DD-PCR			Northern Analysis			Sequence
Clones	Control	STZ	STZ+IN	Control	STZ	STZ+IN	
G2		↑		undetectable			no sequence
G5		↓		11.45	7.33$^{\diamond}$	10.57	S. pombe complete mitochondrial genome
G7		↑		2.12	2.56$^{\diamond}$	2.27$^{\diamond}$	M. musculus mRNA for B-cell specific coactivator
G24		↑		1.76	2.69*	3.98*	Rat mitochondrial gene coding for 16S ribosomal RNA
G25		↓	↓	1.05	0.46*	0.48*	Rat mRNA for ATP synthase subunit e
G35		↑		2.25	2.22	5.85	Human adenosine deaminase
C8$^{(1)}$		↑		1.05	0.82$^{\#}$	0.81$^{\#}$	Rat MLC2 gene for muscle myosin light chain
C8$^{(2)}$		↑		----------			
C9		↑		----------			
C10		↓		undetectable			
C15		↓		undetectable			
C16		↓	↓	7.40	3.39*	2.95*	Rat striated muscle alphatropomyosin mRNA
C30		↑		----------			
C31		↓		5.7	9.42$^{\#}$	12.7$^{\#}$	M. musculus seb4 mRNA
A2		↑		1.41	0.73$^{\#}$	1.25	Human chromosome 12p13 gene
A4		↑		8.4	7.6$^{\#}$	6.64$^{\#}$	non match
A5		↓		4.94	3.87*	4.12	Mus. musculus tyrosine protein
A24		↑	—	undetectable			
A30		↑		5.9	3.65$^{\#}$	3.79$^{\#}$	Mouse ul-2 small nuclear rna
T32		↓	↑	.11	3.99$^{\diamond}$	16.1$^{\diamond}$	Rat glucagon receptor
T34		↑		1.97	3.54$^{\diamond}$	5.34$^{\diamond}$	Rat S-adenosylmethionine de.
T33		↑		1.97	3.54$^{\diamond}$	5.34$^{\diamond+}$	not done

Fig. 2.A, B, C. Differential display (A) and northern analysis (B) of the differentially expressed cDNA fragment G25. (**A**). cDNA signals demonstrating altered expression by mRNA differential display are marked by arrowheads. Total RNA samples from 5 individual rats in each experimental group were analyzed by differential display as labeled. (**B**). Phosphorimager image of Northern analysis. 5 μg /lane poly(A) RNA from control (lane 1-3) and STZ-diabetic (lane 4-6) and STZ-diabetic, insulin-treated rats (lane 7-9) using ^{32}P-dCTP labeled probes generated from G25. The same blot re-probed with a γ- actin cDNA probe as a loading control is shown below. (**C**). Quantitation of mRNA levels from three groups based on signal intensity analysis on the phosphorimager. Data are presented as mean ± SEM (n=3). A 63 % decrease in diabetic and diabetic, insulin-treated groups compared to the control was evident ($P < 0.05$). No significant difference was seen between diabetic and diabetic, insulin-treated groups.

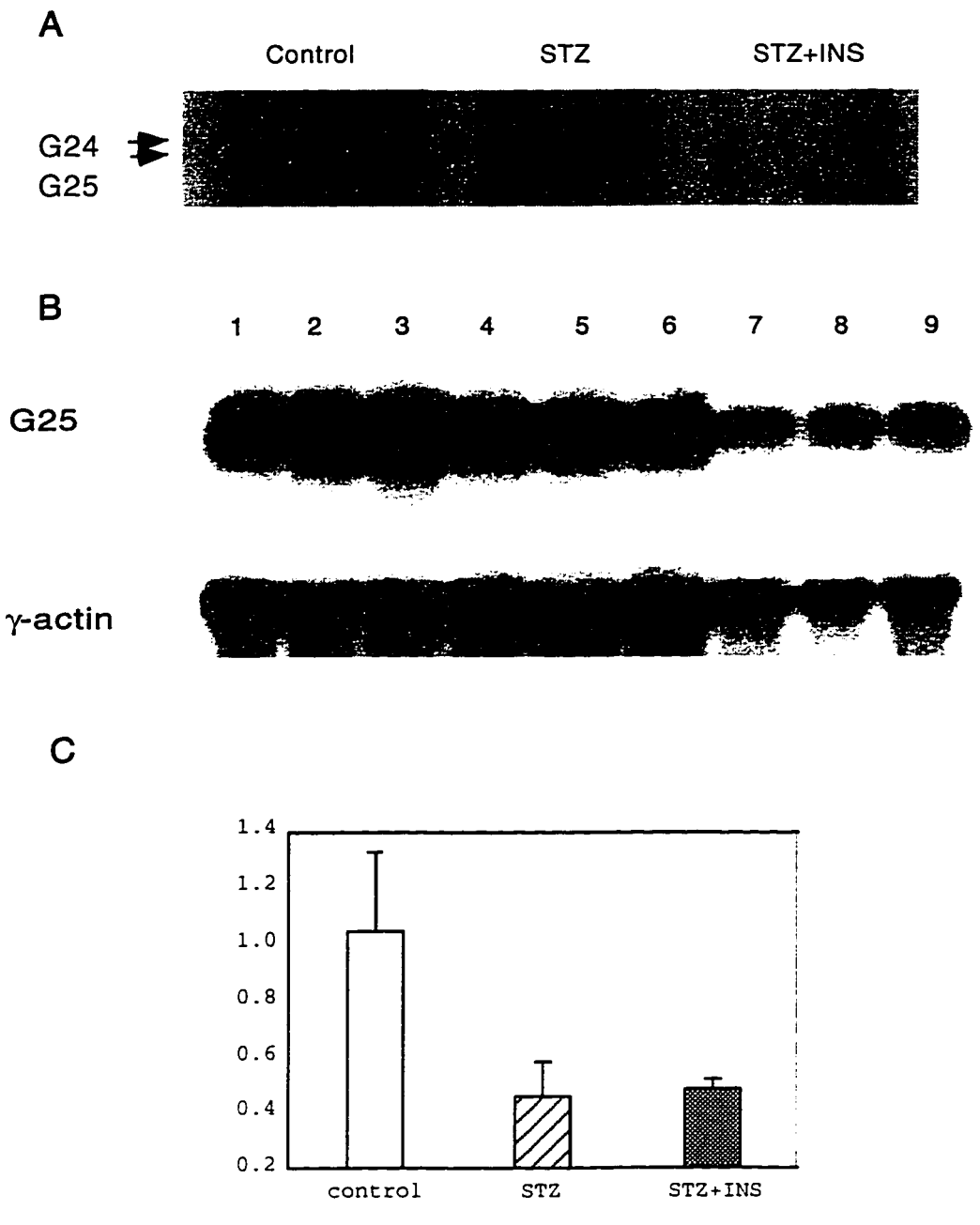

A
Control
STZ
STZ+INS
G24
G25
B
1 2 3 4 5 6 7 8 9
G25
γ-actin
C
1.4
1.2
1.0
0.8
0.6
0.4
0.2
control
STZ
STZ+INS

Fig. 3. Nucleotide sequence of clone G25. Primer sequence is underlined.

