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Regulation of skeletal muscle Glucose transporter 4 expression in fructose-fed exercised rats

By

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DECLARATION

I, **Veeraj Goyaram**, hereby declare that the work on which this dissertation is based is my original work (apart from the normal guidance from my supervisor and except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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PRESENTATIONS AT CONFERENCES

International Sports and Exercise Nutrition Conference 2012

Northumbria University, Newcastle-Upon-Tyne, England, December 2012

Poster session: "Short term fructose consumption attenuates exercise-induced increase in GLUT4: evidence for divergent effects on accessibility of the MEF2A binding domain"

Conference poster presentation abstract: Ojuka, E; Goyaram, V. Short-term fructose consumption attenuates exercise-induced increases in nuclease accessibility to the MEF2 cis-element on the GLUT4 promoter and GLUT4 expression in rat skeletal muscle. *International Journal of Sport Nutrition and Exercise Metabolism*, 2013, 23, S1-S15

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LIST OF ABBREVIATIONS

ACC	Acetyl Coenzyme A carboxylase
ADP	Adenosine di phosphate
Akt	Protein Kinase B
AMP	Adenosine Mono-phosphate
AMPK	AMP-activated protein kinase
apoB	Apolipoprotein B
AS160	Akt substrate 160
ATP	Adenosine tri phosphate
AUC	Area under curve
BSA	Bovine serum albumin
CABP2	Calcium binding protein 2
CaM	Calmodulin
CaMKII	Ca ²⁺ / Calmodulin-dependent protein kinase II
ChIP	Chromatin immunoprecipitation
CHO	Carbohydrate
ChRE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
DAG	Diacylglycerol
DNA	Deoxyribose nucleic acid
DNL	<i>de novo</i> lipogenesis

dNTP	deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EMSA	Electrophoretic mobility shift assays
FAS	Fatty acid synthase
GCGR	Glucagon receptor
GEF	GLUT4 enhancer factor
GLUT2	Glucose transporter isoform 2
GLUT4	Glucose transporter isoform 4
HATs	Histone Acetyl transferases
HDAC	Histone deacetylase
HFCS	High fructose corn syrup
HRP	Horseradish peroxidase
IMCLs	Intramyocellular lipids
IR	Insulin receptor
IRS	Insulin receptor substrate
LSB	Laemmli sample buffer
MEF2A	Myocyte enhancer factor
MNase	Micrococcal nuclease
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaF	Sodium fluoride

NaHCO₃	Sodium bicarbonate
Na₃VO₄	Sodium orthovanadate
Na₄P₂O₇	Sodium pyrophosphate
NEFA	Non-esterified fatty acid
PCR	Polymerase chain reaction
PFK	Phosphofructokinase
PGC-1	Peroxisome proliferator receptor gamma coactivator -1
PI3-K	Phosphatidylinositol 3-kinase
PKCθ	Protein kinase C θ
PKCζ	Protein kinase C ζ
PP2A	Phosphatase-2A
PVDF	Polyvinylidene difluoride
RNA Pol II	RNA polymerase II
SCD	Stearoyl-Coenzyme A desaturase
SDS	Sodium Dodecyl Sulphate
SREBP-1c	Sterol Regulatory element binding protein
TBST	Tris-Buffered Saline and Tween 20
TE	Buffer containing Tris HCL and EDTA
TIC	Transcriptional initiation complex
VLDL	Very low density lipoprotein

ABSTRACT

Several studies have found that the expression of skeletal muscle glucose transporter 4 (GLUT4) is decreased by high fructose consumption but increased by exercise. However, the amounts of fructose used in these studies were extremely high and the effects of moderate feeding protocols are not known. While it is known that exercise enhances GLUT4 expression via increased histone H3 acetylation and binding of the myocyte enhancer factor-2A (MEF2A) transcription factor to its binding domain on the *Glut4* gene promoter, the impact of fructose consumption on this interaction has not been studied. Moreover, there is no direct evidence that an increase in MEF2 binding is due to increased accessibility of the MEF2 binding domain to transcription factors. This study tested the hypothesis that both exercise and high fructose consumption affect GLUT4 expression by altering the accessibility of the MEF2 binding domain on the *Glut4* gene promoter via remodelling of chromatin in that region.

Male Wistar rats (n=30) were randomly assigned to three dietary groups: a) standard Chow, b) Chow + 10% fructose drink and c) Chow + 10% maltodextrin drink. All rats had access to drinking water and chow *ad libitum* for a period of 13 days. In the last 6 days of the experiment 5 animals in each group performed 3 x 17 min daily bouts of intermittent swimming, with a load equivalent to 5% bodyweight attached to their tails. The remaining 5 rats from each group were untrained. Animals were fasted overnight on the last day of the experiment, anaesthetized and sacrificed on the morning of day 14. Triceps muscle were harvested and used: (a) for measurement of total GLUT4 content by western blot, (b) to obtain nuclei for assessment of accessibility of a 350bp region encompassing the MEF2 element on the *Glut4* gene using nuclease digestion assay, and (c) to measure the acetylation of histones H3 and bound MEF2A in the region above using chromatin immunoprecipitation (ChIP) assay. Blood was also collected and assayed for fasting serum glucose, insulin and free fatty acids.

Consumption of fructose or maltodextrin for 13 days did not decrease GLUT4 levels in untrained rats but significantly attenuated the ~70% (water-fed trained vs. untrained controls; $P < 0.05$) increase that occurred due to swimming. Maltodextrin consumption also blunted this GLUT4 increase. Exercise increased histone H3 acetylation and MEF2A binding by ~1.5-fold and ~1.54-fold respectively (vs. untrained; $P < 0.05$). However, this effect was attenuated by fructose consumption as there was no significant difference in histone H3 acetylation (~1.08-fold) and MEF2A binding (~1.12-fold) from the corresponding untrained controls ($P > 0.05$). Similarly, exercise failed to significantly increase histone H3 acetylation (~1.16-fold) and MEF2A (~1.1-fold) binding in maltodextrin-fed rats (vs. untrained controls; $P > 0.05$). Moreover, fructose or maltodextrin feeding in untrained rats did not alter histone H3 and MEF2A binding significantly.

MNase digestion rates, which indicate the accessibility of the interlinker region within the 350bp segment surrounding the MEF2 binding site, show that in untrained rats fructose consumption significantly reduced the accessibility of the interlinker region but this effect was not seen with maltodextrin. Training increased accessibility ~2.75-fold this increase was abolished by both fructose and maltodextrin. In fructose- but not maltodextrin-fed rats, exercise tended to improve accessibility of the interlinker region although to a lesser extent than that seen in the absence of both carbohydrates. Additionally DNase I digestion rates, reflecting accessibility of the nucleosomal region within the 350bp segment surrounding the MEF2 binding site show that the accessibility of the nucleosomal region is decreased by both fructose and maltodextrin in untrained rats. In trained rats exercise increased accessibility but fructose and maltodextrin consumption abolished the exercise effect. In both fructose- and maltodextrin-fed rats exercise tended to improve accessibility of the nucleosomal region although to a lesser extent than that seen in the absence of both carbohydrates. All of the above changes occurred in the absence of any significant change in fasting plasma glucose and insulin.

The absence of any decrease in GLUT4 expression in fructose-fed untrained rats in contrast with previous high-dose studies, suggests a dose-dependency in the effects of fructose. The fact that not only fructose but also maltodextrin consumption blocked the exercise-induced increase in GLUT4 suggests that this effect is not specific to fructose. Furthermore, both fructose and maltodextrin attenuated the exercise-induced increase in histone H3 acetylation, MEF2A binding and nuclease accessibility, showing that the modulatory role of both carbohydrates takes place at the level of chromatin remodelling. Our results suggest that increased chromatin accessibility in exercise may be a result of increased histone H3 hyperacetylation which is known to relax chromatin and increase binding of MEF2A and GLUT4 expression. However, changes in accessibility correlated well with changes in histone H3 acetylation, MEF2A binding and GLUT4 expression only when there were large differences in these parameters. This is because smaller decrements in accessibility in the untrained state due to fructose and maltodextrin were not reflected in histone H3 acetylation, MEF2A binding and GLUT4 expression. Taken together these results suggest that the only overall biochemical effect of our fructose feeding protocol was an attenuation of the GLUT4 adaptive response in trained rats with changes occurring primarily at the level of chromatin remodelling. Secondly, these effects were not unique to fructose as maltodextrin also produced similar effects. Our feeding protocol did not induce any change in fasting serum glucose and insulin in both the trained and untrained rats but changes in chromatin structure were noted, suggesting that changes at the molecular level precede those at the systemic level.

CHAPTER ONE

Objectives of the project

1.1. Introduction to the study

The prevalence of type 2 diabetes worldwide is increasing at an unprecedented rate, making it one of the most serious diseases afflicting humankind. Uncontrolled type 2 diabetes can cause a number of complications like damage to blood vessels, blindness, renal failure or coronary artery disease (84). According to the World Health Organisation, about 300 million people worldwide were estimated to have type 2 diabetes in 2010 (32). This number is expected to reach 350 million by the year 2030 (108) with remarkable increases predicted in Asia (32).

A vast body of evidence links the increased consumption of caloric sweeteners, particularly fructose, to the development of obesity and type 2 diabetes (50-52). Indeed, the consumption of sucrose (50% fructose) and other caloric sweeteners has increased since the 1800s and the consumption of fructose mainly in the form of high fructose corn syrup (HFCS) has drastically increased over the last 30 years (3). To date, HFCS represents more than 40% of caloric sweeteners used in a variety of foods and is almost the exclusive sweetener used in sugar-sweetened beverages in many countries. Fructose is thus a significant component of the modern diet.

Epidemiological as well as experimental studies on animals and humans have associated high fructose consumption with the development of insulin resistance and type 2 diabetes (17; 37; 43; 101). A strong positive association was seen between the increased consumption of HFCS and the prevalence of type 2 diabetes from 1909 to 1997 in the United States (24). Furthermore, chronic fructose overconsumption in both animal and human subjects triggers the development of insulin resistance in a dose-dependent manner.

From a mechanistic perspective, there is evidence that fructose induces insulin resistance principally through adiposity-associated mechanisms. Fructose is a highly lipogenic nutrient mainly because of its unregulated metabolism which leads to a constant flux of precursors for hepatic *de novo* fatty acid synthesis if consumed in high amounts. Interestingly, high fructose feeding also activates transcription factors that upregulate key hepatic lipogenic enzymes like fatty acid synthase (FAS), acetyl-coA carboxylase (ACC), stearoyl-CoA desaturase (SCD) and enzymes involved in very low density lipoprotein (VLDL) packaging (68). In addition, a high fructose diet inhibits enzymes involved in hepatic fatty acid β -oxidation (35). The consequence of increased hepatic production and decreased clearance of fatty acids is lipid accumulation in the liver which eventually leads to hepatic insulin resistance. Subsequently, there is increased secretion of VLDL and free fatty acids into the blood circulation leading to a dyslipidemic profile which brings about an increased lipid uptake by skeletal muscle (100). Metabolism in skeletal muscle of the accumulated lipids, called intramyocellular lipids (IMCLs), then produce metabolites that can disrupt normal insulin signalling and mediate insulin resistance in this tissue, leading to reductions in insulin-stimulated glucose uptake via the glucose transporter isoform 4 (GLUT4) protein (98).

Other potential molecular mechanisms of fructose-induced skeletal muscle insulin resistance have not been completely elucidated. One such mechanism may possibly involve a decrease in the content of skeletal muscle GLUT4. GLUT4 is the main transporter of glucose into skeletal muscle, a major site of glucose disposal in the body and the level of expression of GLUT4 correlates well with whole body glucose disposal (38; 88) and a reduction in GLUT4 content in non-diabetic individuals is often used as an early molecular marker of insulin resistance (42).

To our knowledge, GLUT4 regulation in skeletal muscle under high fructose consumption has not been extensively investigated and the available data is inconsistent. The conflicting observations are possibly due to the use of varying feeding protocols (48):

For example, Shih et al. (89) found that rats fed a 60% fructose diet for 8 weeks had decreased GLUT4 mRNA and membrane protein levels in skeletal muscle. Similarly, in humans, feeding healthy subjects with 1.5 g/kg bodyweight/ day of fructose for 4 weeks decreased skeletal muscle GLUT4 protein content by about 27%. In contrast, Li et al (45) found that rats fed a 60% fructose diet for 6 weeks did not experience any change in GLUT4 protein content, but had impaired GLUT4 translocation to the sarcolemma. It is important to note that the amounts of fructose used in most animal studies were unrealistically high in order to develop non-genetic models of metabolic syndrome and type 2 diabetes (106). As such, the majority of these studies were interventional studies in which dietary and pharmaceutical treatments (89) were evaluated on these animal disease models. As a result, the effect of more realistic fructose feeding protocols on GLUT4 is unknown. The first purpose of this study was thus to re-assess the effect of fructose consumption on GLUT4 expression in rat skeletal muscle using a feeding protocol in which animals are fed a 10% fructose solution in addition to normal feed over a period of two weeks.

Exercise has been considered an important therapeutic tool for type 2 diabetic patients because it increases the activity and expression of GLUT4 (10). Furthermore, exercise stimulates mitochondrial biogenesis in skeletal muscle thereby ameliorating insulin sensitivity (7). The effect of exercise training on GLUT4 expression in animals that consume large amounts of fructose has not been studied. Additionally, it is not known how the GLUT4 adaptive response to exercise is modulated in the presence of high amounts of dietary fructose. Indeed, such data might prove useful to exercise scientists because athletes are often advised to consume energy drinks that have substantial fructose content (12; 33). Hence, the second purpose of the present study was to examine the effects of a 13-day high fructose diet on skeletal muscle GLUT4 expression in rats that were subjected to a swimming exercise protocol.

GLUT4 expression is regulated by myocyte enhancer factor (MEF2) and GLUT4 enhancer factor (GEF) transcription factors (30; 70) which bind to conserved DNA

sequences on the *Glut4* gene promoter to initiate transcription (61). However, changes in *Glut4* gene expression are dependent on the accessibility of the promoter region to MEF2, GEF and other transcription factors, a phenomenon which is in turn dependent on chromatin structure in the region of the binding loci of these transcription factors. It is known that exercise activates the enzymes AMP-activated protein kinase (AMPK) (60) and Ca²⁺/ Calmodulin-dependent protein kinase II (CaMKII) (95), which modulate the activity of histone modifying enzymes like Histone Acetyl transferases (HATs), leading to hyperacetylation of histone H3 (55; 59) protein subunits at the *Glut4* gene promoter. This leads to a remodelling of chromatin in that region to allow a looser interaction between DNA and histone proteins. This is thought to increase (a) accessibility of binding domains for transcription factors such as MEF2 and GEF (b) binding of these transcription factors to their respective DNA sequence and (c) gene transcription (61).

Chromatin immunoprecipitation (ChIP) has previously been used to analyse histone modifications and MEF2 binding at the *Glut4* gene promoter (73; 95) but an assessment of the accessibility of MEF2 binding sites has never been conducted. The third purpose of this study was therefore to assess the changes in accessibility of MEF2 binding domain at the *Glut4* promoter under exercise and fructose consumption and its link with GLUT4 protein expression. Accessibility can be determined by nuclease accessibility assays (13; 23) which measure the time course of the digestion of DNA by nucleases at the desired transcription factor binding domains in isolated intact nuclei.

1.2. Summary of objectives

In summary, the main objectives of this present study were as follows:

1. To assess the effect of consuming a 10% fructose drink on GLUT4 expression in rat skeletal muscle after a 13-day period.

2. To examine the effect of a swimming exercise protocol on skeletal muscle GLUT4 expression in rats that were fed a fructose-rich diet.
3. To evaluate the effects of exercise and fructose feeding on Histone H3 acetylation and the *in vivo* binding of MEF2A to the *Glut4* promoter in skeletal muscle.
4. To assess changes in the accessibility of the *Glut4* gene promoter as a result of exercise and fructose feeding.

1.3. Justification for the study

To the best of our knowledge, all studies that have reported an effect of fructose overconsumption on GLUT4 expression are interventional studies that tested pharmaceutical interventions on fructose-induced metabolic derangements (66; 89). In these studies unrealistically high amounts of fructose for long periods of time were used in order to induce a fructose-fed model of metabolic syndrome. In contrast, our study sheds light on the effects of lower doses of fructose administered for a shorter duration on GLUT4, for which data is seriously lacking.

Secondly, subjecting fructose-fed rats to an exercise protocol will tell us whether any eventual GLUT4 dysregulation can be amended by exercise training. Concurrently, the data will also reveal whether the GLUT4 response to exercise training is modulated by fructose consumption. In the latter case, the findings have significance for nutritional recommendation to sports persons and exercising individuals who are often recommended products containing substantial amounts of fructose.

Finally, *Glut4* gene regulation is well characterised in terms of histone modifications and interaction of transcription factor with DNA but no data exists on chromatin accessibility at the *Glut4* promoter. Our study is the first to investigate changes in chromatin

accessibility of the *Glut4* gene promoter under exercise and fructose consumption. The findings add significantly to existing knowledge of *Glut4* gene regulation.

CHAPTER TWO

Review of Literature

This chapter presents a literature review to provide the necessary background information for the work presented in this thesis. The following are the topics that will be discussed:

- Evidence linking high fructose consumption to insulin resistance and type 2 diabetes.
- An overview of known and potential pathogenic mechanisms in fructose-mediated insulin resistance related to GLUT4 dysfunction and dysregulation.
- Molecular mechanisms in the upregulation of *Glut4* gene expression by exercise.
- Assessment of accessibility of the MEF2 binding domain on the *Glut4* gene
- Exercise as a means to alleviate fructose-mediated GLUT4 dysfunction and dysregulation

2.1. Evidence linking high fructose consumption to insulin resistance and type 2 diabetes.

The most common sugars in human diet are sucrose, glucose and fructose. However, fructose consumption has increased over the last 30 years especially with the development of high fructose corn syrups (HFCS) (25; 105). HFCS is made by hydrolysing corn starch to produce glucose followed by an enzymatic isomerisation of the glucose to produce varying percentages of fructose (53). For example, the commonly used HFCS55 contains 55% free fructose and 43% Glucose. HFCS is preferred by food manufacturers over sucrose mainly because of its relatively low price, high sweetening power and ability to confer a long shelf life to products (25). Consequently, HFCS presently accounts for 40% of all added caloric sweeteners in the modern diet and has substituted sucrose in a number of commonly consumed products like carbonated beverages, baked goods and dairy products (25).

The global increase in consumption of fructose parallels the rise in obesity, hypertension, renal disease and type 2 diabetes (34; 87). In the United States, a strong positive association was seen between the increased consumption of fructose and the prevalence of type 2 diabetes using national data from 1909 to 1997 (24). Furthermore, in a Finnish survey involving 51,522 non-diabetic subjects the intake of fructose from sweetened fruit juices and soft drinks was associated with an increased risk of diabetes (69).

However, such associations do not prove cause-and-effect. In this regard, animal studies using both acute and chronic high fructose feeding (72) have provided considerable insights into whether fructose causes the metabolic syndrome. Fructose feeding in animal models has been shown to induce features of the metabolic syndrome like insulin resistance, hypertension, obesity, dyslipidemia, non-alcoholic fatty liver, renal disease, hyperuricemia, endothelial dysfunction and oxidative stress (79-81). Interestingly, these effects were not seen in animals fed glucose and starch. Similar effects were observed in humans, whereby consumption of large amounts of fructose rapidly led to insulin resistance, postprandial hypertriglyceridemia and high blood pressure (4; 54; 97).

The mechanisms by which high fructose consumption brings about insulin resistance in skeletal muscle are well documented and are closely linked to its ability to disturb lipid metabolism. Indeed, fructose is well known to stimulate the production of lipids via the *de novo* lipogenesis (DNL) pathway in the liver which is the main site of fructose metabolism. When consumed in high amounts fructose stimulates hepatic fat accumulation which leads to hepatic insulin resistance. Secretion of high levels of triglycerides, VLDL-TG, and non-esterified fatty acids (NEFAs) in plasma also takes place, leading to a condition called dyslipidemia (90; 96). Dyslipidemia in turn favours an increased uptake and accumulation of lipids in skeletal muscle where these lipids give rise to fatty acid metabolites known to disrupt normal insulin function in this major insulin-responsive tissue (90; 96). The high lipogenic ability of fructose stems from particularities in its metabolism in contrast with other carbohydrates like glucose, as depicted in Figure 2.1.

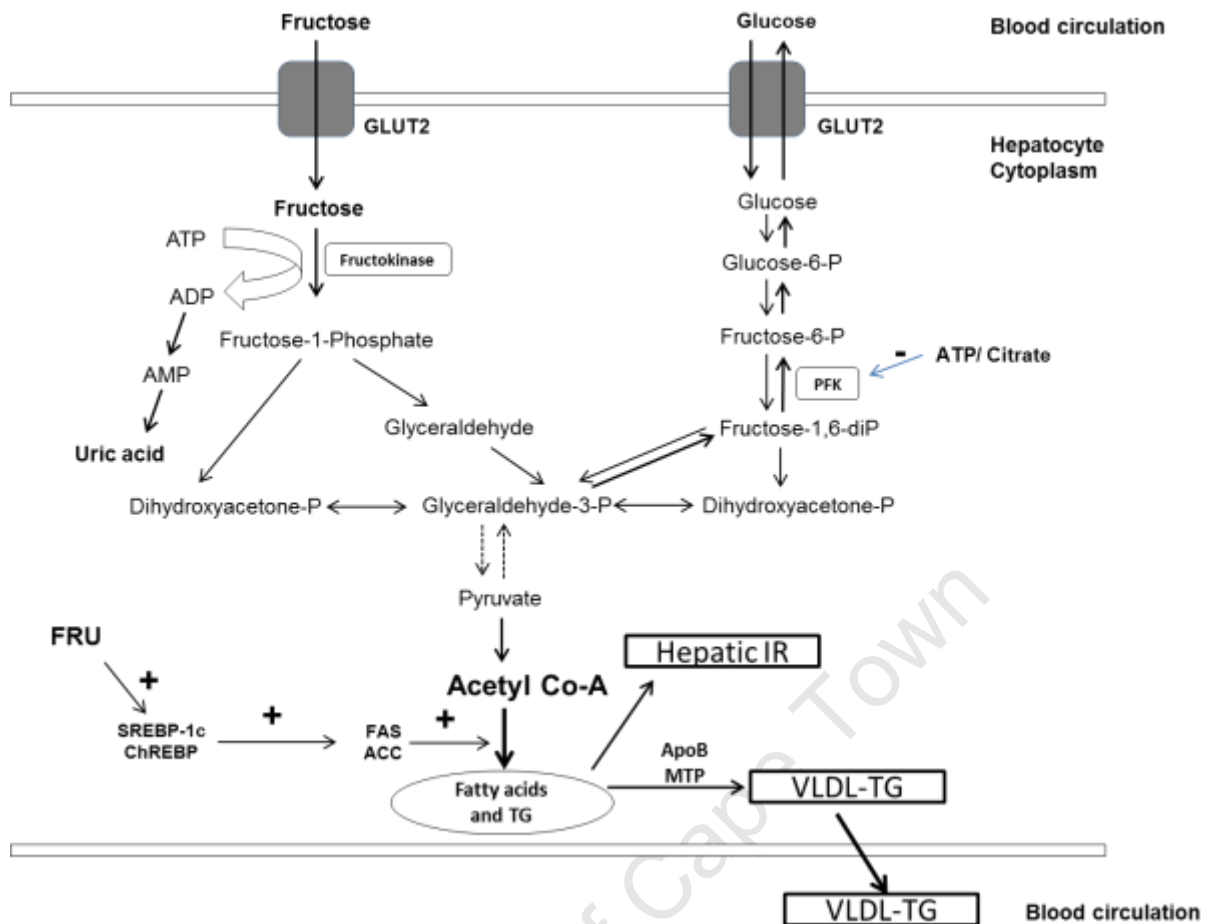


Figure 2.1: The particularities of fructose metabolism and its lipogenic mechanisms.

Fructose enters hepatocytes mainly through GLUT2 transporters and is metabolised by fructokinase to generate fructose-1-phosphate. This initial phosphorylation step brings about ATP depletion, resulting in AMP accumulation and subsequently the stimulation of adenosine monophosphate (AMP) deaminase which generates uric acid. In contrast to glucose whose metabolism is regulated by the rate-limiting enzyme phosphofructokinase (PFK), the metabolism of fructose is unregulated, leading to large increases in acetyl CoA which are precursors for fatty acid synthesis. Fructose also activates transcription factors Sterol Regulatory element binding protein (SREBP-1c) and carbohydrate response element binding protein (ChREBP) which increase the expression of lipogenic enzymes like FAS and ACC. Increased hepatic fatty acids and triglycerides leads to increased formation of VLDL particles as a result of higher substrate availability, increased apoB stability, and higher MTP, the critical factor in VLDL assembly. Eventually, lipids are secreted into the blood circulation, leading to dyslipidemia and deposition of lipids in skeletal muscle

2.2. Fructose-induced impairment of insulin-stimulated Glucose uptake: an overview of known and potential mechanisms.

Normal glucose uptake by skeletal muscle is crucial for maintaining blood glucose homeostasis because skeletal muscle represents a major site of whole body glucose disposal. Glucose is transported into skeletal muscle cells mainly via the GLUT4 transporter protein (36). In the basal state of the cell, GLUT4 is located predominantly in the sarcoplasm within intracellular vesicles in an inactive state (9). In the post-absorptive state insulin is released which binds to the α subunit of the insulin receptor (IR) on the sarcolemma. This triggers the auto-phosphorylation of the β subunit of the IR on tyrosine residues and subsequent activation of a signalling cascade involving several intermediates including insulin receptor substrate (IRS) 1/2, phosphatidylinositol 3-kinase (PI3 kinase), Protein Kinase B (Akt) and Akt substrate 160 (AS160)(Figure 2.2). Activation of this signalling cascade ultimately results in the translocation of GLUT4 to the sarcolemma for increased glucose uptake and restoration of euglycaemia (9).

High fructose consumption is known to lead to a reduction of this insulin-mediated glucose uptake capacity which is characteristic of skeletal muscle insulin resistance and is a prelude to type 2 diabetes. This takes place via (a) a decrease in GLUT4 translocation through disruption of the insulin signalling pathway and (b) a potential decrease in the amount of GLUT4 protein in the skeletal muscle cell through a decrease in *Glut4* gene expression.

2.2.1. Decrease in GLUT4 translocation.

Disruption of the normal insulin signalling pathway which triggers GLUT4 translocation to the sarcoplasm often occurs when there are disorders in cellular lipid metabolism and is thus far the best described mechanism by which fructose affects GLUT4 function in skeletal muscle (86; 114). Fructose-induced dyslipidemia leads to excess deposition and incomplete oxidation of lipids in skeletal muscle cells (104). which may

generate ceramides and toxic lipid-derived metabolites such as diacylglycerol (DAG) and long chain acyl CoA (LCACoA). These lipid metabolites increase the phosphorylation of the IRS-1 on serine/threonine residues via protein kinase C θ (PKC θ) activation. This reduces insulin signaling which is dependent on tyrosine (but not serine/threonine) phosphorylation of IRS-1. Additionally, several other kinases, including IKK, JNK and p38 MAP Kinase may also be activated. These kinases have been postulated to catalyse the phosphorylation of serine residues in IRS-1, thereby inhibiting its activity and targeting it for proteasomal degradation (111).

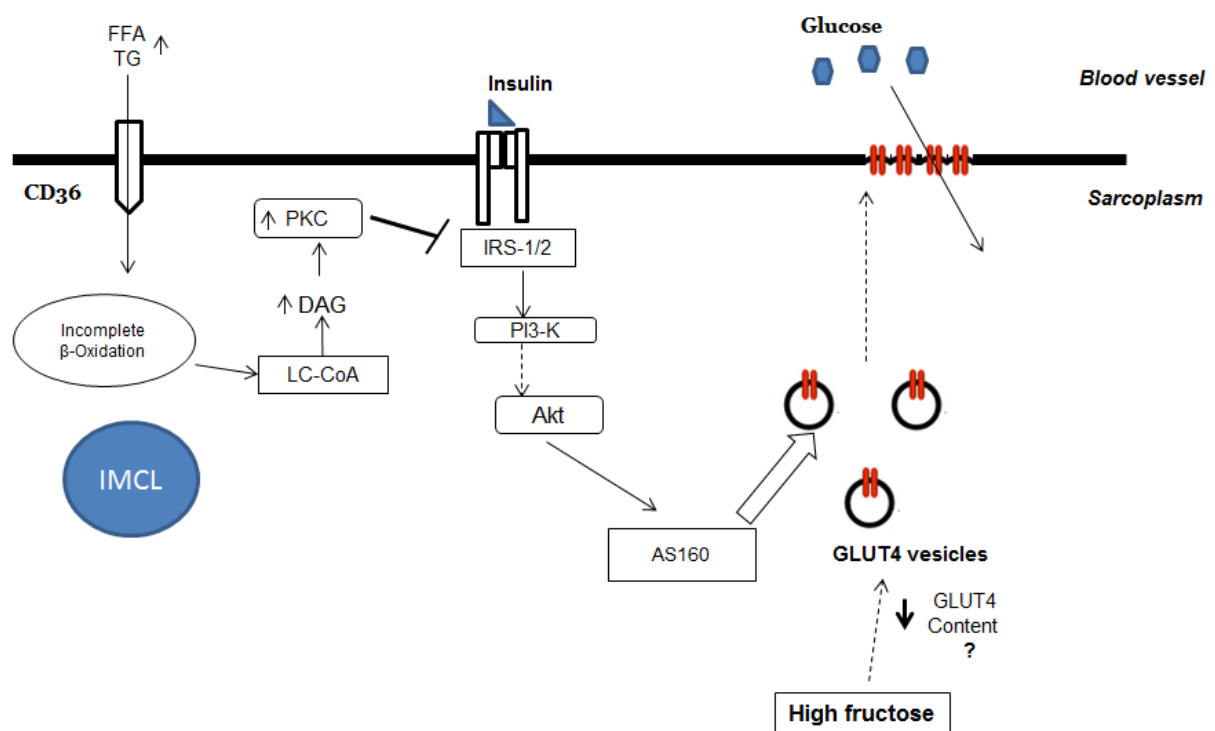


Figure 2.2: Signalling mechanisms of fructose-induced impairment of insulin-stimulated glucose uptake in skeletal muscle.

Excess FFA uptake brings about incomplete fatty acid oxidation resulting in accumulation of LCACoA. These produce intermediates like DAG which activates isoforms of PKC which phosphorylate IRS-1 on serine residues and prevent them from associating with PI3K. This disruption in insulin signalling leads to a reduction in GLUT4 translocation to the sarcolemma for glucose transport.

Ceramides, on the other hand, mediate increases in phosphatase-2A (PP2A) and sequestration of Protein Kinase B (Akt2) by protein kinase C ζ (PKC ζ). Impaired Akt2 activation limits translocation of vesicles containing GLUT4 molecules to the plasma membrane, resulting in impaired glucose uptake (91).

2.2.2. Reduced levels of GLUT4 protein in skeletal muscle.

The cellular level of GLUT4 protein correlates well with whole body glucose disposal capacity (38; 88). There are some reports that high fructose consumption may reduce the cellular levels of GLUT4 protein thus representing a first step towards insulin resistance. However, existing data on the effects of fructose on GLUT4 expression (Table 2.1) is inconsistent because of the lack of standardisation of experimental protocols in terms of the amount of fructose used and duration of feeding experiments. For instance, Meeprom et al. (66) found a 56% decrease in total cellular GLUT4 in rats fed a 63% fructose diet for 8 weeks. Conversely, the same strain of rats fed a 60% fructose diet for 5 weeks failed to show any decrease in GLUT4 but experienced a 20% decrease in GLUT4 translocation to the sarcolemma. In humans, a 27% decrease in skeletal muscle GLUT4 was seen when subjects were fed 1.5g/kg/ day of fructose over a period of 4 weeks. In all reported animal studies, however, GLUT4 translocation to the sarcolemma was affected.

To the best of our knowledge this is the first study that specifically investigated the expression of the *Glut4* gene under high fructose consumption. It is important to note that most of the existing GLUT4 data stem from studies that used fructose-fed rats as a non-genetic model of insulin resistance in order to test different interventions, hence the high amount and long duration of fructose feeding. Consequently, there is no available data on the effect of more moderate intakes of fructose. Table 2.1 summarises the available data.

Table 2.1: Summary of available data on the effect of fructose on GLUT4 expression and translocation

Study (Ref.)	Experimental diet	Duration (weeks)	Subjects	Effect on skeletal muscle GLUT4
Meeprom et al (66)	63% fructose	8	Male Wistar rats	56% reduction in total GLUT4
Shih et al (89)	60% fructose	8	Male Sprague Dawley rats	60% reduction in membrane GLUT4
Liu et al (47)	60% fructose	6	Male Wistar rats	Reduction in GLUT4 mRNA and protein in membrane fraction
Li et al (45)	60% fructose	5	Male Wistar rats	No change in total cellular GLUT4 but 20% decrease in translocation.
Le et al (42)	1.5g/kg/ day	4	Human male	27% decrease in total cellular GLUT4

2.3. Molecular mechanisms in the regulation of skeletal muscle GLUT4 activity and content by exercise.

This section reviews current knowledge of *Glut4* regulation by exercise to provide the background information for our experiments conducted to examine the effects of exercise and concurrent fructose consumption on the *Glut4* gene in skeletal muscle. Exercise exerts both acute and longer term effects on skeletal muscle glucose uptake ~~respectively~~ via an increase in translocation and protein levels of GLUT4, respectively. Figure 2.3 depicts the acute effects of exercise whereby signals activated by muscle contraction trigger the translocation of GLUT4-containing vesicles from intracellular locations to the sarcolemma via a mechanism which is independent of insulin (107). Furthermore, this section presents

the molecular mechanisms of by which exercise increases GLUT4 expression, especially with respect to changes in chromatin structure at the *Glut4* gene locus in order to allow increased transcription rates.

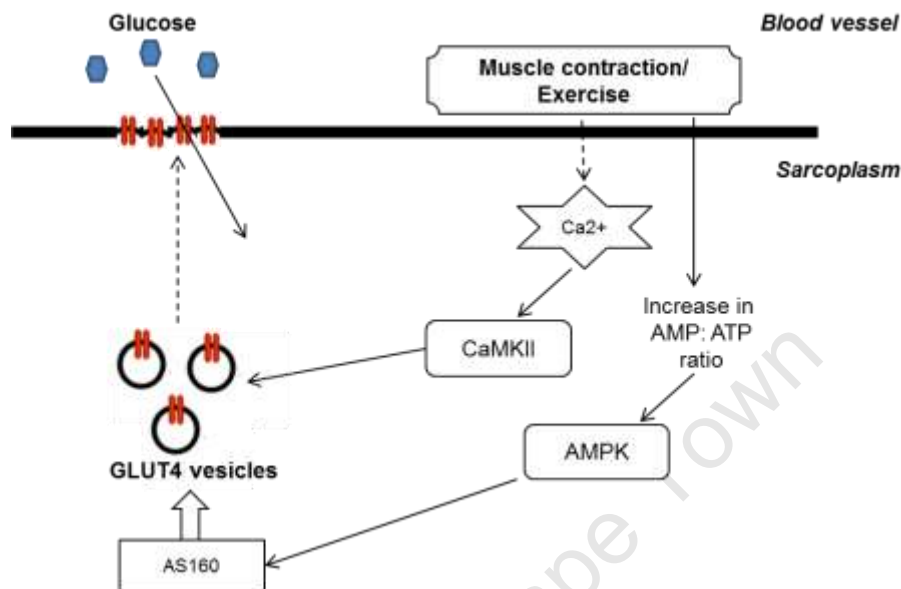


Figure 2.3: Acute effects of exercise on glucose uptake by skeletal muscle

Muscle contraction during exercise leads to increases in Ca^{2+} and ADP:ATP ratio. These signals respectively activate CaMKII and AMPK which trigger mechanisms that lead to the translocation of GLUT4-containing vesicles from intracellular locations to the sarcolemma.