TTTTTTTTTT TTTTTAATGA AGCTTTATTC ATCCTCAACA

ACCAGAGAAA AGCAAGATCA CTNNNNCCTC ACTTGAAAAT

GCTGACATCT TCAGCTTCTG CCAGTTCTCT AAGCCGAATT

CTGCAGATAT CCATCACACT GGCGGGANNG TAGCAGAATT

AGAGGCCNNN AAGAGATAAG GAGTCGATTC AATTCATGGA

TGATTCGAGC AGACATGGAA AAGTATCACA TC

Fig. 4.A, B, C. Differential display (A) and northern analysis (B) of the differentially expressed cDNA fragment G24. (**A**). cDNA signals demonstrating altered expression by mRNA differential display are marked by arrowheads. Total RNA samples from 5 individual rats in each experimental group were analyzed by differential display as labeled. (**B**). Phosphorimager image of Northern analysis. 5 μg /lane poly(A) RNA from control (lane 1-3) and STZ-diabetic (lane 4-6) and STZ-diabetic, insulin-treated rats (lane 7-9) using ^{32}P-dCTP labeled probes generated from G24. The same blot re-probed with a γ- actin cDNA probe as a loading control is shown below. (**C**). Quantitation of mRNA levels from three groups based on signal intensity analysis on the phosphorimager. Data are presented as mean ± SEM (n=3). A 57 % increase in diabetic and diabetic, insulin-treated groups compared to the control was evident ($P < 0.05$). No significant difference was seen between diabetic and diabetic, insulin-treated groups.

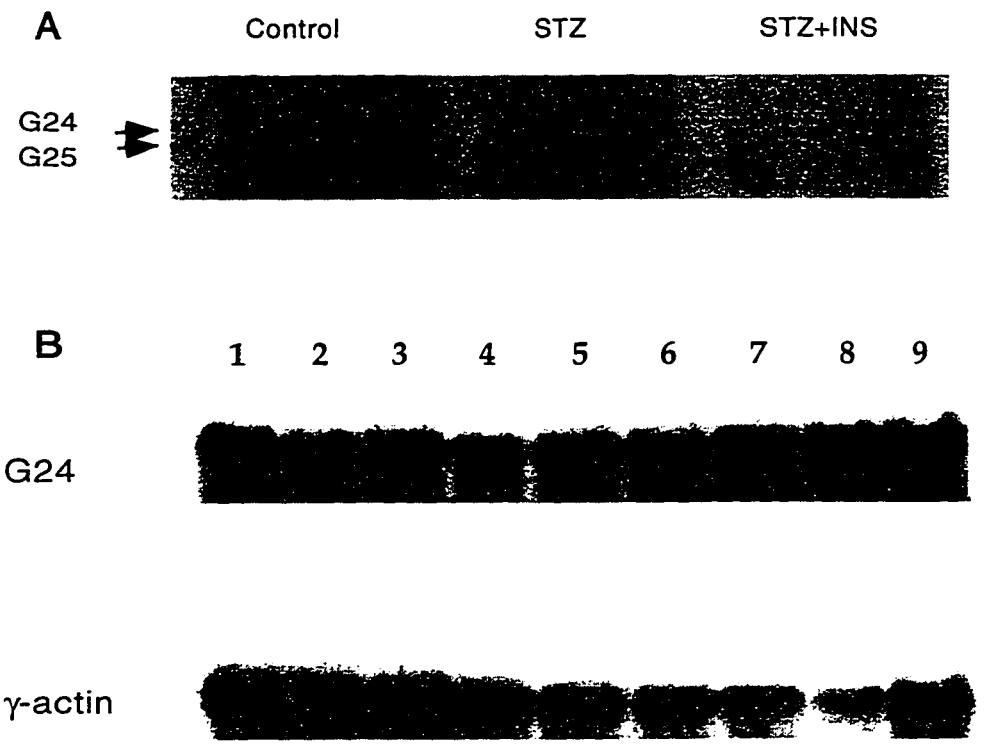

A
Control
STZ
STZ+INS
G24
G25
B
1 2 3 4 5 6 7 8 9
G24
γ-actin
C
45
40
35
30
25
20
15
10
5
control
STZ
STZ+INS

Fig. 5. Nucleotide sequence of clone G24, primer sequence is underlined.

TTTTTTTTTT TTTTTTCAAG AAACTGACCT TTACTCAACA

AAGTTCTACA CGAGTCGGAA TCTCACNCCA CCTAGGCCTA

TAGAGAAAGT CCCGCAAGGA ATGGTGAAAG ACTAATTTAA

AGTAAAAATG ANCAAAGATT AAACCTTGTC CCTTTTGCAT

AATGAATTAA CTAGAAAATT CTTAACAAAA AGAATTTNNN

TAAGANNNCC GANNCCAA

Fig. 6.A, B, C. Differential display (A) and northern analysis (B) of the differentially expressed cDNA fragment C16. (**A**). cDNA signals demonstrating altered expression by mRNA differential display are marked by arrowheads. Total RNA samples from 5 individual rats in each experimental group were analyzed by differential display as labeled. (**B**). Phosphorimager image of Northern analysis. 5 μg /lane poly(A) RNA from control (lane 1-3) and STZ-diabetic (lane 4-6) and STZ-diabetic, insulin-treated rats (lane 7-9) using ^{32}P-dCTP labeled probes generated from C16. The same blot re-probed with a γ- actin cDNA probe as a loading control is shown below. (**C**). Quantitation of mRNA levels from three groups based on signal intensity analysis on the phosphorimager. Data are presented as mean ± SEM (n=3). A 60 % decrease in diabetic and diabetic,insulin-treated groups compared to the control was evident ($P < 0.05$). No significant difference was seen between diabetic and diabetic, insulin-treated groups.