2.3.1. MEF2 transcription factors and the MEF2 binding site on the *Glut4* gene.

It is now well established that there are two conserved regions on the *Glut4* gene promoter which are essential for normal expression of GLUT4 in skeletal muscle. The first proximal region is found between base pairs -463 and -473 (in rat or human???) and contains a binding site for dimers of the MEF2 transcription factor isoforms (103). On the *Glut4* gene promoter MEF2 binds to its binding domain as a MEF2A/D heterodimer (71). The second region is a distal region containing the binding domain for GEF which is located between base pairs -712 and -742 (78).

2.3.2. Exercise increases MEF2 binding activity

Exercise increases MEF2A binding activity to its binding element on the *Glut4* gene, as revealed by electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays. Studies by Smith et al (94) from our laboratory showed that MEF2A binding increases about two-fold in rat triceps muscle following 5 x 17min bouts of swimming. Likewise, a 1.6-fold increase in binding was observed in human muscle following a 60-minute bout of cycling (57). These findings substantiate the importance of MEF2 in mediating the exercise-induced increase in GLUT4 and therefore regulatory mechanisms upstream of MEF2 binding have been extensively studied and characterised.

In the basal state, MEF2 is associated with the class II HDAC isoform 5 (HDAC5) transcriptional repressor and this MEF2-HDAC5 physical association inhibits MEF2 mediated transcription (65). HDAC5 inhibits transcription by deacetylating lysine side chains on histones, thus inhibiting transcription because it physically restricts the access of transcriptional activators to DNA. This is reversed by the action of HATs. However, for HATs to acetylate histones HDAC5 must be dissociated from MEF2. Various mechanisms triggered by exercise bring about this MEF2-HDAC5 dissociation by phosphorylating HDAC5 followed by its export from the nucleus. In human skeletal muscle a single bout of exercise reduced MEF2 associated HDAC5 and nuclear HDAC5 abundance. These changes were associated with an increase in MEF2A binding to its cis-element on the *Glut4* gene and GLUT4 mRNA (56). The next section will review the mechanisms triggered by muscle contraction during exercise which lead to the above changes.

As shown in figure 2.4, muscle contraction triggers signalling cascades that increase GLUT4 expression by regulating MEF2-HDAC interactions. These mechanisms are triggered by an increase in cytosolic calcium (Ca^{2+}) concentration due to increased release from the sarcoplasmic reticulum and a decrease in high energy phosphate levels (increase in AMP: ATP ratio).

Muscle contraction brings about an increase in cytosolic Ca^{2+} levels. This rise in Ca^{2+} is transduced by the protein calmodulin (CaM) which binds Ca^{2+} and in turn binds to and activates Ca^{2+} /Calmodulin dependent protein kinase II (CaMKII) (31). The Ca^{2+} message is relayed by CaMKII by phosphorylating target substrates on serine or threonine residues (31). Activated CaMKII phosphorylates HDAC5 on Serines-259 and -498 within HDAC/ MEF2 complexes. The phosphorylated HDAC5 then dissociates from MEF2 and is exported from the nucleus by chaperone protein 14-3-3 (63). Once dissociated from the HDAC5, MEF2 is then able to form complexes with histone acetyl transferases (HATs) such as p300 and co-activator molecules such as peroxisome proliferator receptor gamma coactivator -1 (PGC-1). This association leads to acetylation of histone 3 (Lys ^{9/14}) (95) and chromatin relaxation in the region of the MEF2 binding domain on the *Glut4* gene promoter. Increased DNA-binding activity of MEF2 (as a MEF2A/D dimer) takes place followed by increased GLUT4 mRNA (56). There is an abundance of studies that substantiated the importance of CaMKII using research techniques that alter CaMKII activity in vitro as well as in vivo exercise models and pharmacological interventions (76). For instance, our laboratory showed that CaMKII activation is apparently critical for histone acetylation and increased MEF2 binding to the GLUT4 gene in rats after swimming (95).

AMP-activated protein kinase (AMPK) is another important HDAC kinase which is activated during exercise by an increase in AMP: ATP ratio through allosteric mechanisms (62). Furthermore, following exercise AMPK increases in nuclear abundance and activation in skeletal muscle. AMPK regulates GLUT4 expression by phosphorylating HDAC5 on Serine 259 and 498. This reduces the interaction of the transcriptional repressor with the GLUT4 promoter, increases its nuclear export (62) and increases MEF2 binding to the *Glut4* gene promoter (22). Therefore both CaMKII and AMPK may be involved in the regulation of nuclear HDAC4 and 5 and histone acetylation during exercise. There is a redundancy in the control of GLUT4 expression by these two pathways because the inhibition of one pathway is compensated for by the other (74). However, the relative contribution of these

two kinases could be dependent on factors like exercise intensity, mode, duration and muscle fibre type.

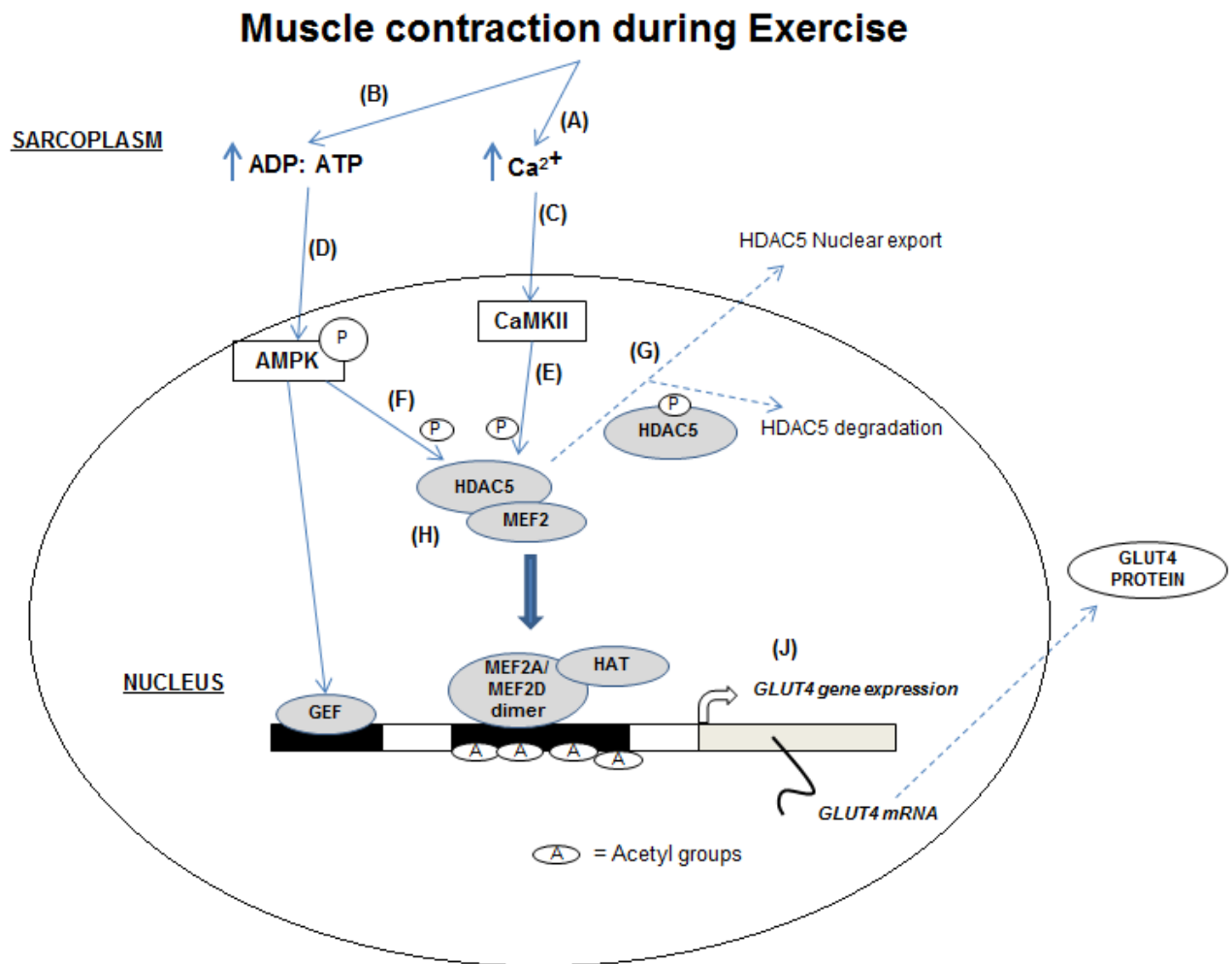


Figure 2.4: Schematic diagram of the regulation of MEF2 activity and GLUT4 expression in contracting skeletal muscle.

Muscle contraction during exercise increases cytosolic Ca^{2+} levels (A) and decreases cellular ATP levels (B). These respectively lead to the activation of CaMKII (C) and AMPK (D) which phosphorylate HDAC5 (E and F) within MEF2/HDAC complexes. HDAC5 then dissociates from MEF2 and is exported from the nucleus (G) by chaperone proteins. MEF2 is then able to form complexes with HATs like P300 as well as recruit co-activators like PGC-1 α . This leads to acetylation of histone proteins in the neighbourhood of the MEF2 binding site on the GLUT4 gene, allowing a relaxed chromatin conformation which leads to greater accessibility to MEF2 and increased binding of the transcription factor as a MEF2A/ D dimer (I) and increased GLUT4 gene expression (J)

2.3.3 Exercise remodels chromatin to increase *Glut4* expression.

It has become increasingly clear that remodelling of chromatin is an important process in the regulation of exercise-responsive genes like *Glut4* (55; 58; 59). Indeed, significant insights have been gained about the spatial relationships between genomic DNA and the histone core that influence the access of transcriptional regulators to promoter regions of exercise-responsive genes (59). Of note has been the elucidation of the various post-translational modifications to histone proteins that alter the DNA-histone interactions which in turn affect whether chromatin is in a compact repressive conformation or a relaxed transcriptionally permissive conformation (62).

At a structural level, DNA is packaged around histone proteins to form chromatin. Chromatin is made up of functional units called nucleosomes (Figure 2.5). One nucleosome unit consists of 146bp of DNA wrapped in ~1.7 turns around a histone octamer made up of two molecules of the histone subunits H2A, H2B, H3 and H4 (39; 41). DNA is bound to histones by means of electrostatic forces and each nucleosome is separated from adjacent nucleosomes by a linker DNA region (49). Once thought to be a static structure serving mostly at packaging and compaction, the nucleosome is now known to have dynamic characteristics that play a role in the regulation of gene expression.

Many genes which are in a transcriptionally repressed state have their promoters and enhancers in regions of the nucleosome which are inaccessible to transcription factors. In this condition, formation of transcriptional initiation complex (TIC) does not occur and RNA polymerase II (Pol II) is restricted; therefore transcriptional initiation is prevented (6) (40). In this condition, chromatin needs to undergo modifications that alter the spatial relationship between histone proteins and DNA in order to allow access of these factors to DNA. Subsequent to binding, transcription factors assist the assembly of the TIC at the promoter region (44).

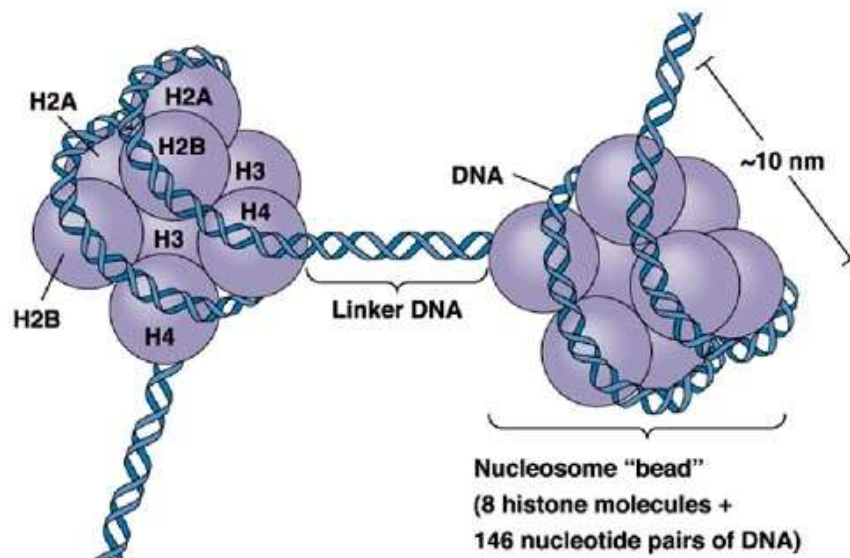


Figure 2.5: Basic nucleosome structure

One nucleosome is made up of an octamer of histone subunits (2 units of each H2A, H2B, H3 and H4). 146bp of DNA is wrapped in ~ 1.7 turns to histones by means of electrostatic interactions between the negatively charged DNA and the positively charged histones. A segment of DNA (average of 20bp) called the Linker DNA joins one nucleosome to adjacent nucleosomes.

There are many mechanisms by which chromatin structure can be modified to allow a relaxed conformation. These involve post-translational modifications to histone proteins and include acetylation, phosphorylation and methylation (44). For example, when the N-terminal tails of lysine amino acid residues on histone H3 and H4 are hyper-acetylated, the positive charge of the lysine is neutralised thus weakening the electrostatic interaction between histones and the negatively charged phosphate backbone of DNA. The result is a relaxed chromatin structure which allows access to transcription factors and assembly of the TIC (44) (Figure 2.5).

Acetylation/deacetylation are important mechanisms that influences chromatin structure at the *Glut4* gene and are regulated by contrasting enzymes, namely HATs and HDACs (64). HDACs are identified as transcriptional corepressors because they remove negatively charged lysine residues from histones thus making histone tails more positively

charged. This cause histones to interact more tightly with the negatively charged DNA backbone and therefore restricts the access of transcription factors to their binding domains (44). Conversely, HATs catalyse the addition of acetyl groups to lysine residues on histones which neutralises the positively charged side chain on these residues and weakens the electrostatic interactions with the negatively charged DNA phosphate backbone. The resulting change in the spatial relationship between the nucleosome and DNA increases the accessibility of cis-regulatory elements to transcription factors to initiate the transcriptional cascade. It is understood that the acetylation state, spatial relationships between DNA and histone and transcriptional activity are dependent on a net balance in HAT/HDAC activity at a gene promoter (44).

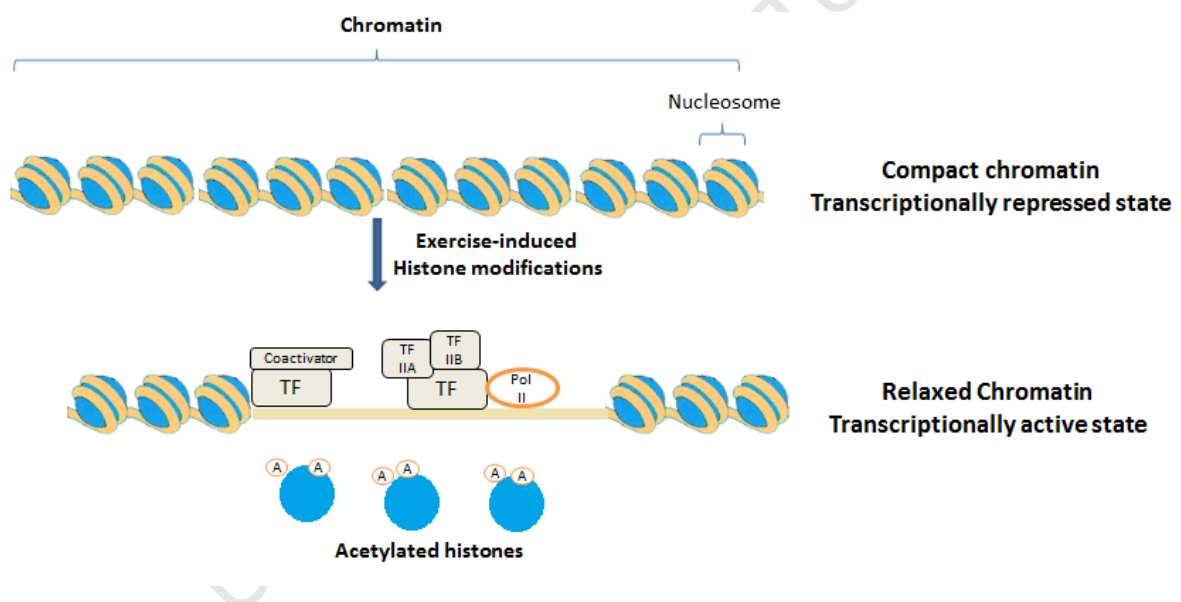


Figure 2.6: Regulation of transcription by histone modifications

Histones in the native (unmodified) state interact tightly with DNA at the gene promoter and coding regions, leading to a transcriptionally repressed state. Histone modifications like (in this example) acetylation disturbs this tight DNA-protein interaction, leading to a relaxed chromatin state. This exposes promoter and coding regions to transcriptional regulators, like RNA polymerase (Pol II) and the transcription factors (TFIIs), resulting in transcription.

2.4. Methods used for the structural analysis of chromatin in gene regulation.

In view of the major impact of chromatin structure on gene transcription, structural analyses of chromatin are commonly performed in gene expression studies (73; 95). In these studies histone modifications are commonly analysed using the chromatin immunoprecipitation (ChIP) assay for measuring histone modifications and transcription factor binding at binding domains.

With regard to the *Glut4* gene, the ChIP assay, described in section 3.10, has been highly useful for the identification of histone H3 acetylation and MEF2A binding at the MEF2 binding site on the *Glut4* gene (73; 95). An increase in MEF2A binding and Histone H3 acetylation are indicative of relaxation of chromatin at the MEF2 binding site on the *Glut4* gene (73; 95). Therefore, changes in histone acetylation and MEF2A binding are used as an indirect predictor of whether the gene is in an open transcriptionally permissive conformation or a closed transcriptionally repressed conformation. Consequently, the ChIP assay only provides indirect information on chromatin structure rather than a direct quantification of changes in chromatin structure (13; 23).

Direct analysis of changes in chromatin structure in transcriptional regulation is achieved by using the nuclease digestion assay. This assay makes use of micrococcal nuclease (MNase) and DNase I to digest DNA from intact nuclei. The principle of the nuclease digestion assay is that the nucleases will be able to enter nuclei and cleave DNA at a particular locus of interest at a rate which is proportional to the accessibility of that particular region to transcription factors. (14) Therefore, in time-course digestion experiments DNA will be digested by nucleases at a faster rate in a transcriptionally active state compared to a transcriptionally repressed state. Digestion of DNA in accessible chromatin will result in fragments that are less likely to contain primer binding sites for PCR and therefore the amount of PCR products correlates with extent of digestion and thus chromatin accessibility.

Of note, DNase I and MNase each exhibit different DNA digestion characteristics in the sense that MNase cuts the internucleosomal linker regions but not DNA wrapped around nucleosomes. DNase I, in contrast, cleaves DNA wrapped around the nucleosome and at positions where the minor groove faces away from the nucleosome (93). Interestingly, there are regions in the genome called DNase I hypersensitivity sites which are nucleosomal regions known to likely contain binding sites of transcription factors (13; 14; 23). Transcription usually induces hypersensitivity to DNase I at these sites suggesting that nucleosome arrays are disrupted there, resulting in greater accessibility based on a higher rate of digestion.

The Nuclease digestion assay has been used in several gene expression studies particularly in plant science research in order to link increased gene expression, histone acetylation and transcription factor binding to increased accessibility of chromatin in promoter regions. Our study represents the first to describe the use of the nuclease digestion assay to directly assess changes in chromatin structure in *Glut4* gene regulation and therefore represents a milestone in the use of this method for the study of *Glut4* transcriptional regulation.

2.5. Exercise training and concurrent high fructose consumption: implications on GLUT4 expression.

As reviewed earlier, exercise increases skeletal muscle GLUT4 content by various mechanisms thus improving whole body glucose disposal in both healthy and diabetic individuals (10) and as a result is a valuable therapeutic tool for type 2 diabetes. The use of exercise as a therapeutic modality to correct fructose-induced metabolic derangements is well documented in literature (2; 109). For example, Botzelli et al. (2; 109) found that a swimming exercise protocol counteracted fatty liver disease in rats that were fed a fructose-rich diet. Similarly, Fiebig et al (19) found that treadmill running training attenuated the fructose-induced upregulation of hepatic lipogenic enzymes in fructose-fed rats. However,

there are no studies that used exercise as a modality to correct fructose-induced GLUT4 dysregulation. Therefore, one of the aims of our study was to assess whether an eventual dysregulation of GLUT4 expression by fructose can be amended by exercise training.

Moreover, it is not known how the consumption of fructose, an ingredient in sports drinks, modulates exercise-induced increases in GLUT4 content. There is substantial evidence that nutrient availability serves as a strong modulator of the many acute and chronic adaptations to exercise (27) by altering the activities of many exercise-responsive genes with established roles in training adaptation (1). For instance, there is evidence that muscle glycogen concentration, determined by carbohydrate availability, has a regulatory effect on the increase in GLUT4 expression after exercise: It is known that commencing exercise with low muscle glycogen level results in greater transcriptional activation of GLUT4 compared with when glycogen content is normal (26; 83).

Overall, there is not yet enough athlete-specific research and studies done on animal exercise models to investigate the effect of fructose on exercise responsive genes and other metabolic genes like *Glut4*. Therefore, our study represents a breakthrough in this field. For that purpose we devised experiments to elucidate the molecular mechanisms by which exercise and concurrent fructose consumption influence *Glut4* gene expression particularly at the level of histone modifications and chromatin accessibility at the *Glut4* gene promoter.

The effect of fructose consumption on skeletal muscle GLUT4 expression has not been studied in sufficient detail for any data to be available about its impact on histone modifications and changes in accessibility at the *Glut4* promoter. Nevertheless, the ability of fructose to regulate a number of genes by altering histone modifications is known. For example, the consumption of high amounts of fructose was found to increase the expression of the intestinal glucose/ fructose transporter GLUT5 by a mechanism involving trimethylation of Histone H3 at Lysine 4 (H3K4) at the *Glut5* gene promoter (110). Additionally, using the ChIP assay, Douard et al (15) showed that high fructose consumption

also induces trimethylation of H3K4 at the promoter of the fructose-inducible genes *Cabp2* (Calcium binding protein 2) and *Gcgr* (Glucagon receptor) in the rat small intestine. However, transcription factor binding was not measured in these *Glut5* expression studies (110). Moreover, fructose is known to induce lipogenic genes in the liver by increasing the binding of the transcription factor ChREBP to the carbohydrate response element (ChRE) on lipogenic genes like FAS (18; 37). However, none of these studies undertook any direct measure of accessibility.

Therefore, our study was the first to conduct a direct measurement of accessibility of the *Glut4* promoter and to relate changes in histone H3 acetylation and MEF2A binding to changes in GLUT4 expression under conditions of high fructose consumption in both trained and untrained rats.

University of Cape Town

CHAPTER THREE

Experimental Methods

3.1. Introduction

The expression of the *Glut4* gene under a high-intensity intermittent exercise protocol and high fructose consumption was assessed in Wistar rats. Experiments were performed over a period of 14 days and skeletal muscle and blood were harvested at the conclusion of the experiment. Serum was analysed for fasting glucose, insulin and free fatty acid levels. GLUT4 protein content, histone H3 acetylation and nuclease accessibility of the MEF2 binding site on the *Glut4* gene as well as bound MEF2A were measured from skeletal muscle. This chapter provides a justification for the use of rats, a description of the feeding and exercise protocols and the laboratory methods used for these investigations.

3.2. Justification for the use of rats as an experimental model.

According to the National Institute of Health (NIH) Meeting on Rat Model Priorities (<http://www.nhlbi.nih.gov/resources/docs/ratmtg.htm>), the rat model is very suitable for the study of human health and disease, particularly the link between genes and biological function. There exists vast amount of data on rat behaviour as well as physiology, the biochemistry and genetics of this experimental model. Furthermore, rats have been used extensively for research on exercise and GLUT4 gene regulation. In many cases, the rat is the most suitable experimental model for the study of human disease.

Wistar rats were used because they were easily available, very easy to handle and are not aggressive. These were desirable traits because our experiments involved a fair amount of handling. It is well known that Wistar rats are able to complete the swimming protocol and their muscles respond to exercise by increasing GLUT4 expression (20).

Male rats were used instead of female rats in order to prevent the influence of factors initiated by the oestrous cycle, like reduced food consumption, weight loss and increased physical activity levels. All these factors could potentially affect results. Rats weighing 250-300g were used because pilot studies in our laboratory have shown that they were able to complete the swimming protocol with no sign of distress. Younger rats of 150-200g were unable to complete the protocol. Older rats were not used because the rats become more aggressive with age and difficult to handle. Furthermore, 5 rats per experimental group were used in the present study to provide enough statistical power to detect treatment effects.

3.3. Animal care.

Ninety day old male Wistar rats (n=30), weighing 250-300g, were used in the study. All procedures involving the use of animals were performed in accordance with the rules and regulations of the Animal Research Ethics Committee of the University of Cape Town. The experiments were conducted at the Animal Facility. The animals were housed individually in polycarbonate cages in an environmentally controlled room (temperature: $25^{\circ} \pm 1^{\circ}\text{C}$ and humidity: 40-60%) with a set 12 hour light-dark cycle. All animals had ad libitum access to standard rat chow and tap water throughout the experiment. Bedding consisted of wood shavings and shredded paper and was changed every 3 days or more frequently whenever necessary. The welfare of the animals was checked on a daily basis by the investigator and the animal facility staff.

3.4. Experimental groups and dietary treatment.

On the day of arrival in the research facility, rats were assigned to one of six treatment groups (n=5 per group; Table 3.1) and familiarised to human handling for 4 days. Each animal was provided with 200g of chow, 200ml of water and 250ml of CHO drink (10% fructose or maltodextrin solution in water) at 08:00-09:00 daily. The amount of water, chow and CHO consumed per day was calculated at the same time the next day by subtracting the weight of left over water, chow and CHO respectively from the amount initially provided. To ensure accurate chow intake calculations, all pieces of chow that were found within the

bedding were collected and weighed together with the left over chow. The choice of 10% fructose or 10% Maltodextrin (w/v in tap water) for these experiments was made to ensure that the caloric density of these solutions were comparable to the caloric density of common sugar-sweetened carbonated beverages (~0.4 kcal/mL). Furthermore, maltodextrin (a glucose polymer) was used as a control for fructose and calories

Table 3.1: Experimental groups and details of treatment

Experimental Group	Details of treatment
Water untrained (WU)	These rats had access to standard rat chow and tap water <i>ad lib</i> and were kept sedentary (Untrained) for the duration of the experiment. (n=5)
Water trained (WT)	These rats received standard rat chow and tap water <i>ad lib</i> and were submitted to a swimming training protocol. (n=5)
Fructose Untrained (FU)	These were given access to standard rat chow, 10% fructose solution and tap water <i>ad lib</i> and were kept were sedentary for the duration of the experiment. (n=5)
Fructose trained (FT)	This group had access to standard rat chow, 10% fructose solution and tap water <i>ad lib</i> and received swimming training.
Maltodextrin untrained (MU)	They had access to standard rat chow, a 10 % maltodextrin solution and tap water <i>ad lib</i> and were kept sedentary. (n=5)
Maltodextrin trained (MT)	They had access to standard rat chow, 10% maltodextrin solution <i>ad lib</i> and tap water and were submitted to swimming training. (n=5)

3.5. The swimming exercise protocol.

After 4 days of familiarization to handling, rats that were randomly assigned to the swimming groups were made to adapt to swimming in a deep water bath using a protocol previously described (94). Briefly, rats performed 2 bouts of 17 minute swims on day 5 and each subsequent day, until day 7, an additional bout was added. At the end of this adaptation period, the rats could swim for 4 bouts of 17 min without any sign of distress. On days 8-13, these animals were submitted to a swimming exercise consisting of 3 bouts of 17 min carrying a load equivalent to 5% body weight. The load was securely attached to the base of the tail. Throughout the training period, rats swam in water maintained at $31 \pm 1^\circ\text{C}$ and were given 3 minutes of rest between bouts. They were dried and kept warm during the rest periods using clean and dry towels.

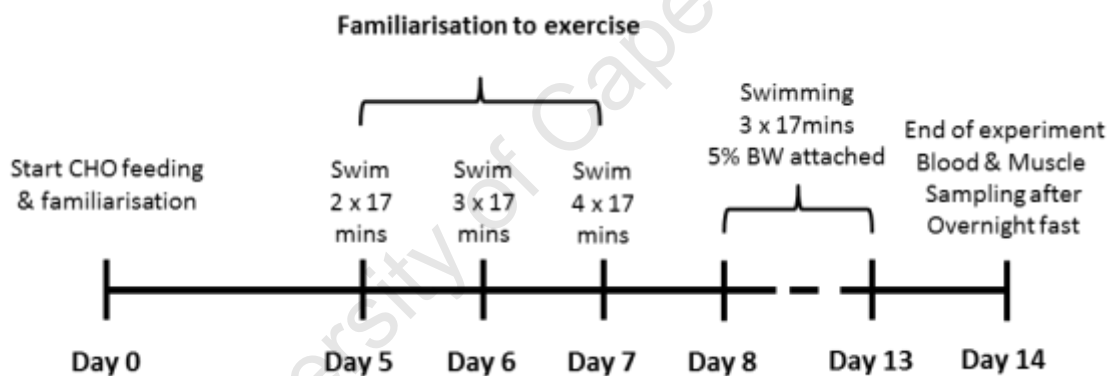


Figure 3.1: Swimming protocol for the study.

The use of swimming in these studies is justifiable because it has a number of advantages over treadmill running in terms of the simple and inexpensive equipment required and the rodents' natural swimming abilities (29). The protocol is also justifiable based on findings from previous studies which have shown that the high intensity intermittent swimming protocol was able to increase GLUT4 expression to a similar extent as the traditional long duration protocol (102). The protocol is also more time-efficient and subjects the rats to less stress. The exercise familiarisation as well as the incremental

exercise protocol was included in order to reduce anxiety and stress responses that could confound our results.

3.6. Tissue harvesting and blood sampling.

On day 13, chow, fructose and maltodextrin solutions were removed from all cages at 1800h, leaving only water. At 0800–0900h the next day, rats were weighed and then anaesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Kyrion laboratories, Johannesburg). Triceps muscles were excised, rapidly frozen in liquid nitrogen and stored frozen at -80°C until analyzed. Triceps muscle was used for analysis because they adapt well to exercise with a good increase in GLUT4 protein content. Moreover, triceps provides enough material for analysis.

About 5ml of mixed arteriovenous blood was obtained by cardiac puncture and aliquoted into two test tubes for a) lipid and insulin measurements and b) glucose assay. Blood for fasting insulin and lipid measurements was collected in tubes containing a clot activator (Greiner Bio-One, Austria) while blood for fasting glucose determinations was collected in tubes containing fluoride oxalate, a glycolytic inhibitor. Immediately after collecting blood the tubes were inverted few times and kept on ice before spinning 3000g for 15 min. The serum was kept at -80°C until further analysis.

3.7. Measurement of plasma glucose, insulin and free fatty acids.

Fasting serum glucose was measured using YSI 2300 Glucose-Lactate Analyser (YSI Corp, Ohio, USA) according to manufacturer's protocol. Insulin was quantified using the Advia Centaur XP Immunoassay Analyser (Siemens, Erlangen, Germany). Non esterified free fatty acids (NEFA) were quantified using the Roche free fatty acids half micro test colorimetric assay (Roche Diagnostics, Indianapolis, USA) according to manufacturer's instructions.

3.8. Measurement of proteins by Western blot.

Approximately 50 mg of frozen muscle was ground to a powder in liquid nitrogen, transferred to an eppendorf tube and mixed with 1 ml of HES buffer [20 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid), 1 mM EDTA (ethylene diamine tetra-acetic acid) and 250 mM Sucrose) containing 1 x Complete protease inhibitors (Roche)]. The samples were homogenised on ice for 30 sec using a Teflon tip dounce homogeniser attached to drill press at slow speed. Muscle homogenates were centrifuged at 8000 x g for 10 min and the protein concentration of the supernatants determined using the Bradford assay.

Approximately 5 µl of the supernatant was incubated with 1 ml of Bradford reagent (0.02% coomassie brilliant blue G250; 4.75% ethanol; 8.5% phosphoric acid) for 5 minutes at room temperature and the absorbance at 595 nm was measured. Concentrations of unknown samples were calculated based on a standard curve generated using bovine serum albumin (BSA) solutions of known concentrations. Crude protein extracts were solubilised in Laemmli sample buffer (LSB) (250 mM Tris-HCl, pH 6.8; 2% SDS, 10% glycerol; 0.01% bromophenol blue and 5% β-mercaptoethanol) and incubated at 95°C for 5 minutes. The samples were either used immediately for western blots or stored at -82°C for later use.

3.8.1. GLUT4 assay: Twenty (20) µg of total protein were separated on a 10% SDS polyacrylamide gel at 120V for about 1.5 hours. 5µl of a protein molecular weight standard was also loaded onto one lane. After the run, protein was transferred onto a Polyvinylidene difluoride (PVDF) (Amersham, UK) using a transfer apparatus at 30V overnight in a transfer buffer (25 mM Tris-HCl; 192 mM glycine; and 15% methanol). The electro transfer tank was kept on ice and the run was performed inside a cold chamber.

After the overnight transfer membranes were blocked for 1 hour at room temperature in a blocking buffer consisting of 5% non-fat dry milk in TBST. Membranes were then rinsed 3 times in TBST (50 mM Tris. HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and incubated with 10ml of anti-GLUT4 primary antibody (Abcam, USA) (diluted 1:5000) overnight at 4°C.

Following primary antibody incubation membranes were washed 3 x 5 mins in TBST and incubated for 1hr at room temperature with HRP-conjugated goat anti rabbit secondary antibody, diluted 1:10000 in blocking buffer. Membranes were washed 3x 5mins in TBST and incubated with enhanced chemilumescence (ECL) solution for 1 minute followed by exposure to radiographic film (Kodak, USA) for 2 minutes. Films were developed according to the procedure described by Kodak and then scanned at 300 dpi in 8 bit grey scale mode. The intensities of the bands were quantified using UN-SCAN-IT gel 6.1 software (Silk Scientific, USA). The signal intensities, representing the protein content, were normalised to α -tubulin and expressed relative to a control in each blot. Measurement of α -tubulin for normalisation purposes is described in section 3.8.2.

3.8.2. Stripping and reprobing membranes: Membranes that had been used for western blots were stripped using a harsh stripping protocol to remove bound antibodies. Membranes were washed briefly with TBST and then incubated with 10ml of a harsh stripping buffer (0.5 M Tris HCl, pH 6.8; 10% SDS; 0.8% β -mercaptoethanol) at 55-60°C with gentle rotation for 30 minutes. Membranes were then washed under running water until odourless. Membranes were then rehydrated briefly with methanol and then washed with TBST for 10 minutes. Membranes were then blocked and re-probed for α -tubulin as explained in section 3.8.1.

3.9. Nuclease Digestion Assay

The Nuclease digestion assay was used to measure the accessibility of the 350bp section of DNA on the *Glut4* gene promoter that contains the MEF2 binding domain after various treatments. The assay measures the rates of degradation of DNA by MNase and DNase I in this region from isolated intact nuclei. The assay is based on the premise that endonucleases will enter intact nuclei and digest DNA in a region of interest (e.g. the MEF2 binding domain on the GLUT4 gene) at a rate which is proportional to the accessibility of that region to transcription factors. MNase and DNase I exhibit different DNA digesting

properties. MNase preferentially cuts at the interlinker regions of nucleosomes but DNase I cleaves nucleosomal DNA at positions where the minor groove faces away from the nucleosomes. By using both MNase and DNase I one is able to assess the accessibility of both inter-linker and nucleosomal DNA at the region of interest.

The assay requires intact nuclei to ensure a valid and reliable assessment of the accessibility of the DNA section of interest. For this reason a nuclear integrity assay, described in section 3.9.2, was performed to confirm that our nuclei extraction protocol did not compromise the integrity of nuclei.