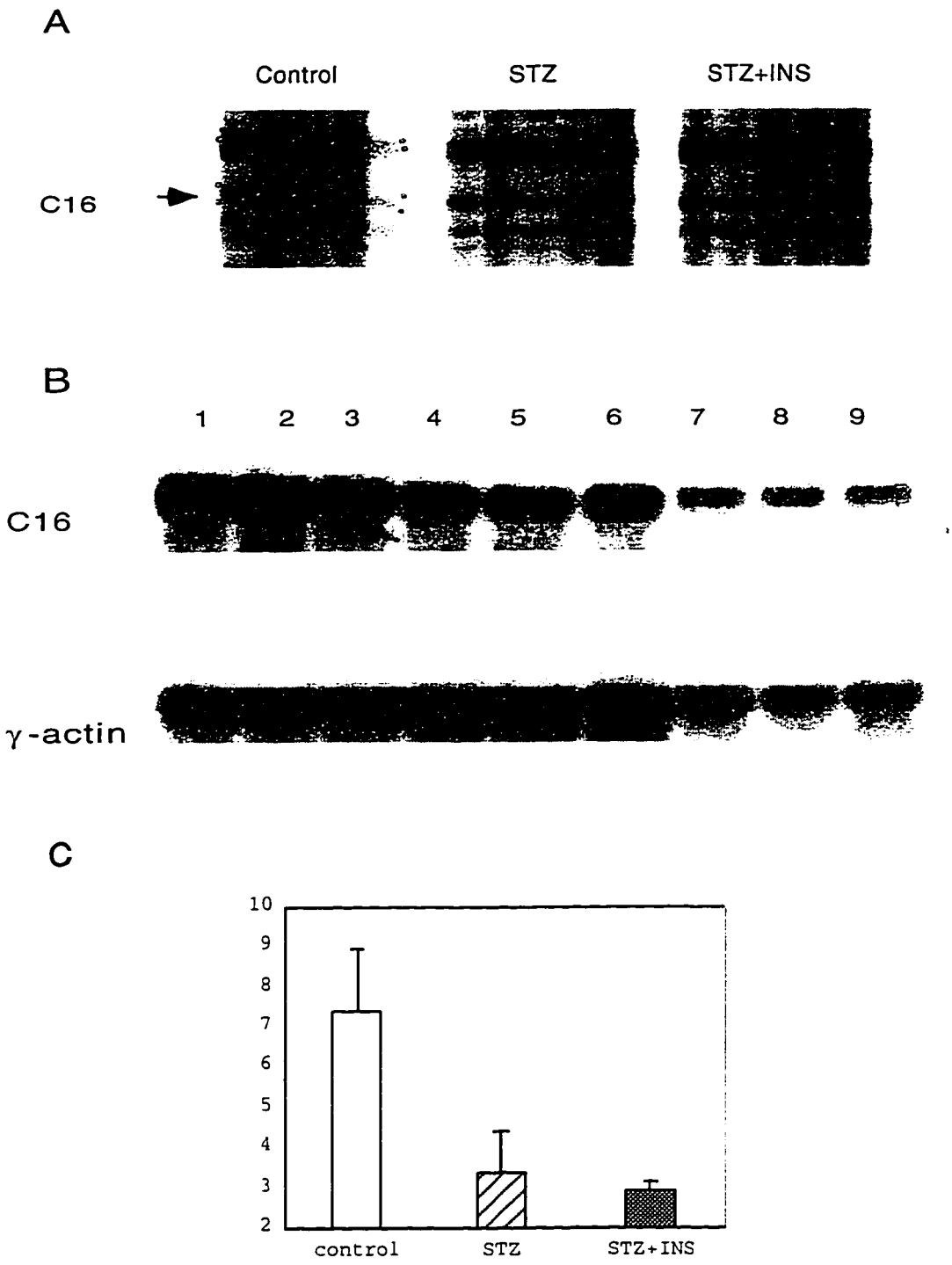

A
Control
STZ
STZ+INS
C16
B
1 2 3 4 5 6 7 8 9
C16
γ-actin
C
10
9
8
7
6
5
4
3
2
control
STZ
STZ+INS

Fig.7. Nucleotide sequence of clone C16, primer sequence is underlined.

TTTTTTTTTT TTTGCACAAA GCAGAACTGT AGTTTTATTG AGCGCTGAGA

CAAAAATGTC AATAAATAAG GAGCAGAGGA ATAGCAAGAG GGAGAGGAGG

AGAGTGTCAA GGAGAGAATG TAGGGTCAGT AGTTTCTCAA GCATCCTAAC

AGAGACCTAT TATCTTCCAC CAGTCTTCAA GTTTAGGTTC TTTTCTCTGT

TACTAAGTTG TTGGGAATCT TAGTGTGTAC ATACGGGCTT TAATGCAGTA

TTACTCTGAG TTAAAGCAAT CATTCGGGAA

DISCUSSION

I. GENERAL

Insulin resistance is a characteristic of both type I and type II diabetes. In type I diabetes, it is considered to be a consequence of insulin deficiency and hyperglycemia. In type II diabetes, although primary genetic factors play a predominant role in the pathogenesis of insulin resistance, it is generally believed that the metabolic derangements of the diabetic state, particularly hyperglycemia, also contribute to this defect. Unfortunately, studies performed in humans cannot separate genetic factors from secondary factors in the etiology of NIDDM. Many *in vitro* studies have been used to isolate the effects of chronic hyperglycemia. These studies have shown that chronic hyperglycemia can cause dysfunction of a variety of tissues and organs including heart, retina, kidney, blood vessel and kidney by alteration of gene expression [126,128]. Chronic hyperglycemia also has a toxic effect on pancreatic beta-cells, impairing insulin secretion by decreasing mRNA expression of the insulin gene [96,97]. It is possible that the insulin resistance caused by the chronic diabetic state may also be caused by altered gene expression which may confound the search for the primary factors of this defect. Skeletal muscle is the major insulin target tissue, hence the major aim of this project was to investigate the secondary pathogenic mechanisms of insulin resistance in skeletal muscle at the level of gene expression, using a streptozotocin-induced diabetic model.

In this study, there were 40 differentially expressed cDNAs identified on differential display gels. Northern analysis verified and quantified 22 of them, and 3 of these were significantly altered in streptozotocin-treated groups (diabetic and diabetic with insulin treatment). Since the changes in gene expression appear to be due to STZ administration per se, rather than resulting from the diabetic state, the following questions were raised: Does streptozotocin cause any damage to skeletal muscle? Does streptozotocin induce insulin resistance? Do the differentially expressed genes we identified from our study relate to insulin resistance?

II. STREPTOZOTOCIN TOXICITY ON SKELETAL MUSCLE

A: Streptozotocin

Streptozotocin, a naturally occuring antibiotic, produced by Streptomytes achromogenes [105], was first isolated in 1959-1960 [131]. A unexpected diabetogenic property of streptozotocin was first discovered in dogs and rats in1963 [106], and later it was also found to have chemotherapeutic [132,133] and carcinogenic properties [134,135]. The structure of streptozotocin was determined to be a methyl-nitrosourea side chain linked to the C2 position of a D- glucose molecule [107,108]. Since the primary diabetogenic effect of this compound is of a pancreatic origin [106,136,137], studies into the mechanisms by which it exerts its toxic effect have mainly focused on the beta-cell.

The earlier evidence that D- deoxyglucose inhibited streptozotocin induced diabetes [109] suggested that the glucose moiety of STZ is the key element for its toxic effect. This

glucose moiety targets STZ to glucose-utilizing tissues. Pancreatic beta-cells are believed to be more sensitive to STZ than other tissues because these cells express a low affinity isoform of the glucose transporter (GLUT2) which is not expressed in most other tissues.

In the1980s, experimental evidence suggested a "NAD depletion hypothesis" as the mechanism of the diabetogenic effect of STZ on beta-cells. After entering the cell, streptozotocin was degraded to form carbonium ions that alkylate DNA at various positions. To remove those alkylated lesions, cells initiate excision repair mechanis leading to the activation of the nuclear enzyme poly(ADP-ribose) synthetase which is responsible for the DNA repairment. This enzyme forms poly(ADP-ribose) with NAD as a substrate, so NAD (which is a very important coenzyme in cells) becomes critically depleted, resulting in a cessation of cellular function and ultimately cell death [110,138,140].

Although this hypothesis might explain the acute effect of streptozotocin damage on the beta-cell, it was questioned by later observations. Exposure of the islets to different alkylating agents, such as the aglucone moiety of streptozotocin (nitroso-N-methylure, NMU), and methyl methanesulfonate (MMS) have been studied. Islets were examined after exposure to these drugs at different time points. On day 0, STZ, MMS and NMU induced a similar initial response, an acute decrease in $NAD^+$and NADH content, a decrease in glucose oxidation and an impaired insulin release. On day 6 after exposure to STZ, there was still impaired glucose oxidation and insulin release, but the NAD and

NADH content were again similar to the control group. On the other hand, islets exposed to MMS and NMU were able to regain normal beta-cell function [113]. These findings suggest that NAD depletion is not the only factor responsible for beta-cell damage. Since substrate metabolism and ATP generation by mitochondria are crucial for glucose-induced insulin release [114], impaired glucose oxidation at the mitochondrion could explain the decrease in insulin release [113].