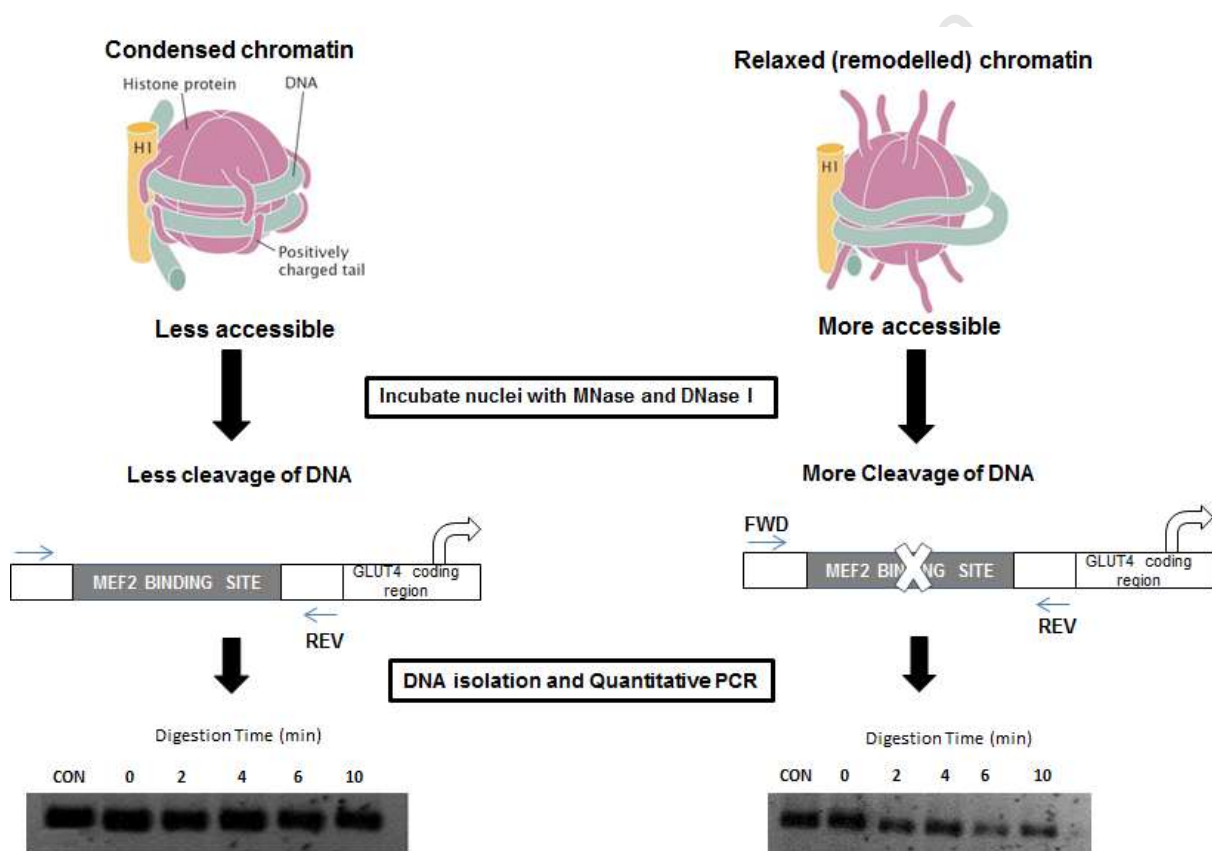


Figure 3.2: Principle of the Nuclease Digestion Assay

Isolated nuclei are digested with the endonucleases MNase and DNase I for various times between 0 and 10 minutes. Condensed chromatin would be less accessible to the nucleases resulting in a low rate of cleavage of DNA at the region encompassing the MEF2 binding site on the *Glut4* gene promoter.

3.9.1. Isolation of nuclei: About 75mg of triceps muscle was weighed and broken into small pieces in liquid nitrogen and then transferred to a homogenising tube kept on ice. Cold Homogenisation buffer (850 μ l) (Tris 10mM, EDTA 1mM, $MgCl_2$ 5mM and sucrose 1.4M) was added and allowed to stand on ice for 10 minutes. The muscle was then homogenised on ice using a hand-held glass tissue homogeniser with a pestle clearance of 0.7mm for 25 strokes.

The resulting homogenate was then centrifuged for 10 minutes at 600g and 4°C. The supernatant was discarded and the pellet was resuspended in 850 μ l of cold homogenisation buffer. Pellet was resuspended by repeated gentle pipetting instead of vortexing in order to avoid mechanical damage to the nuclei. The suspension was then filtered on one layer of cheesecloth and the filtrate centrifuged again at 600g and 4°C for 5 minutes. The supernatant was discarded and the pellet resuspended in 300 μ l of cold homogenisation buffer. The suspension was again centrifuged at 600g and 4°C for 5 minutes and the final pellet was resuspended in 200 μ l nuclei suspension buffer (Glycerol 18.5%, $MgCl_2$ 11.1mM, HEPES 14.8mM, NaCl 0.33mM, KCl 75mM and RCPI 1x). The entire procedure was performed on ice and nuclei were used on the same day for the nuclease digestion.

3.9.2. Validation of the nuclei extraction protocol: Because undamaged nuclei is required for the nuclease digestion assay the nuclei extraction protocol described in section 3.9.1 was validated with a nuclear integrity assay adapted from Czybryt et al (11). In this assay, nuclei were incubated with increasing concentrations of sodium chloride in the range of 0 to 1000mM for 30 minutes. The increase in salt concentration breaks the nuclear envelope by creating osmotic shock (5) or extracting proteins from the membrane (21). DNA and other nuclear material can then leak from the nucleus during this disruption and the nucleic acid release quantitatively measured spectrophotometrically. Nucleic acid released out of intact nuclei displays a sigmoid relationship with respect to increasing [NaCl].

For this assay about 3g of muscle was used to extract nuclei as per the protocol in section 3.9.1. Nuclei were used for the assay on the same day as extraction and were resuspended in nuclear suspension buffer. The nuclear membrane integrity assay was carried out in 8 polypropylene eppendorf tubes containing 0, 50, 100, 150, 200, 250, 300 and 1000mM NaCl in 1 ml nuclei homogenising buffer. To these reaction tubes 100 μ l of nuclei suspension (corresponding to 500 μ g of total protein) was added and the tubes capped, gently inverted a few times to mix the contents and incubated on ice for 30 minutes. A set of blank tubes, which contained NaCl-HB but not nuclei, were prepared and treated the same way as the tubes that contained the nuclei.

After incubation, the tubes were centrifuged at 7500g for 10 minutes at 4°C and the supernatants collected. The absorbance was spectrophotometrically measured at 260nm using UV-absorbing quartz cuvettes. The readings were standardised using the corresponding salt-only control tubes. The wavelength of 260nm was chosen because DNA exhibits peak absorption at this wavelength. The absorbance values were expressed as a percentage of the maximum A_{260} (i.e. relative to the sample containing 1000mM NaCl). The results are shown in Figure 3.3. The values represent the means of 3 separate samples \pm SD.

From Figure 3.3 it is seen that the plot has a sigmoid shape as well as a plateau at high salt concentrations. The plateau means that all releasable nucleic acid material has exited the nucleus. Furthermore, even in the absence of salt a small amount of nucleic acid is released from the nucleus (13.2%) which is in the acceptable range.

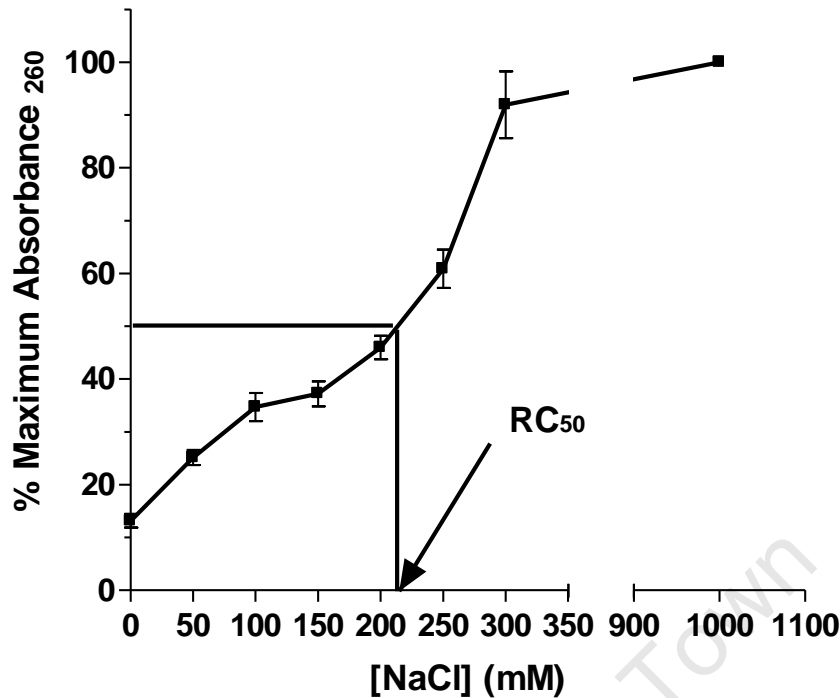


Figure 3.3: The normalised absorption (A₂₆₀) curve for supernatants from isolated rat muscle nuclei following the nuclear membrane integrity assay.

From this plot, it is also possible to interpolate a concentration of sodium chloride at which 50% of nucleic acid has been released from the nucleus. This concentration, called the releasing concentration or RC₅₀, is 215 mM in our experiment and is within the acceptable range (11). Conversely, non-intact nuclei could result from factors in the extraction procedure such as mechanical or chemical damage that compromise nuclear membrane integrity. In this case a lower RC₅₀ value would be expected because of the requirement of less salt to cause an equivalent amount of DNA to be released. A greater amount of nucleic acid would be released from the nucleus (>13.2%) (11) in the absence of salt. Overall, the results mean that our extraction procedure did not compromise the integrity of the nuclei.

3.9.3. Measurement of DNA content in nuclei using Bradford assay:

DNA concentration was estimated in intact nuclei in order to determine the optimum amount of nuclei and nucleases to be used in the nuclease digestion reactions. The protein concentration of the nuclei suspension was first estimated using the Bradford assay by adding 5 μ l of nuclei suspension to 1 ml of Bradford reagent, mixing, incubating at room temperature for 5 minutes and reading the absorbance at 595nm. The DNA concentration was obtained by multiplying the protein concentration by a factor of 0.17, as determined by Edelman et al. (16). Our nuclei extraction protocol, using 75mg of muscle as starting material, yielded nuclei equivalent to about 175 μ g of DNA.

3.9.4. Digestion of nuclei with endonucleases: samples of nuclei containing 80 μ g DNA in nuclear suspension buffer was used for digestion with each enzyme. The nuclei were then diluted with either MNase digestion buffer (50 mM Tris-HCl, 5 mM CaCl₂, pH 7.9) or DNase I digestion buffer (10 mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH 7.6) to a final volume of 480 μ l. The suspension was then incubated for 1 minute at 37°C. A control 80 μ l aliquot (non-nuclease control) was removed and added to a tube containing 8 μ l of 250mM EDTA, thoroughly mixed and kept on ice.

Either endonuclease was then added to a concentration of 0.25U/ml for MNase and 0.3U/ml for DNase I. Immediately after addition of enzyme an 80 μ l aliquot is taken for the zero time point and added to a tube containing 8 μ l of 250mM EDTA to stop the reaction and processed as above. The remaining nuclei were then allowed to digest for 2, 4, 6 or 10 minutes. At each of these time points, 80 μ l of the digest was taken and added to another tube containing 8 μ l of 250mM EDTA and kept on ice. The volume of each time point aliquots was then brought to 200 μ l with distilled water to ease handling.

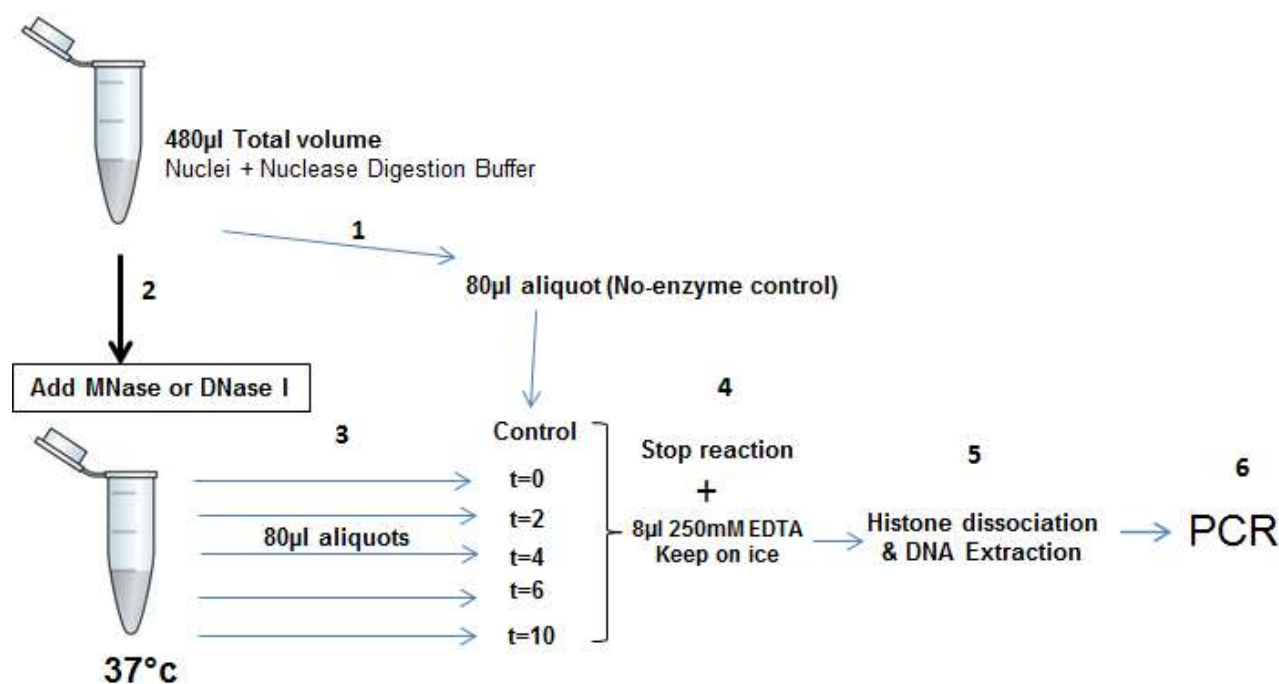


Figure 3.4: The Nuclease Digestion Assay Protocol

For each sample, nuclei suspension containing 80µg DNA was diluted to 480µl in either MNase or DNase I digestion buffer. Aliquots were taken for non-nuclease control followed by addition of MNase or DNase I was added to a concentration of 0.25U/ml and 0.3U/ml respectively. A zero time point aliquot was immediately taken. Digestion was then allowed to take place at 37°C for 2, 4, 6 and 10 minutes. At each time point aliquots were mixed with EDTA to stop the enzyme reaction. Samples were mixed and then placed on ice until further processing.

3.9.5. DNA extraction: forty (40) µl of 2.5mM NaCl was added to each of the digested samples and placed on ice for 10 minutes after mixing to dissociate nuclear proteins. DNA was then extracted using the Phenol/ Chloroform/ Isoamyl method. Equal volumes of phenol was added to the digested samples and rotated at room temperature for 5 minutes on a rotary shaker, followed by centrifugation at 2000g for 1 minute. The top layers were recovered and transferred to a separate tube to which equal volumes of Phenol/ chloroform/ Isoamyl (25:24:1) were added. The mixtures were rotated for 5 minutes at room, followed by centrifugation at 2000g for 1 minute. The supernatants were again transferred into new tubes followed by the addition of equal volumes of chloroform/ Isoamyl (24:1),

rotation for 5 minutes at room temperature and centrifugation at 2000g for 1 minute. The supernatants were transferred to new tubes and 2µl of 2µg/µl glycogen, 20µl 2.5M sodium acetate (pH 5.2) and 500µl 100% ethanol. The samples were incubated overnight at -20°C.

Following incubation, the samples were centrifuged at 2000g for 15 minutes at 4°C and the supernatants were discarded. The DNA pellets were then washed by adding 500µl of cold 70% ethanol followed by incubation on ice for 30 minutes and centrifugation again as above. Finally, the ethanol was removed and the pellets were allowed to air dry for a few hours before being reconstituted in 20µl sterile distilled water. DNA was then stored at -20°C until further analysis.

3.9.6. Polymerase Chain Reaction (PCR): DNA that was extracted from nuclei was used as template to amplify a 350bp region on the GLUT4 gene promoter containing the MEF2 binding domain. The following primers were used:

Forward 5'-GACACGGTTCTCAGACACACG-3';

Reverse 5'-CTGAGAGGTGGAAGAGGAGG-3'.

PCR was carried out in 30µl reactions containing 0.4µM forward and reverse primers, 2.5mM MgCl₂, 1x reaction buffer, 0.5U Taq polymerase, 2mM deoxynucleotide triphosphates (dNTPs) and 5µl of template DNA. The PCR was run for 35 cycles with each cycle consisting of 30 seconds of denaturing at 94°C, 30 seconds of annealing at 56°C, and 45 seconds of extension at 72°C. In the first cycle denaturing occurred for 10 min at 94°C and in the last cycle a final extension was carried on for 5 min at 72°C. Following PCR, the products were resolved on a 2% agarose gel by electrophoresis. 5µl of PCR product and 5 µl DNA ladder were mixed with 2µl of SYBR gold, loaded into the gel followed by a run for 50 minutes at 120V.

3.10. Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using triceps muscle for the assessment of (a) the level of acetylation of Histone H3 on Lysine 9 and 14 surrounding the MEF2A binding site on the *Glut4* gene promoter and (b) the binding of MEF2A to its binding site on the *Glut4* gene promoter. The principle of the ChIP assay is depicted in Figure 3.5 below.

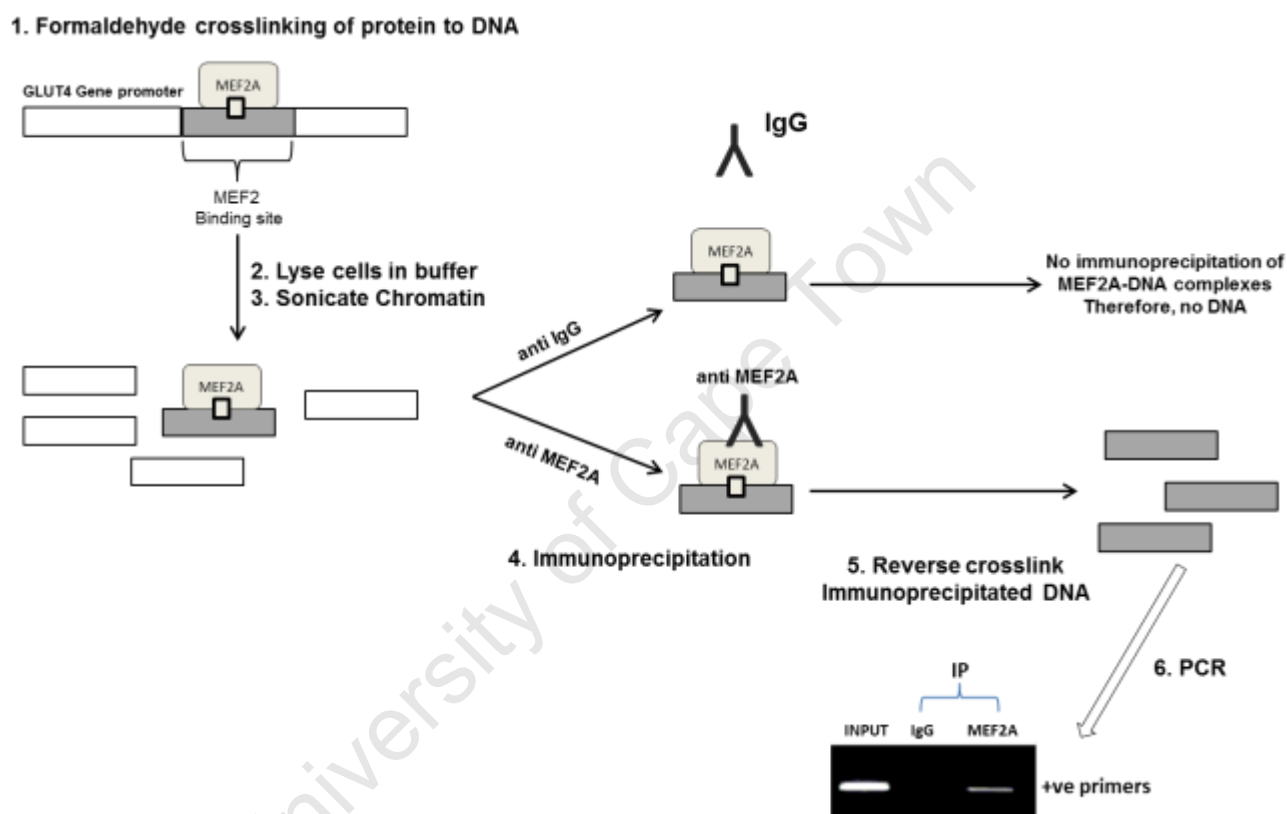


Figure 3.5: The principle of ChIP assay to measure MEF2A binding to the GLUT4 gene promoter

Frozen rat muscle was ground in liquid nitrogen and incubated in formaldehyde to cross link protein to DNA. The samples were then lysed in SDS Lysis buffer and sonicated to obtain DNA fragments of 300-1000bp. Lysates were then precleared of endogenous antibodies before immunoprecipitation with either anti-MEF2A or IgG. Co-immunoprecipitated chromatin was then reverse cross-linked and DNA was purified for use as template in PCR using primers that amplify a 360bp region containing the MEF2 binding site on the *Glut4* gene. Control experiments were also performed using negative primers that amplify a region 5kb downstream in the GLUT4 gene which does not contain a MEF2 binding domain. An aliquot of the sample that did not undergo immunoprecipitation (the “Input”) was reverse cross-linked and DNA isolated for use in PCR. PCR products were stained with SYBR Gold®, run on a 2% Agarose gel and visualised under UV.

3.10.1. Formaldehyde crosslinking: about 100mg of frozen muscle was crushed to a fine powder in liquid nitrogen and then transferred to a 15ml falcon tube. 10ml of 1% formaldehyde in PBS (pH 7.4) was added and incubated for 10 minutes at room temperature on a bench top rotator. This was done to crosslink protein-DNA interactions. Cross-linking was stopped by adding 500 μ l of 5M Glycine (final concentration 0.125M) followed by incubation with rotation for 5 minutes at room temperature. The mixture was then centrifuged at 2000g for 2 minutes and the pellet was washed twice with 10ml of cold PBS. The washed pellets were lysed in 500 μ l SDS Lysis buffer (1% SDS, 10mM EDTA, 1x RCPI, 50mM Tris, and pH 8.1) for 10 minutes on ice with frequent mixing.

3.10.2. Chromatin sonication: the lysates were sonicated in order to shear chromatin to fragments of approximately 300-1000bp using a Sonica Q125 sonicator (QSonica, Newtown, CT) set at 33% of maximal power. In order to determine the number of bouts of sonication needed to obtain the desired fragment lengths, samples were sonicated for varying numbers of bouts of 15 seconds. Importantly, samples were sonicated on slushy ice at all times and a period of at least 1 minute was allowed between bursts of sonication to prevent samples from overheating.

Following sonication, the samples were centrifuged at 13000g at 4°C for 10 minutes. Aliquots of the supernatants were then taken for Bradford Assay for determination of protein concentration and for assessment of sonication efficiency. Briefly, 5 μ l of lysate sample (diluted x5) was added to 1ml of Bradford reagent, mixed and incubated at room temperature for 5 minutes. Absorbance was read at 595nm and protein concentration was calculated from a standard curve generated using a series of standard solutions of BSA.

Assessment of sonication efficiency was achieved by reverse cross-linking the sample, followed by DNA isolation and finally agarose gel electrophoresis. Samples were reverse cross-linked by adding 0.2M NaCl and incubating for 6 hours at 65°C. DNA was isolated and purified using phenol-chloroform extraction as per the protocol in 3.9.5, stained with SYBR

Gold and then run on a 1% agarose gel for 1 hour. Figure 3.6 shows that 7 bursts of sonication of 15s at 33% of maximal power were sufficient to shear chromatin to a fragment length of 300-1000bp which is optimal for ChIP assay. Ten (10) and 12 bursts of sonication at the same power produced fragments of 500bp or less. Therefore, for all subsequent experiments chromatin was sonicated for 7 x 15s bursts at 33% of maximum power.

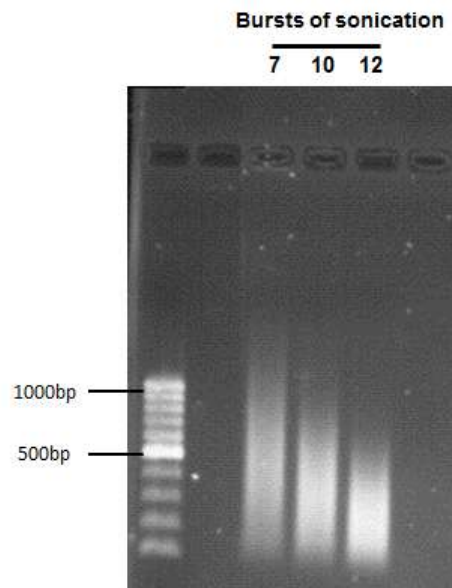


Figure 3.6: Optimising chromatin shearing for the ChIP assay.

Muscle was cross-linked for 10 minutes with 1% formaldehyde, homogenised in SDS Lysis buffer and sonicated for 7, 10 and 12x 15 second bursts at 33% of maximum sonicator intensity as described in section 3.10.2. Chromatin was reverse cross-linked followed by DNA isolation, staining with SYBR Gold® separation on a 1% agarose gel by electrophoresis.

3.10.3. Pre-clearing and Immunoprecipitation: A ChIP assay kit from Millipore was used and kit reagents were used unless otherwise stated. Following Bradford assay, 100µl of lysate containing 200µg of protein was diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris HCl pH 8.1 and 167mM NaCl). A pre-clearing step was performed to remove endogenous antibodies by adding 40µl (about 80µg protein) of salmon sperm DNA/ Protein A agarose beads and incubating for 1 hour at

4°C. Following incubation, agarose was pelleted by centrifugation at 1000g for 1 minute at 4°C.

A 300µl aliquot of the resulting supernatant, called the “input” (IN) was saved to be used as control for the ChIP assay. DNA-protein complexes were immunoprecipitated with antibodies for 36-48 hours at 4°C with gentle rotation. The antibodies used were as follows:

(a) For Acetyl H3 ChIP for measurement of histone H3 acetylation at Lys 9 and 14): 5µl of Anti Acetyl H3 (Lys9/15) antibody (Cell Signalling).

(b) MEF2A ChIP for measurement of MEF2A binding to its binding site on the GLUT4 gene): 25µl of anti-MEF2A antibody (Santa Cruz Biotechnology).

Negative control ChIP experiments were conducted in parallel using an anti-rabbit immunoglobulin G (IgG) antibody and agarose beads only (no antibody control) in order to control for non-specific binding (Section 3.10.7)

3.10.4. Washing and elution of immune complexes: following antibody incubation with antibody, immune complexes were precipitated by incubating with 40µl (≈80µg protein) of salmon sperm DNA/ Protein A agarose beads for 1 hour at 4°C. The complexes were centrifuged at 1000g for 1 minute at 4°C and the pellets were washed sequentially with the following buffers: Low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl pH 8.0, and 150mM NaCl), High salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl pH 8.0 and 500mM NaCl), Lithium chloride wash buffer (250mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA and 10mM Tris-HCl pH 8.0) and TE buffer (10mM Tris-HCl pH 8.1 and 1mM EDTA). Washes were performed by adding 1ml of buffer to the pellet, followed by gentle rotation for 3 minutes at 4°C and centrifugation at 1000g for 1 minute. All washes were performed once except for TE buffer which was performed twice and at room temperature.

Immune complexes were eluted from the agarose beads by adding 150 μ l freshly prepared elution buffer (1% SDS, 100mM NaHCO₃), incubating with rotation at room temperature for 15 minutes followed by centrifugation at 1000g for 2 minutes. The supernatant (eluate) was transferred to another tube and the above elution step was repeated. Eluates from the two steps were combined to produce a total volume of ~300 μ l.

3.10.5. Reverse crosslinking and DNA isolation: histone-DNA cross-links were reversed by incubating the Immunoprecipitated (IPs) and Input (IN) samples with 0.3M NaCl for 6 hours at 65°C. Protein in the samples was then digested in 0.01M EDTA, 0.08M Tris-HCl (pH 6.5) and 0.7U/ml for 1 hour at 45°C. DNA was isolated and purified as per the protocol in section 3.9.5.

3.10.6. Polymerase chain reaction: five (5) μ l of purified DNA from both IN and IP samples was amplified by PCR in 30 μ l reactions containing 0.4 μ M forward and reverse primers, 2.5mM MgCl₂, 1x reaction buffer, 0.5U Taq DNA polymerase, 2mM deoxynucleotide triphosphates (dNTPs). The sequences of the forward and reverse primers are given in table 3.2.

The PCR thermal cycling program consisted of an initial denaturation step for 10 min at 94°C followed by 35 cycles, each consisting of 30 seconds of denaturing at 94°C, 30 seconds of annealing at 56°C, and 45 seconds of extension at 72°C. A final extension was carried on for 5 min at 72°C. Following PCR, the products were resolved on a 2% agarose gel by electrophoresis. Five μ l of PCR product and 5 μ l DNA size marker were mixed with 2 μ l of SYBR gold®, loaded into the gel followed by a run for 50 minutes at 120V. Optimisation trials were performed to determine the optimal concentration of MgCl₂, the number of PCR cycles and the annealing temperature for each PCR.

Table 3.2: Primers and the size of products generated in PCRs using rat genomic DNA

Primer	Template DNA	Amplicon size (bp)	Forward (F) and Reverse (R) primer sequence
MEF2-Glut4 (+ve)	Rat GLUT4 promoter (from MEF2A and Acetyl H3 ChIP-IN and IP DNA)	350	F 5'-GACGCATGGTCTCCAGATACAC-3' R 5'-CTGAGAGGTGGAAGAGGAGG-3'
MEF2-Glut4 (-ve)	Rat GLUT4 promoter (-ve control for MEF2A and Acetyl H3 ChIP)	285	F 5'-GACGGACACCTTCTCTCTTAGC-3' R 5'-CCACAGCCTAGCCACAACA-3'

3.10.7. Assessment of the specificity of the ChIP assay: Control ChIP assays were performed on cross-linked muscle homogenates according using anti-MEF2A, anti-acetyl Histone H3 or anti-IgG antibody and no antibody (agarose beads only). DNA was then isolated and purified from immunoprecipitated chromatin and used in PCR reactions using primers that span the MEF2 binding site on the GLUT4 gene (+ve primers).

As shown in Figure 3.7A amplicons of 350bp were detected when the MEF2A or Acetyl Histone H3 (Lys 9/14) antibodies were used for immunoprecipitation while no amplification was noted when no antibody or IgG was used, indicating that the signals seen are due to immunoprecipitation by the MEF2A or Acetyl H3 antibodies and not from experimental artefacts. A second set of control PCR experiments were also performed on both IN and IP (using MEF2A antibody) samples using primers that span a 5kb region downstream of the GLUT4 promoter which does not contain a MEF2 binding site (-ve primers) (Table 3.2). A 268bp product was obtained in IN samples but not in IP samples. This validates the specificity of the assay to within 5kb of the MEF2 binding site of the GLUT4 gene promoter. The MEF2A antibody therefore does not non-specifically precipitate chromatin. This shows that DNA segments that do not contain the MEF2 binding site are absent in complexes that are immunoprecipitated by the MEF2A antibody, indicating that

this pulled down MEF2A-bound chromatin. Collectively, these observations validate the specificity of the ChIP assay.

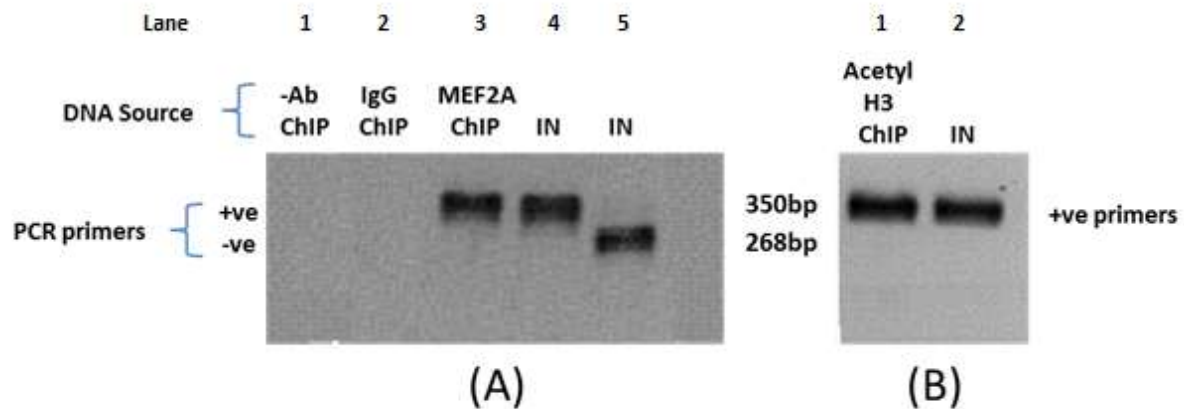


Figure 3.7: Control ChIP experiments

A: ChIP assay performed on cross-linked muscle samples using no antibody (beads only) (lane 1), IgG antibody (lane 2), anti-MEF2A (lane 3). ChIP assay results on IN samples using +ve primers (lane 4) and -ve primers (lane 5) showing amplification of a 350bp and 268bp fragment respectively. **B:** Lanes 1 and 2 are blots obtained from ChIP assay using anti Acetyl-H3 antibody and +ve primers.

3.11. Statistical analysis

Data generated from our experiments were presented as means \pm SD. Statistical differences between all treatments were determined using a one-way ANOVA. Significance was accepted at $P < 0.05$. A Fisher's least significant difference test was used for post hoc analysis when ANOVA showed a significant difference. The STATISTICA Version 10 Software (Statsoft Incorporated, USA) was used for the statistical analyses.

CHAPTER FOUR

RESULTS

4.1. Introduction

The aims of this study were to: (a) assess the effect of fructose consumption over a period of 13 days on GLUT4 expression in rat skeletal muscle, (b) examine the effect of a swimming protocol on skeletal muscle GLUT4 under conditions of high fructose consumption, (c) evaluate the effects of exercise and fructose feeding on histone H3 acetylation and MEF2A binding at the MEF2 binding site on the GLUT4 gene and (d) analyse changes in accessibility of the MEF2 binding site in response to the fructose feeding and exercise.

4.2. General characteristics of animals

4.2.1: Body weight gain: Table 4.1 shows the initial and final bodyweights of rats from all experimental groups over the 13 days of the experiment. As expected, increases in bodyweight were recorded in all groups but the amount of weight gain was modulated by the different treatments. In the WU group, a 16.8% weight gain was noted, which is consistent with normal growth of Wistar rats over a 13 day period (ref).

Table 4.1 shows that among untrained rats, the % weight gain was significantly higher (* $p < 0.05$) in fructose-fed (FU) rats compared to WU controls. However, no difference in weight gain was observed in maltodextrin-fed rats (MU vs. WU); showing that fructose but not maltodextrin exaggerates weight gain. Among the fructose-fed rats, training (FT) significantly attenuated weight gain (# $P < 0.05$) compared to the untrained (FU); showing that exercise training attenuates the weight gain caused by fructose. Likewise, exercise training also significantly reduced weight gain in maltodextrin fed rats (MT vs. MU; † $P < 0.05$). In contrast, exercise did not alter weight gain in rats that drank water only (WT vs. WU; $p > 0.05$).

Table 4.1: Baseline, final and % bodyweight gain among treatment groups.