Based on evidence that chronic impairment in mitochondrial function is observed after STZ exposure [114,141], an alternate hypothesis based on damage to mitochondrial DNA was proposed. Nitrosourea can alkylate both nuclear and mitochondrial DNA (mtDNA)[142,143]. STZ treatment induces a decrease in the mitochondrial-encoded cytochrome b mRNA [111,112]. The formation of methylated DNA after exposure to STZ is about 4-fold higher in the mitochondrial DNA compared to the nuclear DNA [149]. The mitochondrial DNA is particularly vulnerable to damage because of its own characteristics: it has a highly compacted structure consisting almost entirely of coding regions [144]. Mitochondrial DNA codes for 13 subunits of the respiratory chain complexes, 22 transfer RNAs and 2 ribosomal RNAs [145]. The mitochondrial DNA exhibits a very high mutation rate because of its higher evolving rate and lack of histone protection [146,147], and as well, mitochondrial DNA polymerase-γ for DNA replication has a relatively high insertion error-rate of about 1/7000 bases [148]. Based on experimental evidence and the characteristics of the mtDNA, " a targeting hypothesis" has been proposed that mtDNA is the preferential target for streptozotocin, and

incomplete repair of DNA damage at the mitochondrial level causes the long-lasting effect on the beta-cell [113].

B. Streptozotocin toxicity on skeletal muscle

The question still remains whether streptozotocin can cause any damage in skeletal muscle. Most studies examining pharmokinetic and systematic toxicity of STZ only monitor the damage to liver and kidney, two major organs involved in the metabolism and excretion of this drug. It appears that STZ has some effect on the liver and kidney [115]. Evidence suggests that STZ alters both structure and function of multiple tissues rather than beta-cells. It was shown that STZ changed the structure of many tissues including kidney tubules, hepatocytes, and lymphoid tissues. As well, a variety of host functions have been shown to be altered, including immune function, renal function, lipogenesis, liver enzyme function and adrenal steroid metabolism [134,151,153]. In tissue distribution studies, where mice were injected with ^{14}C- STZ and subsequently sacrificed at different time points post drug administration, radiolabeled STZ was detected by autoradiography in different tissues. Liver, kidney, and gastrointestinal contents were high, but did not exceed the level seen in the islet. Other tissues, like salivary glands, Harder's gland, bone marrow, gastrointestinal mucosa and the red pulp of the spleen also showed radioactivity [116]. Results from this distribution study demonstrated that STZ is not only directed to the pancreatic islet, but is directed to other tissues as well, and this raises the possibility that STZ toxicity may affect multiple sites.

It is reasonable to assume that the glucose moiety of STZ may target the glucose transporters in peripheral tissue promoting damage at these sites as well.

It is surprising that no single study has been done to assess the effect of STZ on skeletal muscle, but evidence from two studies implies the possibility. The first study was performed by Paoli et al [154]. They examined the early effects of the administration of STZ on muscle glycogen, by measuring muscle glycogen contents at 0, 2, 4, 6 and 8 hours post-STZ injection under fasting and refeeding conditions. Following an initial hyperglycemic response at 2-4 hours post STZ injection, hypoglycemia occurs, with a marked increase in plasma insulin due to degranulation of the beta-cell. At the same time, liver glycogen increases and muscle glycogen content is decreased in the STZ - treated group compared to the control. The degree of the decrease of glycogen strongly depends on the dosage of STZ, suggesting a dose dependent impairment of glycogen synthesis. After refeeding, similar results were obtained. The STZ-treated group had a slower and reduced maximal accumulation of glycogen. The authors remarked that this impairment of glycogen synthesis may be important in the primary hyperglycemia which follows streptozotocin administration. Another important implication from this study was that insulin resistance was present in skeletal muscle. When comparing glycogen changes between 4 and 6 hours in the control versus STZ-treated rats, it becomes clear that the rate of glycogen accumulation at similar insulin levels was significantly lower in the treated rats, in spite of the higher blood glucose. This study showed that there was an impairment of glycogen synthesis in skeletal muscle, and the impairment strongly

depended on the dosage of STZ. It is reasonable to speculate that STZ may cause damage in skeletal muscle.

Another study done by Armstrong [155] determined the activity of succinate dehydrogenase (SDH) in rat skeletal muscle following two doses of STZ. SDH is a mitochondrial protein responsible for oxidizing pyruvate or palmitate. SDH activity in skeletal muscle was decreased and exhibited a more rapid decline in animals receiving a larger dose of STZ, although insulin and glucose levels were similar. The authors cautioned that extrapancreatic effects of the drug might be partially responsible for the effects on SDH in this study.

Our study demonstrates three genes that have altered expression by STZ in skeletal muscle. Two of these genes are of mitochondrial origin. In combination with the hypothesis that STZ preferentially targets mitochondrial genes of the beta-cell, we can speculate that a high dose of STZ can affect skeletal muscle independently of its effect on beta-cells, and that this effect may be responsible for the defect in insulin action.

III. STREPTOZOTOCIN EFFECTS ON INSULIN SENSITIVITY

Since STZ is generally used under experimental conditions to induce insulin deficiency, the possibility that STZ might directly cause insulin resistance has not been addressed. Although there has not been a study performed solely to investigate this possibility, observations from studies using STZ provide some insight into this question.

Rats injected with STZ at 6 weeks of age exhibit frank hyperglycemia as adults and have shown marked insulin resistance as measured by the glucose- insulin tolerance test. In this test, exogenous insulin and glucose were given, blood samples were frequently obtained to characterize the glucose and insulin profile, and the glucose disappearance rate as a parameter of reflection of insulin action was quantitated between 2-20 min after the injection [156]. The same conclusion was drawn from STZ-treated dogs studied by a euglycemic clamp [157]. In these studies, marked hyperglycemia is present, and hyperglycemia and other metabolic consequences of the diabetic states have been considered as the major contributors to this insulin insensitivity. Insulin treatment, which normalizes glycemia, can improve insulin sensitivity, however, it cannot completely reverse insulin sensitivity to normal by ameliorating the metabolic derangement [157]. This observation suggested that some factors rather than hyperglycemia are also contribute to the insulin resistance present in STZ-diabetic model.

Moreover, evidence from studies using a neonatal STZ model shows that insulin resistance is present prior to the onset of hyperglycemia and leads to the concept that the insulinopenia caused by STZ may be directly related to insulin resistance. One study which supports this idea was performed by McCulloch [160]. It was demonstrated that in the STZ-treated baboon, the decrease in insulin secretive function associated with the STZ administration was correlated with a decline in insulin sensitivity, while fasting plasma glucose remained normal. On the other hand, the study performed by Tsuji et al. [161] raised the possibility that STZ itself may cause a decrease in insulin sensitivity.

This study demonstrated insulin resistance is present in the STZ-diabetic model independent of hyperglycemia and insulin deficiency. 1.5-day-old neonate rats which received 90mg/kg STZ had a normal fasting plasma glucose as adults. When the oral glucose tolerance test was performed in this group of animals, there was a similar insulin response observed as that of controls, but a mild impairment in glucose tolerance was seen in these animals. The authors remarked that a decrease in insulin action in peripheral tissues may contribute to this glucose intolerance; however, no interpretation is offered to explain the origin of the insulin resistance in this group. Since insulin secretion and fasting glycemia were the same in this group as in the control rats, what could be the reason for glucose intolerance in these animals, if not STZ itself?

Another piece of evidence, which supports the possibility that STZ itself induces insulin resistance is based on the fact that animals treated with high doses of STZ display insulin resistance before the onset of fasting hyperglycemia [156,157]. In contrast, when diabetes is generated by autoimmune destruction or by partial pancreatectomy, decreased insulin secretion per se is not accompanied by insulin resistance [45,163,164]. Moreover, the evidence described earlier in this thesis [36] also supports this possibility since impairment of glycogen synthesis occurs before the hypoinsulinemia and the rate of glycogen accumulation at the same insulin levels is significantly lower in STZ-treated rats compared with control rats. These findings strongly suggest that STZ has direct effects on skeletal muscle and contributes to the insulin insensitivity in these animals.