Values are means \pm SD. * $P < 0.05$ vs. WU; # $P < 0.05$ vs. FU; † $P < 0.05$ vs. MU

Baseline and final bodyweight						
Variables	WU	WT	FU	FT	MU	MT
Baseline bodyweight (g)	286.4 \pm 7.4	258.4 \pm 6.1	278.4 \pm 14.2	274.9 \pm 14.1	281 \pm 6.0	279.6 \pm 16.2
Final bodyweight (g)	334.6 \pm 9.4	287.4 \pm 17.5	343.1 \pm 18.5	319.5 \pm 24.4	329.6 \pm 15.5	312.2 \pm 21.1
Bodyweight gain (g)	48.0 \pm 5.5	29.0 \pm 12.9	64.7 \pm 5.3	44.6 \pm 10.7	48.1 \pm 8.5	32.6 \pm 21.6
% Bodyweight gain	16.8 \pm 3.8	13.3 \pm 0.93	23.2 \pm 1.7*	16.1 \pm 9.8#	17.2 \pm 3.6	11.8 \pm 8.0†

4.2.2. Food and carbohydrate solution consumption: In Figure 4.1 the daily average consumption of 10% fructose and 10% maltodextrin solution for the various groups is given. As shown, similar amounts of fructose and maltodextrin (~140 ml) were consumed by untrained rats per day (FU vs. MU). Interestingly, exercise training significantly decreased consumption of both fructose and maltodextrin (FU vs. FT and MU vs. MT; $p < 0.05$). The average daily chow consumption for the various treatment groups is shown in Figure 4.2. Untrained rats that were fed 10% fructose (FU) or 10% maltodextrin (MU) consumed significantly more chow (31.2 \pm 6.3g/ day and 24.2 \pm 4.4g/day, respectively) compared to the WU (control) group (21.2 \pm 4.4g/day) ($P < 0.05$). However, exercise training caused a significant reduction in chow consumption in both fructose and maltodextrin-fed rats compared to the corresponding untrained group (22.4 \pm 5.7 vs. 31.2 \pm 6.9 and 20.6 \pm 8.3 g/day vs. 27.20 \pm 8.30, respectively; $p < 0.05$).

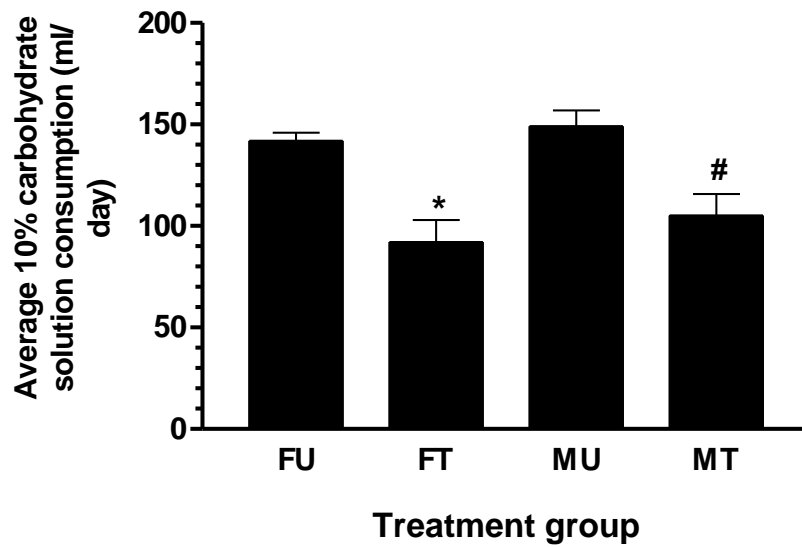


Figure 4.1: Average daily consumption of fructose and maltodextrin solutions in the various treatment groups. Data presented as mean \pm SD (n=5 per group). * $P < 0.05$ FT vs. FU. # $P < 0.05$ MT vs. MU

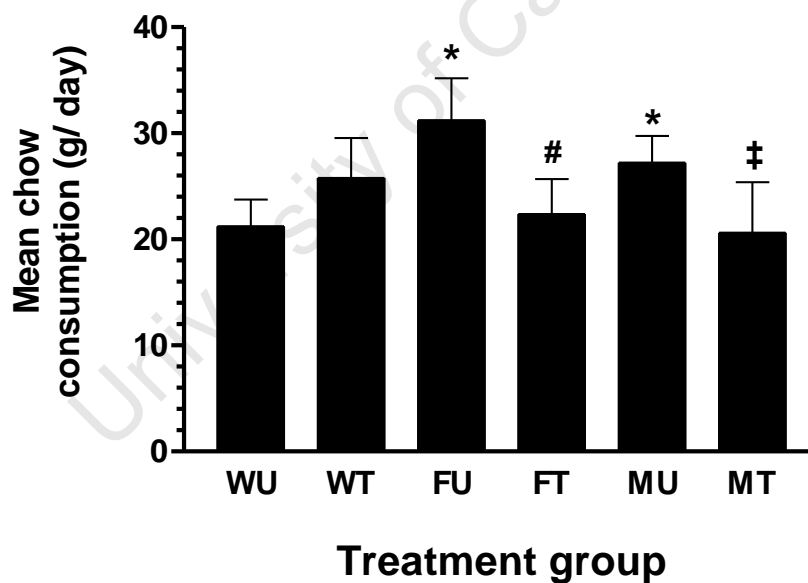


Figure 4.2: Average daily chow consumption in the various treatment groups. Data presented as means \pm SD (n=5 per group) * $P < 0.05$ FU vs. WU and MU vs. WU, # $P < 0.05$ FT vs. FU, ‡ $P < 0.05$ MT vs. MU

4.3. Fasting Serum Glucose, Insulin and free fatty acids

The fasting serum chemistry variables analysed are presented in Table 4.2. No significant difference in fasting glucose and insulin was noted among the groups. In untrained rats fructose and maltodextrin feeding did not induce any change in circulating levels of FFA compared to controls. In water and fructose-fed rats it was noted that exercise training significantly decreased circulating FFA levels (0.106 ± 0.024 vs. 0.196 ± 0.037 and 0.175 ± 0.12 mmol/L vs. 0.196 ± 0.024 , respectively; $p < 0.05$) compared to untrained controls ($P < 0.05$). However, this effect of exercise training on FFA levels was not seen in maltodextrin-fed rats.

Table 4.2: Fasting serum glucose, insulin and free fatty acids at the end of the experiment

Serum Glucose, Insulin and Free fatty acid in the fasted state						
Variables	WU	WT	FU	FT	MU	MT
Fasting glucose (mmol/L)	6.56 ± 1.4	6.32 ± 1.3	6.35 ± 0.81	7.00 ± 1.5	6.34 ± 1.5	4.87 ± 1.2
Fasting Insulin (pmol/L)	122.0 ± 24	156.2 ± 14	124.1 ± 37	125.3 ± 37	162.9 ± 28	126.8 ± 10
Fasting FFA (mmol/L)	0.196 ± 0.037	0.106 ± 0.024*	0.196 ± 0.084	0.175 ± 0.12 #	0.243 ± 0.025	0.242 ± 0.047

Results are expressed as mean ±SD, n=5, * $P < 0.05$ vs. WU, # $P < 0.05$ vs. FU

4.4. Skeletal muscle GLUT4 protein levels

The effect the various treatments on the expression of total GLUT4 in triceps muscle as measured by Western blotting is shown in Figure 4.3. As expected, swimming training significantly increased total GLUT4 content by ~66% in triceps muscle of rats that did not receive fructose or maltodextrin solutions (WT vs. WU; $P < 0.01$). However, this training-induced increase in GLUT4 expression was significantly attenuated by fructose consumption (FT vs. FU; $p > 0.05$). FT rats experienced only a ~7% increase in GLUT4 content relative to FU. Of note, maltodextrin feeding also suppressed the exercise-induced increase in total GLUT4 such that training induced only a ~6.6% increase in GLUT4 content in maltodextrin fed rats (MT vs. MU; $p > 0.05$). Interestingly, no change in total GLUT4 was noted as a result of fructose or maltodextrin feeding in untrained rats (FU vs. WU and MU vs. WU, respectively; $p > 0.05$). These results show that excessive consumption of both fructose and maltodextrin solutions counteract the exercise-induced adaptive response in GLUT4 expression in rat triceps muscle.

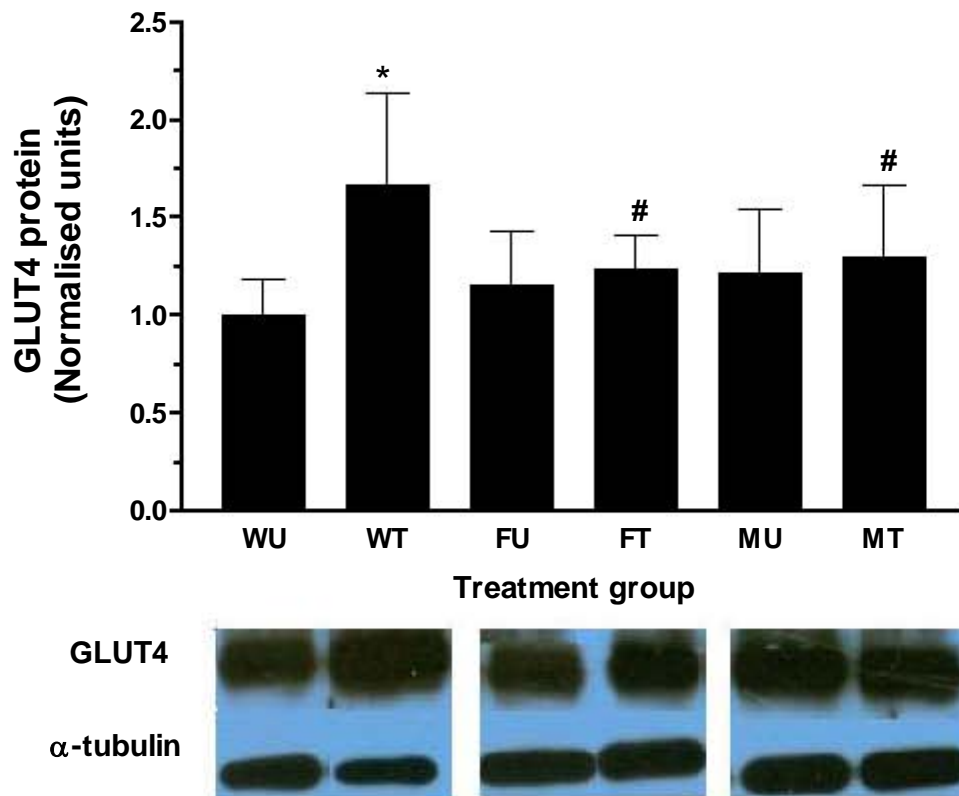


Figure 4.3: Fructose and maltodextrin consumption decrease the exercise-induced upregulation of GLUT4 in skeletal muscle.

Total cellular GLUT4 content was determined in isolated triceps muscle from all treatment groups using the protocol described in section 3.8. Signals for GLUT4 in the histogram were normalised to signals for α -tubulin. Results are presented normalised to WU. Shown are representative GLUT4 and α -tubulin blots. * $P < 0.001$ vs. WU, # $P < 0.001$ vs. WT. Data are presented as mean \pm SD. $n=5$

4.5. MEF2A binding and Histone H3 acetylation at the MEF2 binding site on the *Glut4* gene.

As discussed in section 2.5.5, MEF2A binding to its binding site on the *Glut4* gene is essential for expression of GLUT4 and previous studies have shown that the exercise-induced increase in GLUT4 expression is associated with increased MEF2A binding to the *Glut4* gene promoter which in turn is preceded by increased histone H3 acetylation at the MEF2 binding domain. (give references). To assess whether fructose exerts its modulatory effects on GLUT4 expression under both rest and exercise by altering MEF2A binding activity and histone H3 acetylation at the region surrounding the MEF2 cis element, ChIP assays were performed on skeletal muscle using antibodies directed against (a) MEF2A and (b) Acetyl histone H3. Immunoprecipitated chromatin was amplified using primers that amplify a 350bp section of the *Glut4* promoter containing the MEF2 binding site as described in section 3.10.

As expected, exercise increased bound MEF2A significantly (~54%) compared to untrained rats (See Figure 4.4; WT vs. WU; $P < 0.001$). In contrast, MEF2A binding was significantly attenuated in trained rats fed fructose or maltodextrin (FT vs. WT and MT vs. WT; $p < 0.05$). There was no significant difference in MEF2A binding between fructose-fed and the untrained controls (FT vs. FU; $P > 0.05$). Similarly, exercise did not significantly increase MEF2A binding in maltodextrin fed rats. (FT vs. FU; $P > 0.05$). Fructose or maltodextrin feeding in untrained rats did not alter MEF2A binding (FU vs. WU and MU vs. WU; $P > 0.05$).

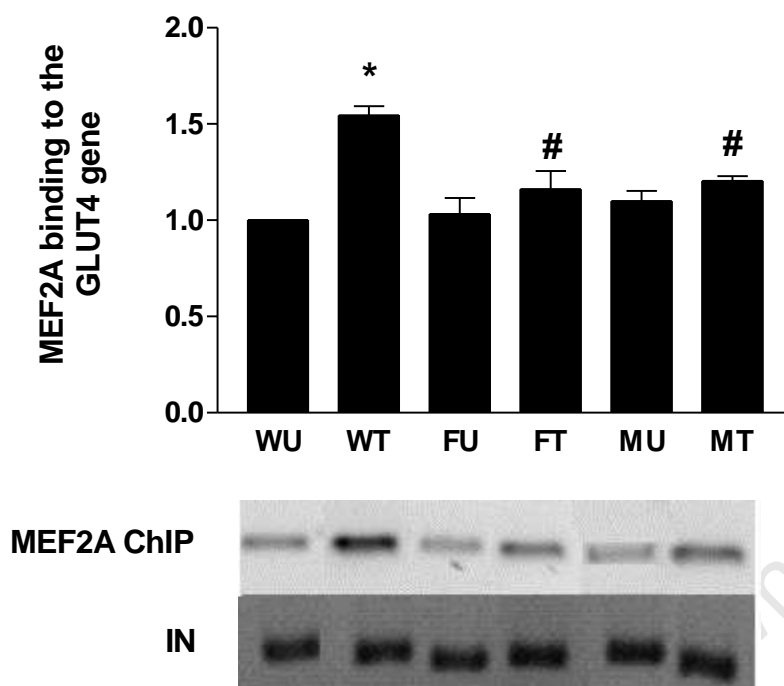


Figure 4.4: Fructose and maltodextrin consumption attenuates the exercise-induced increase in MEF2A binding to the *Glut4* gene

Triceps muscle isolated from the various treatment groups were cross-linked with formaldehyde, lysed and sonicated. ChIP assays were performed using an anti-MEF2A antibody. Co-immunoprecipitated DNA was then amplified by PCR using primers which span the MEF2A binding site on the *Glut4* gene promoter (+ve primers). DNA from an aliquot of crosslinked sample that did not undergo immunoprecipitation (Input-IN) was amplified using +ve primers as described in section 3.10. The histogram represents the ratio of immunoprecipitated DNA (IP) to input DNA (IN) (IP/IN) normalised to WU. n=5. * $P < 0.05$ v/s WU, # $P < 0.05$ v/s WT. Data is presented as means \pm SD.

Acetylation of Histone H3 in the region containing the MEF2 binding domain within the *Glut4* gene promoter followed a similar trend to that of MEF2A binding, as shown in Figure 4.5. Fructose did not decrease histone H3 acetylation in untrained rats compared to water fed rats (FU vs. WU; $P > 0.05$) but attenuated the ~1.5-fold increase in histone H3 acetylation (WT v/s WU; $P < 0.001$) induced by exercise to about ~1.08-fold (FT vs. FU; $P > 0.05$). Maltodextrin feeding did not change histone H3 acetylation in untrained rats (MU vs. WU; $P > 0.05$) but attenuated the increase in histone H3 acetylation triggered by exercise such that trained rats only experienced a ~1.16-fold increase (MT vs. MU; $P > 0.05$) in

contrast to the ~1.5-fold increase in water fed trained rats (WT vs. MT; $P < 0.05$)

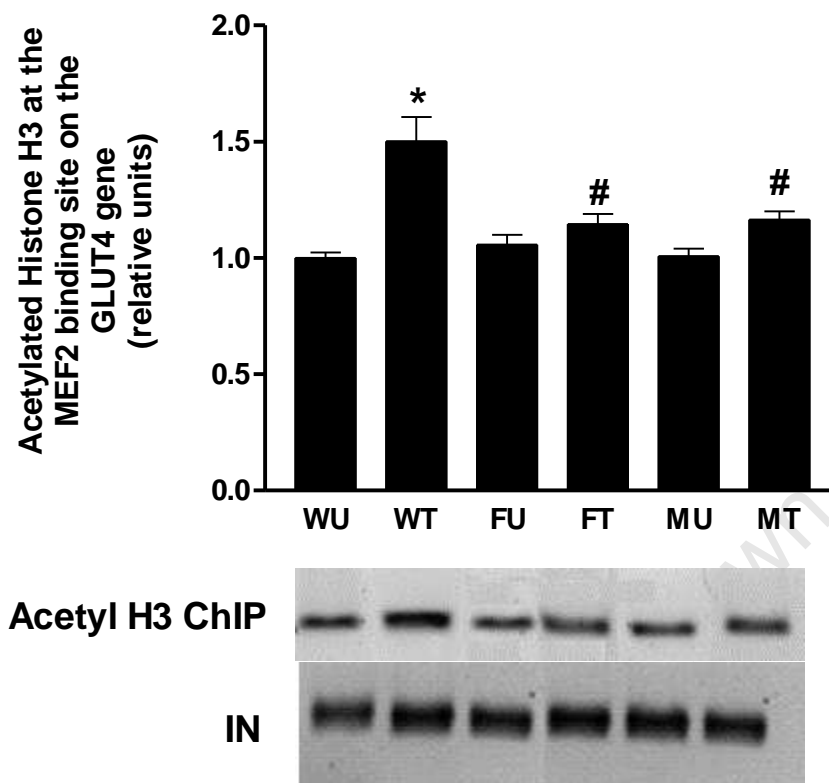


Figure 4.5: Fructose and maltodextrin consumption attenuates the exercise-induced hyperacetylation of histone H3 at the MEF2 binding site on the GLUT4 gene

Triceps muscle was isolated from rats subjected to different treatments and crosslinked with formaldehyde, lysed and sonicated. ChIP assay was performed using an anti Acetyl Histone H3 antibody (Lys 9/14) followed by PCR on the co-immunoprecipitated DNA using primers that amplify the MEF2 binding site on the GLUT4 gene promoter. DNA from an aliquot of crosslinked sample that did not undergo immunoprecipitation (Input-IN) was amplified using +ve primers as described in section 3.10 and used for normalisation. The histogram represents the ratio of signals from IP (Immunoprecipitated)/ IN (Input) normalised to WU. $n=5$, * $P < 0.05$ v/s WU, # $P < 0.05$ v/s WT.