Taking all this evidence together, it is reasonable to hypothesize that STZ itself can induce insulin resistance, but further investigation is needed to address this issue. The results of our study show that the mRNA products of 3 genes are altered by STZ treatment in skeletal muscle. Two of the genes are encoded by the mitochondrial DNA which might be more susceptible to the damage caused by STZ. These observations add more evidence to the hypothesis that STZ induces muscle damage and results in insulin insensitivity, at least in our particular model and at the dosage of STZ administered.

IV. ATP SYNTHASE AND PERIPHERAL INSULIN ACTION

One of the genes we identified in STZ-treated animals is mitochondrial mRNA for an ATP synthase subunit. ATP synthase serves as the gatekeeper for the final step of oxidative phosphorylation during ATP production. A decrease of this enzyme would have significance in both glucose homeostasis and energy generation.

A: Oxidative Phosphorylation and ATP Synthase

A great proportion of the chemical reactions in cells are devoted to harnessing energy from foods thus making it possible for the cells to remain viable. The energy needed for physiological processes of the cells is mostly found in a special chemical compound called adenosine triphosphate (ATP) which contains high energy bonds between two phosphate radicals. Upon loss of one phosphate radical from ATP, the compound becomes adenosine diphosphate (ADP) and 12,000 calories of energy are released under standard conditions of temperature and concentration of reactants in the body [55].

Essentially all of the physiological mechanisms that require energy for operation obtain this directly from ATP. The development of mitochondrial oxidative phosphorylation is a great step in the evolution of the cells for energy generation. Without mitochondria, cells would be dependent on anaerobic glycolysis to make all of their ATP, and only a small portion of the energy potentially available from glucose can be extracted by glycolysis.

Mitochondria are bounded by specilized phospholipid bilayers that play a crucial role in their functional activities [55]. The outer membrane contains many copies of a transport protein called porin. Porin spins the lipid bilayer and forms large aqueous channels which is permeable to molecules of less than 10,000 daltons. Porin also plays a very important role in terms of glucose metabolism regulation and insulin action via binding to hexokinase which will be discussed in detail later. The inner membrane is highly specialized and impermeable to most molecules and especially ions. The inner membrane contains a variety of proteins which perform three major functions: (i) transporter proteins which transport substances necessary for mitochondrial function; (ii) three protein complexes which are the enzymes for the respiratory chain; (iii) ATP synthase which converts the electron energy to ATP.

The two membranes separate two mitochondrial compartments, an internal matrix space and an intermembrane space. The internal matrix space contains enzymes including those that oxidize pyruvate (products of glycolysis) and fatty acid to produce acetyl-

CoA and those that oxidize acetyl-CoA in the citric acid cycle. The end products of this oxidation are CO_2, NADH and $FADH_2$ which transiently hold high energy electrons generated from the oxidation and pass them to the respiratory chain in the inner membrane. Electron transport begins by removing hydride ions from NADH to regenerate NAD^+ and the hydride ion is converted into a proton and two electrons (H^-: $\rightarrow H^+ + 2e^-$). There are 15 different electron carrier proteins in the respiratory chain, formed as three large enzyme complexes. Each complex in the chain has a greater affinity for electrons than its predecessor and electrons pass sequentially from one to another until finally they reach oxygen. Moreover, the transfer of electrons results in allosteric changes in some protein molecules in respiratory chain, which are permeable to H^+. As the electrons move through the complexes, the energy from NADH is used to transport protons across the inner membrane from the inside to the outside of the mitochondrion generating both a pH and voltage gradient called the electrochemical proton gradient. The energy in this proton gradient will be used by another protein complex located in the innermembrane, ATP synthase, to phosphorylate ADP into ATP which is the final step of oxidative phosphorylation and ATP synthesis .

ATP synthase is a knob and stalk structure. The F_0 component (stalk) is the proton channel that spans the inner membrane, while the F_1 component (knob) contains the catalytic subunit. The complex catalyzes the phosphorylation of ADP to ATP as the protons flow down the electrochemical gradient [159]. Moreover, the proton gradient has

another important role: it can change the porin structure in the outer membrane leading to the higher affinity for hexokinase [165,166](Figure 8).

B. Hexokinase and Its Activation

There are four hexokinase isozymes present in different mammalian tissues according to their electrophoresic mobility on starch gels [194]. Hexokinase I-III are 100kda monomeric proteins, have low K_m value for glucose, and are inhibited by physiological concentrations of glucose-6- phosphate (G-6-P). The remaining isozyme, hexokinase IV is present in liver and beta cells, and differs from the others by having a small molecular mass of 50 kda, a K_m for glucose of >5 mmol/l, and a lack of inhibition by G-6-P [195].

Hexokinase II is the predominant isozyme in insulin- sensitive tissues, skeletal muscle and adipose tissues [194,195]. Hexokinase is an important enzyme for glucose metabolism. It catalyzes the irreversible phosphorylation of glucose once glucose enters the cells and maintains the glucose concentration gradient across the plasma membrane which is the force for facilitated glucose transport across the plasma membrane. The product of this phosphorylation, glucose-6-phosphate (G -6 P), is at the switch position of glycolysis, oxidation, glycogen synthesis and the pentose phosphate shunt. More importantly, G-6-P is not only the substrate for glycogen synthase but is also the allosteric activator of glycogen synthase which plays an important role in glucose homeostasis in the postprandial state.

Mitochondrial Oxidative Phosphorylation

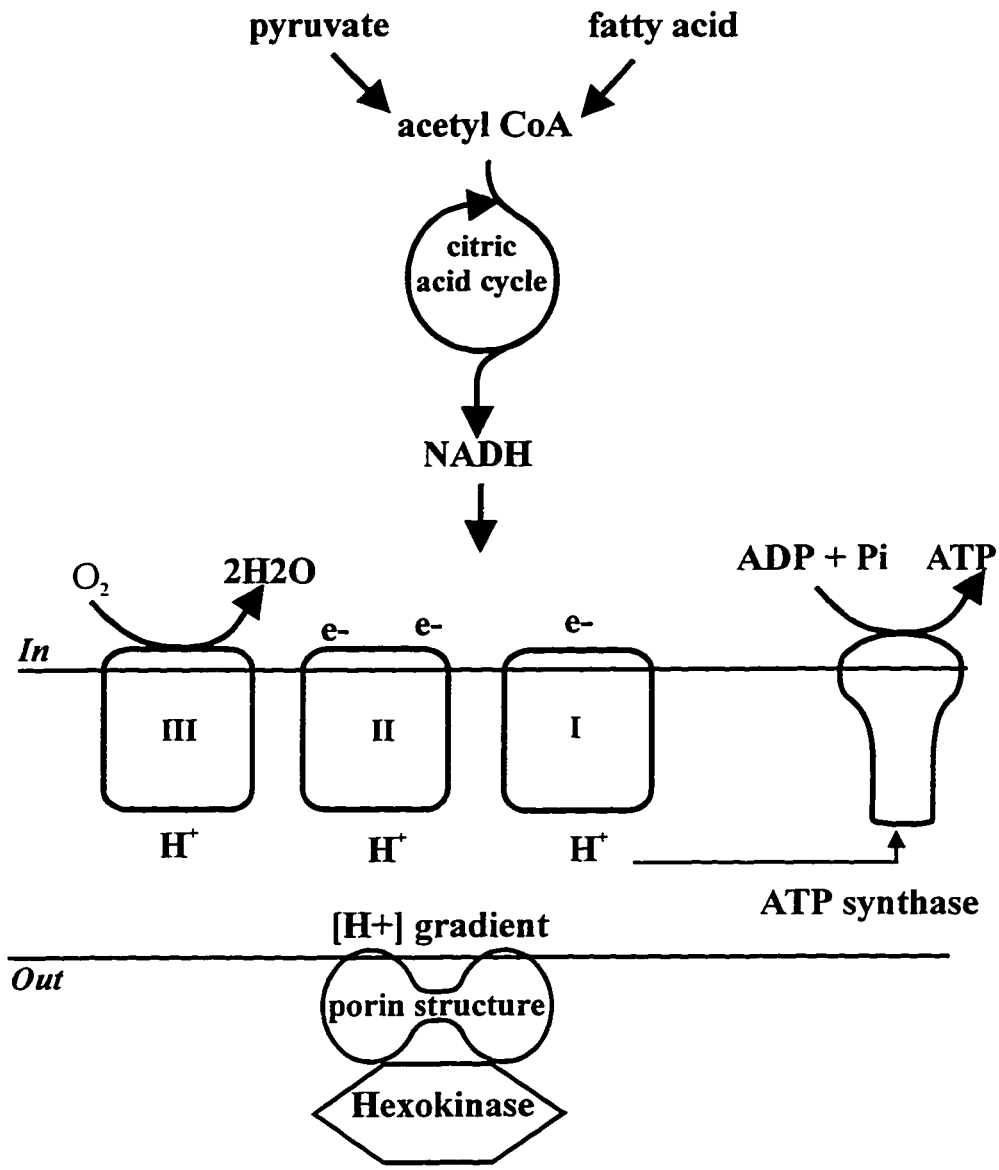


Fig.8. Overview of oxidative phosphorylation. Pyruvate and fatty acid are oxidized into acetyl CoA which enters citric acid cycle and generates high energy electrons transiently held by NADH. As electrons flow through the I, II, III protein complexes of the electron-transport chain, the free energy released from electrons is used to pump protons across the mitochondrial inner membrane and this generate a [proton$^+$] gradient which is used by ATP synthase to phosphorylate ADP into ATP, coupled with the reentry of protons into the mitochondria. [proton$^+$] will modulate the porin structure, leading to higher affinity to hexokinase .

Activation of hexokinase is dependent on its binding to mitochondrion as recognized by the following experiments. First, the activation of hexokinase is induced by the association of the enzyme to the surface of muscle mitochondria [196]. Second, insulin increases the activity of hexokinase II in mitochonrial bound form in fat [197], skeletal muscle [196] and cardiac muscle [198]. Also in these tissues glucose, in the absence of insulin, stimulates the binding of this enzyme to the mitochondrion [196,197]; thus it is suggested that the insulin effect is possibly mediated by the increase in intracellular glucose [196]. Besides this indirect effect through glucose-induced mitochondrial binding, insulin can also exert a direct effect on hexokinase activity [199].

When plasma glucose rises, the elevated glucose and insulin will accelerate the uptake of glucose in the muscle. The binding of hexokinase II to the mitochondrion and consequent activation accelerates formation of both ADP and G-6-P. ADP enters the mitochondrion and serves as a substrate of oxidative phosphorylation, while G-6-P is an important intermediate of glucose metabolism and will enter either pathways of glycolysis, glycogen synthesis, or the pentose-phosphate shunt, depending on the metabolic situation. In the metabolism of glucose, hexokinase binding to mitochondria is a key step and evidence has shown that the mitochondrial contact site and porin are the factors determining the binding and activation of hexokinase, and different mitochondrial metabolic functional states will determine the frequency of the contact sites and porin structure [193].

C. Contact Site and Porin in the Mitochondria

Hackenbrock [167] observed that there was contact between the inner and outer membrane in thin sections of rat liver mitochondrion, and suggested that these sites of contact might relate to the activity of certain kinases localized in the intermembrane space. At present, there is compelling evidence in support of his original suggestion. Moreover, it has become evident [168] that three major functions are located in contact sites. Contact sites are the sites of import for most mitochondrial precursor proteins [169,170] and the sites where flow of phospholipids take place [171,172]. These two functions are necessary for maintaining the mitochondrial function. The third, most important function, is that contact sites contribute to energy transfer from mitochondrion to cytosol through a number of kinases localized in the mitochondrial periphery [173,178]. Contact sites create a condition that mitochondria facilitate peripheral kinases can gain preferential access to ATP generated by the oxidative phosphorylation [177,178]. There are two lines of evidence supporting this. First, functional experiments suggest bound hexokinase I and mitochondrial creatine kinase preferentially use of matrix ATP over cytosolic ATP [179,180]. Secondly, subfractionation of the mitochondrial membranes using sucrose density centrifugation showed that bound hexokinase and creatine kinase were enriched in a membrane fraction between the inner and outer membrane. Immunogold labeling and immunocytochemical staining electron microscope studies have also shown a preferential localization of bound hexokinase I in contact sites [181,182]. It has been shown that the binding at the contact sites and the activation of the enzyme is a cooperative process [184]. In vitro evidence showed that interaction with the

porin protein resulted in a similar activation of the enzyme as that observed by binding to contact sites in intact mitochondrion [183]. Therefore the binding to the porin in contact sites of the mitochondrion may lead to activation of hexokinase [185,186]. Distribution of porin in the outer membrane was found to be random, but electron microscopy and subfraction of liver mitochondria indicated that bound hexokinase was only distributed at the contact sites, thus it was postulated that the preferential binding of hexokinase to the porin in the contact sites is because the inner membrane potential affects the structure of the porin in these sites due to its voltage sensitivity [187,188].

Contact sites are dynamic structures of mitochondria and formed under the regulation of mitochondrial metabolic conditions. Both the outer-membrane and inter-membrane spaces contain components which are capable of forming contacts in response to the metabolic functional state of the mitochondrion [189,210]. The frequency of contact sites is significantly greater in phosphorylating mitochondria compared to mitochondria in other states [190,191]. Using an uncoupler of mitochondria which allows oxidation to occur without phosphorylation caused a further reduction of contact sites because it changed the surface charge of the two adjacent membranes [190]. The frequency of contact sites is correlated with the activity of surface-bound hexokinase and depends on the metabolic states of the mitochondrion [190,209,211].

Therefore, mitochondria regulate the formation of the hexokinase-porin complex and consequently the activation of hexokinase by changing both the frequency of contact sites and the porin surface charge and structure [192,193].

D. Deficiency of ATP Synthase and Activity of Hexokinase

In our study, ATP synthase is downregulated by streptozotocin treatment, but the consequence of this downregulation remains to be elucidated. As mentioned earlier, mitochondrial functional state can regulate hexokinase activation by altering the frequency of contact sites and porin structure. Moreover, mitochondrial bound hexokinase preferentially use intramitochondrially generated ATP.

Our results showed that the messenger RNA for ATP synthase was 62% decreased in skeletal muscle of streptozotocin-treated animals as compared to control animals. We assume that the protein level and activity of this enzyme is also decreased. As a consequence of this decreased enzyme activity, the mitochondria would not have efficient machinery for phosphorylation in the last step of the oxidative phosphorylation. This could not only affect ATP generation, but the frequency of contact sites could also be lower than normal and energy saved in the proton electrochemical gradient could not be efficiently converted into ATP. Consequently, the charge on the surface of the mitochondrial membrane might be affected as so would the structure of the voltage sensitive protein- porin.

Importantly, the downregulation of ATP synthase not only provides less ATP for phosphorylation of glucose substrate by hexokinase, but it also affects the binding/activation of hexokinase by altering the frequency of contact sites and porin structure.

Skeletal muscle is the major site for insulin action and insulin-stimulated glucose uptake. Skeletal muscle glycogen synthesis in the postprandial state is the major mechanism for maintaining the euglycemia in that condition due to its larger volume compared with other insulin target tissues. As mentioned earlier, hexokinase plays an essential role in glucose uptake and glycogen synthesis, thus a deficiency of hexokinase activation will result in a defect of insulin action in skeletal muscle.

We hypothesize therefore that Streptozotocin affects mitochondrial function by downregulating ATP synthase which consequently alters the frequency of the contact sites and porin structure in the mitochondria. This, in turn, leads to decreased activation of membrane-bound hexokinase causing a defect of insulin-stimulated glucose uptake and glycogen synthesis which would be manifest as insulin resistance.

In streptozotocin-diabetic animals, evidence has shown that insulin action is impaired [156,157,160], glucose oxidation pathways and glycogen synthesis are decreased as well [45,150,200]. Although insulin deficiency may account for a proportion of the insulin

resistance, streptozotocin by itself may also be responsible for these defects by damaging the function of mitochondrial ATP synthase.

V. STREPTOZOTOCIN AND MITOCHONDRIAL GENE EXPRESSION

In our studies, two of the differentially expressed genes are mitochondrial genes. 16S ribosomal RNA was upregulated whereas the mRNA for ATP synthase subunit e was downregulated. It is unclear what the mechanism could be for the altered expression of these genes. It was not the aim of this project to determine the mechanism for differential expression, so we can only speculate on the possible mechanism, based on research reported in the literature.

The mammalian mitochondrial genome is a closed, circular, double-stranded DNA about ~16.5 kilobases and has a highly compact structure consisting almost entirely of protein coding sequence. The leading strand has been named the heavy (H) strand due to its greater buoyant density in alkaline cesium chloride gradient, while the opposite strand of the DNA helix has been termed the light (L) strand. There is a short piece of nascent DNA strand located upstream at the origin of the H-strand replication, called the displacement-loop (D-loop) which is the location of promoters of transcription. Both strands of the mitochondrial DNA are transcribed. The heavy (H) strand encodes genes for 12S and 16S mitochondrial ribosomal RNA, 12 polypeptides and 14 tRNAs, while the light strand encodes 1 polypeptide and 8 tRNAs.

The transcription of mitochondrial DNA differs from that of genomic DNA [201]. First, one transcription unit extends over the whole H-strand and another covers the whole light (L)-strand resulting in the complete symmetrical transcription of this DNA, generating a polycistronic RNA molecule from each strand. All mitochondrial genes are expressed in the same direction and tRNA genes lie between the genes coding for rRNA or protein, separating adjacent coding regions. These tRNA genes plays a significant role in posttranscriptional polycistronic RNA processing. Cleavage of the primary polycistronic transcript occurs on either site of each tRNA gene [202]. Since the same transcription units contain genes for tRNA, rRNAs and protein, differential gene expression of mitochondrial DNA must be largely controlled by posttranscriptional mechanisms. Second, there are two overlapping H-strand transcription units. One appears to start ~25 bp upstream of the $tRNA^{Phe}$ (the first tRNA after the promoter) gene and terminate at or near the 3′ end of the 16S rRNA, under the control of the P_{HR} promoter and is responsible for the synthesis of the bulk of rRNA. The other transcription unit starts near the 5′ end of the 12S rRNA gene under the control of promoter P_{HT}, and proceeds beyond the 3′ end of 16S rRNA and synthesizes a polycistronic molecule from the entire H-strand, which is finally processed to generate the mRNAs and most of the tRNA encoded in the heavy strand. These two transcription units with distinct promoters with different initiation efficiency are the basis for the differential regulation of expression of rRNAs and heavy-strand-coded mRNAs and are the main factor responsible for the higher rate of synthesis of rRNA than that of mRNA synthesis in mitochondria (10-30 fold)[203].

The regulation of mitochondrial gene expression remians poorly understood, although some mechanisms have been identified. As noted above, transcriptional mechanisms play an important role in the differential regulation of gene expression mainly through the different activity of the two overlapping transcription units. Two distinct transcription units with different efficiency of promoters and termination position result in a higher rate of synthesis of rRNAs relative to that of mRNAs.

As mentioned earlier, apart from this transcription control, gene expression in mitochondria is largely regulated by posttranscriptionnal mechanisms. Differences in RNA stability accounts for a 20-150 fold higher copy number of the tRNAs compared to the mRNAs derived from the whole H-strand polycistronic transcripts [204,205]. Moreover, unlike nuclear-encoded genes, mitochondrial-encoded gene expression may also be regulated by the changes in mitochondrial DNA copy number, because the nuclear encoded mitochondrial RNA polymerase participates in both mitochondrial DNA replication and transcription, and the promoter and replication origin are almost the same site in mitochondrial DNA [206,207].

It has been demonstrated that streptozotocin alkylates mtDNA in beta cells and consequently recruits DNA repair mechanisms with partial repair of mtDNA causing dysfunction of mitochondrial oxidation which may be responsible for the long-lasting defect of insulin secretion. Isolated beta cells from neonates treated with streptozotocin have a significantly decreased expression of cytochrome b mRNA and 12S rRNA

[111,112]. To investigate the mechanism of this, the sequence of the promoter region of H-strand was fully analyzed. No mutation was found, but the content of mitochondrial DNA was decreased. It seems likely that STZ induced depression of mtDNA and consequently lowered mitochondrial gene expression [112].

In our study, 16S rRNA is upregulated, while mRNA for the ATP synthase subunit is downregulated. A decrease of mtDNA definitely cannot explain this. We postulate that STZ induced damage which might cause premature termination of the transcription unit for the whole H-strand at a position somewhere before the ATP synthase subunit. ATP synthase mRNA level is decreased, and cells might increase transcription of this unit to compensate for the decrease of mRNA through a feedback mechanism. rRNA levels might then be upregulated because of this compensation. Perhaps this mechanism occursin different muscle cells. Another possibility may be that STZ randomly damages mtDNA in such a way as to initiate the transcription unit for rRNA and inactivate the transcription for the whole H-strand including mRNA for the ATP synthase subunit. Perhaps the best explanation would be an alteration in mRNA stability for these different transcripts, although the mechanism for this is unknown. Further investigation is needed to find out the mechanism for regulation of these genes by STZ.

VI. TROPOMYOSIN AND DIABETES

In striated muscle, tropomyosin is a thin filament sitting on the myosin protein and is responsible for the regulation of contraction by transducing the effects of free calcium in

contractile protein activation and inhibiting this activity when calcium is absent [64]. Striated muscle actomyosin ATPase (uses ATP energy for contraction) is activated by calcium ions in the presence of this regulatory component. Studies have shown that cardiac myofibrillar ATPase exhibits a depressed enzymatic activity in STZ-diabetic rats at all concentrations of calcium [209,211]. When actomyosin ATPase from these diabetic animals was combined with tropomyosin, the regulatory component from control animals, the enzyme activity was partially reversed yet when the converse experiment was carried out, the actomyosin had a diminished calcium sensitivity [200]. It was thus suggested that the abnormality in the regulatory complex of tropomyosin is responsible for the cardiomyopathy in diabetic rats. This was consistent with evidence as determined by Malhotra et al that there was a downregulation of the tropomyosin component in the diabetic heart by immunoelectrophoretic assay [162].

Studies have shown that myosin ATPase in skeletal muscle and muscle contractility in diabetic animals were also decreased compared with normal controls [150,158]. It is unclear whether this is due to a decrease of tropomyosin or myosin ATPase itself. In our experiments striated muscle alphatropomyosin mRNA is decreased 60% in skeletal muscle in both STZ-diabetic and STZ-diabetic, insulin treated groups compared with controls. It is possible that this decrease of messenger RNA causes a subsequent protein translation decrease, and is responsible for abnormalities in the contractile apparatus in diabetes. There are similar decreases in both STZ treatment animals independent of the insulin and glucose levels; thus it is likely that STZ causes this abnormality.

VII. LIMITATIONS OF THE TECHNIQUE

Since it was first reported five years ago, differential display has been widely applied to many fields of biology in order to identify differential expression of genes under different physiological and pathological conditions, because of its sensitivity and simplicity over traditional subtractive hybridization method. The most exciting aspect of this technique is the possibility of systematically searching for genes that are differentially expressed under different conditions. Although the advantages of differential display are significant, one troublesome aspect of this method is the high incidence of false positive signals, indicated by no detectable signal or no difference in signal on northern analysis. The authors (Liang and Pardee) suggested a repeat display can reduce the frequency of false positive signals [121,122], although this does not correct the problem.

In our study, 5 independent samples from each group were simultaneously displayed on the same gel. Only those which showed consistent differences in all 5 samples compared to the controls were considered as the differentially expressed cDNAs. In 22 successfully cloned bands from a total of 40 cDNAs identified by differential display, 4 did not show a signal on northern analysis, and 18 showed no significant change by northern analysis. There is still a high rate of false positives detected as a difference in quantitation between differential display and northern analysis, even though 5 independent displays were performed. The same problem was experienced in several other studies which used differential display to identify variations in quantitative expression of genes [126,128].

The major reason for the false positives in quantitation is likely due to the PCR conditions used in the differential display method. This drawback was partially characterized by the inventors [122], when they used RNA between 2 and 0.02ug as a template. The majority of bands on differential display did not exhibit much change in pattern despite the different concentrations of templates; the intensity of some bands even exhibited an inverse relationship with input RNA. The quantitative aspect of this method was further addressed by Bauer et al [129]. PCR conditions under this differential display only detect qualitative changes, quantitative analysis will be possible by running limited numbers of PCR cycles. PERKIN ELMER designed a new PCR system which has a fluorescent probe detecting real-time quantitation of PCR amplification. In their results, the number of PCR cycles is critical for the quantitative amplification, generally less than 28-30 cycles will reflect the quantitative differences in the template of a reaction. In our experiments, we adapted the original differential display method with 40 cycles of PCR. This nonquantitative PCR amplification is probably responsible for the majority of false positives.

Whether the reverse transcription step can proportionally transcribe all the mRNA to cDNA and accurately generate a proportional quantity of cDNA which is then used as the template for PCR amplification, is another possible source of false positive signals. Although northern analysis is reliable for quantitation of steady state mRNA, many steps are involved in this technique, including poly(A) RNA isolation, capillary transfer,

hybridization, washing, stripping and reprobing. Some messenger RNA may be lost during these steps, particularly rare transcripts. In my experiments, most of the clones were detected by northern analysis on a poly (A) RNA blot. It is possible that some sequences found by differential display are not reflected by northern analysis due to limited sensitivity of the latter.

The last aspect we may consider as a potential source for the false positive signals is due to the tissue processing and storage. mRNAs are not stable molecules and will degrade even in frozen tissues with time. Differential display was performed one year earlier than the northern analysis. Perhaps some of the differential expression of mRNA detected by differential display had degraded by the time northern analysis was done.

Another aspect for consideration of drawbacks of this technique was whether all the messenger RNA can be displayed by these primers, specially rare messages. Most published data unambiguously demonstrate that differentially displayed PCR bands indeed correspond to differential expression of mRNAs and this is the prerequisite for the promise of this method to generate a complete pattern for all the mRNA. However, it cannot be excluded that loss of detection of rare mRNAs occurs when the RNA concentration is lower [122]. In our experiments, 5 cDNA bands excised from the gel displayed by one primer set but at different positions, all proved to be muscle myosin mRNA. This result raises the possibility that the primers preferentially pick up the high abundance messages and individual bands do not actually represent individual mRNA

sequences. Maybe some transcripts were not displayed by this particular primer set, so some differences in the non-reverse transcribed and hence non-amplified messages may not be identified.

DNA contamination in the RNA samples used for differential display is another reason for the high incidence of false positive in this technique. In our experiments, an RNA sample without reverse transcription was amplified by PCR and loaded on the same differential display gel as a negative control to control for the presence of residual genomic DNA in the RNA sample following DNase I treatment. No bands were seen on this control which indicated there was no DNA contamination in the RNA sample. Thus DNA contamination is an unlikely reason for the false positive signals in our experiments.

Finally it is worth mentioning the problems in cloning these PCR products. Some sequence heterogeneity in the purified PCR products obtained by the differential display has been observed before by Bauer [129] and Callard [208]. This phenomena is a big obstacle to identifying the right clone for the PCR insert in our experiments. Several cDNAs corresponding to differentially expressed mRNA were reamplified, purified and cloned into the vectors, although all the insert bearing plasmids contain an insert of the expected size, the sequences of these inserts were heterogeneous. Northern analysis to check individual clones is very time consuming. Sequencing the insert to find the primers sequence and using southern analysis to identify the clones are two alternative

strategies to make the experiment easier. It is possible that the actual correct cDNA which is differentially expressed might be lost during this cloning step.

The best aspect of differential display is being able to use specific primers to define and identify the transcripts, reverse transcribe them into cDNA, and amplify them by PCR. The whole design of the technique makes the analysis of changes in gene expression much easier and more rapid. However, further effort is needed to make this technique a more sensitive, specific and reliable tool which can be used to detect both qualitative and quantitative changes of gene expression.

VIII. SUMMARY

We have identified three genes which were altered in skeletal muscle from streptozotocin treated rats at the dosage we used for our diabetic model (55mg/kg). They are all independent of the plasma glucose and insulin level. Two of them represent mitochondrial genes. We hypothesize that streptozotocin can damage skeletal muscle by downregulating the expression of ATP synthase and affecting mitochondrial oxidative phosphorylation, and perhaps subsequently causing a decreased activation of hexokinase through an alteration of the frequency of contact sites and the structure of porin. By this hypothetical mechanism, insulin-stimulated glucose uptake and glycogen synthesis would be decreased. In this way streptozotocin itself can cause an impairment of insulin action, in addition to hyperglycemia and hypoinsulinemia, and may contribute to the insulin-resistance seen in this chemical-induced diabetic model.

IX. FUTURE DIRECTION

A. There are 18 differentially expressed bands from differential display which remain to be cloned and verified by northern analysis. It remains to be seen if differential expression of these may be responsible for the mechanism of insulin resistance causing by diabetic states.

B. The fragments we identified (G24, G25, C16) are all about 200 base pairs long. Although they have a high percentage of homology with genes in the database, they are not complete cDNA sequences. We need to isolate the full length sequence of these gene by screening a rat muscle cDNA library. Using cDNA and antibody probes, we need to do in situ studies to detect the protein levels in study animals to further test our hypothesis.

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