4.6. Nuclease accessibility of the MEF2 binding domain on the *Glut4* gene

After investigating histone acetylation states and MEF2A binding to the MEF2 binding domain we proceeded to examine the accessibility of a 350bp region of the *Glut4* promoter containing the MEF2 binding domain. We measured the rate of digestion of the

above segment by MNase and DNase I in intact nuclei isolated from muscle from the different treatment groups according to the protocol in section 3.9. The rate of digestion, an index of accessibility, was calculated by measuring the area under the curve. (Fig 4.6 and 4.7)

Figures 4.8 and 4.9 respectively show the percentage digestion with respect to time by MNase and DNase I respectively in the untrained state. Figure 4.8 shows that the area under the FU but not the MU curve is significantly lower than the WU curve, indicating that fructose reduces accessibility of the interlinker region within the 350bp segment surrounding the MEF2 binding site. This effect is not seen with maltodextrin. Figure 4.9 represents digestion with DNase I in the untrained state. It is seen that the area under the curve for both FU and MU are significantly lower than that of the WU curve. This indicates that the accessibility of the nucleosomal region is decreased by both fructose and maltodextrin.

Figure 4.10 and 4.11 respectively show the percentage digestion with respect to time by MNase and DNase I in both the trained and untrained states. Figure 4.10 shows that the area under the WT curve is significantly higher than the area under the WU curve, indicating that training increases accessibility of the interlinker region within the 350bp segment surrounding the MEF2 binding site. This increase is abolished by fructose and maltodextrin, as shown by the lack of difference among FT, MT vs. WU. The area under the FT curve is significantly higher than the area under the FU curve, indicating that exercise tends to improve accessibility of the interlinker region although to a lesser extent than that seen in the absence of fructose (WT). This improvement in accessibility due to exercise is not seen in the MT group.

Figure 4.11 shows that the effect of DNase I digestion exhibits a similar trend, indicating that exercise increases the accessibility of the nucleosomal region of the segment investigated but fructose and maltodextrin feeding abolished the exercise effect. However, the area under the FT and MT curve are respectively higher than that of the FU and MU

curves. This indicates that exercise tends to improve accessibility of the nucleosomal region in animals fed these carbohydrates although to a lesser extent than that seen in the absence of fructose and maltodextrin.

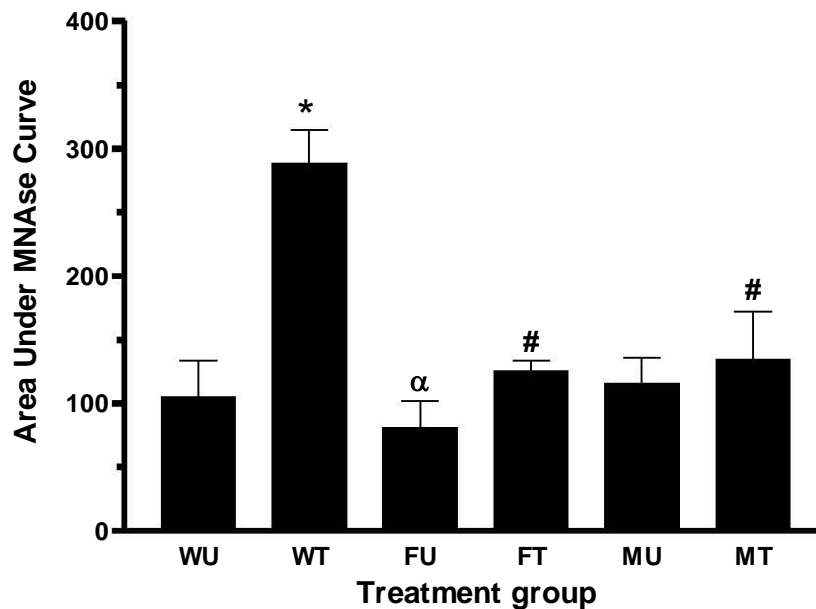


Fig 4.6: Area under the MNase curve for all treatment groups.

* $P < 0.05$ v/s WU, # $P < 0.05$ v/s WT and α $P < 0.05$ v/s WU and MU. (n=5 per group).

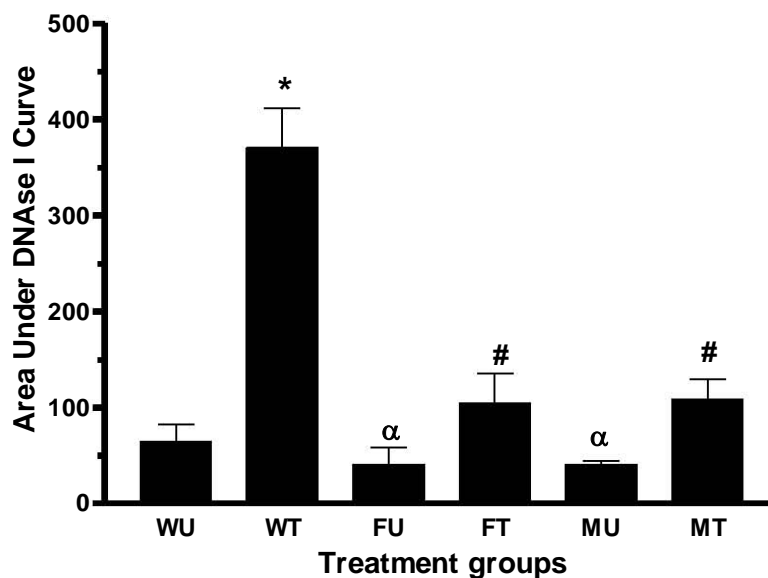


Fig 4.7: Area under the DNase I curve for all treatment groups.

* $P < 0.05$ v/s WU, # $P < 0.05$ v/s WT and α $P < 0.05$ v/s WU. (n=5 per group).

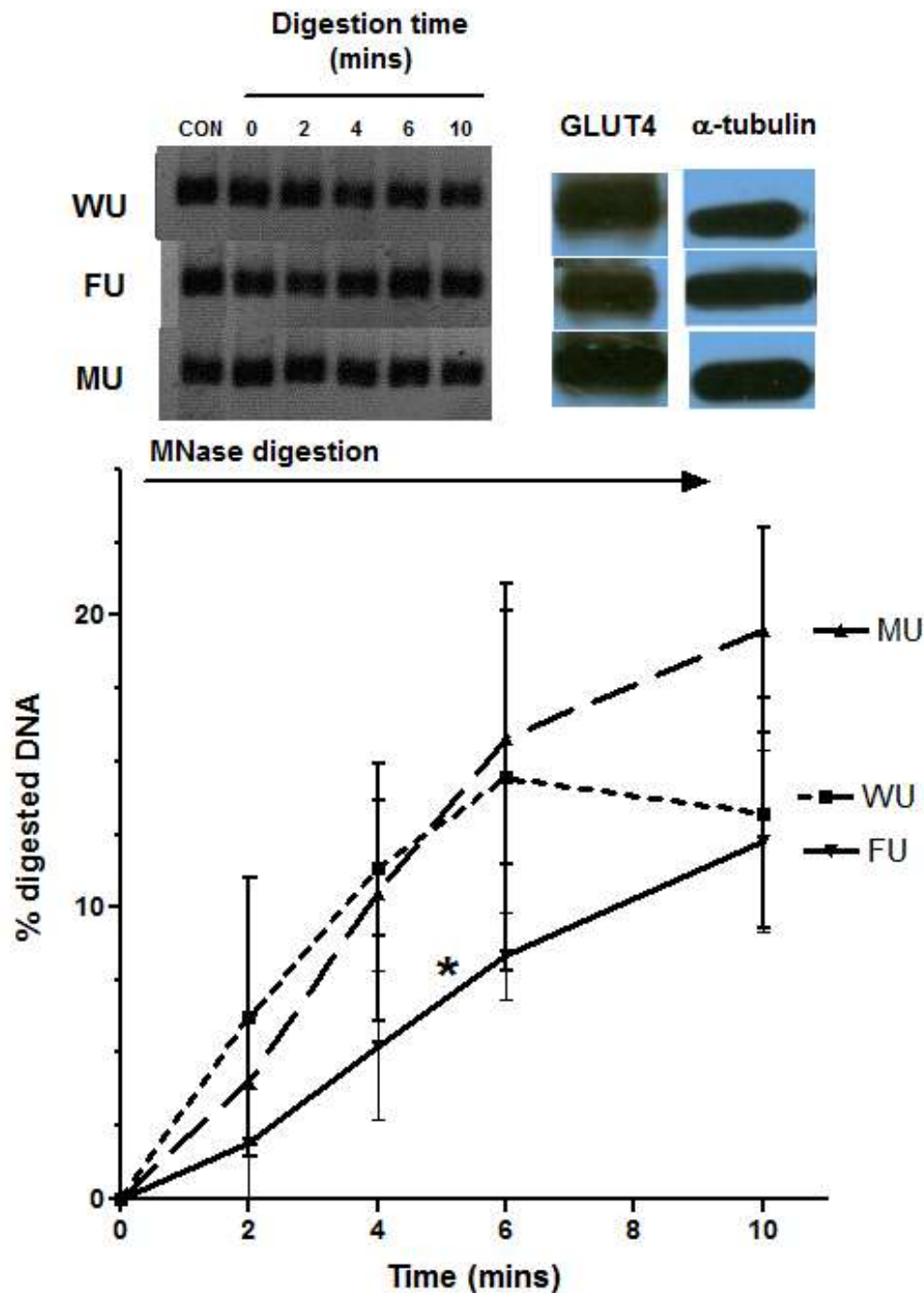


Figure 4.8: Accessibility of the 350bp region containing the MEF2A binding site on the *Glut4* promoter to MNase in untrained rats.

Nuclei isolated from triceps muscle from different treatment groups were incubated with MNase for the times indicated. Control represents a reaction without added MNase. The amount of DNA present at each time was determined by PCR. The amount of DNA present at each time was normalized to that at time 0 and plotted against time to compare degradation rates. * $P < 0.05$ vs. WU. (n=5 per group).

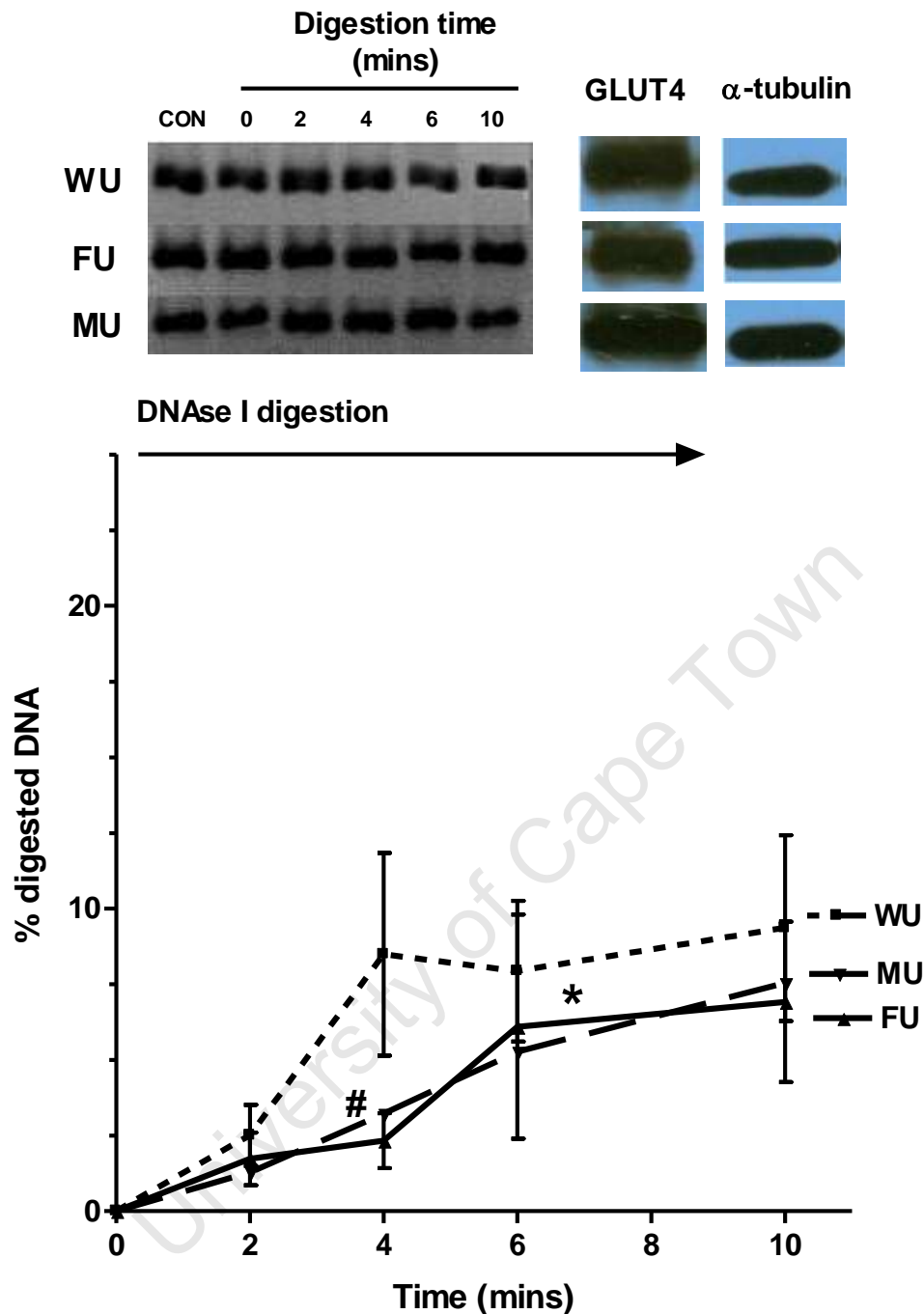


Figure 4.9: Accessibility of the 350bp region containing the MEF2A binding site on the *Glut4* promoter to DNase I in untrained rats.

Nuclei isolated from triceps muscle from different treatment groups were incubated with DNase I for the times indicated. Control represents a reaction without added DNase. The amount of DNA present at each time was determined by PCR. The amount of DNA present at each time was normalized to that at time 0 and plotted against time to compare degradation rates. * $P < 0.05$ vs. WU. (n=5 per group).

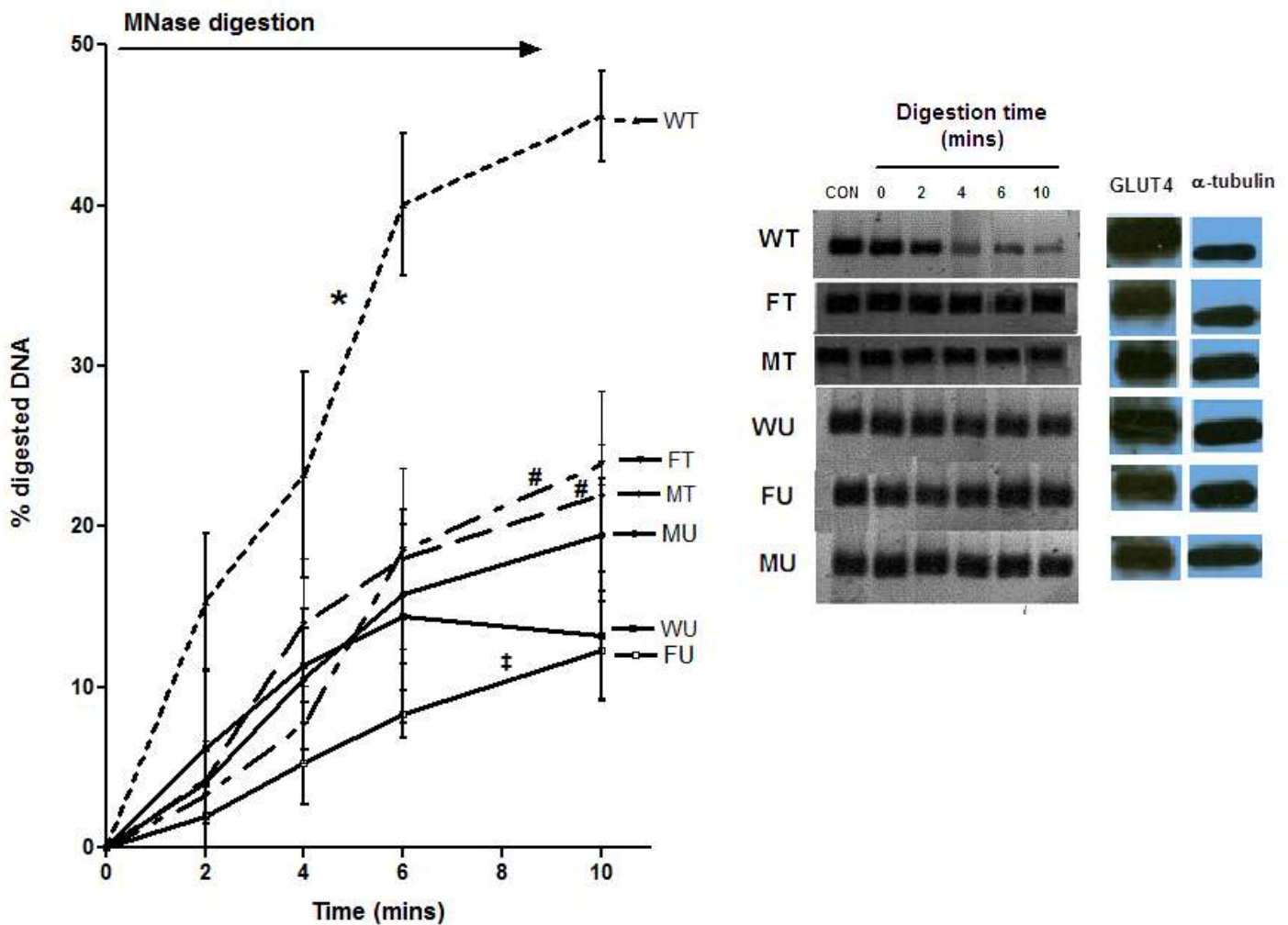


Figure 4.10: Accessibility of the 350bp region containing the MEF2A binding site on the *Glut4* promoter to MNase in trained and untrained rats.

Nuclei isolated from triceps muscle from different treatment groups were incubated with DNase I for the times indicated. Control represents a reaction without added DNase. The amount of DNA present at each time was determined by PCR. The amount of DNA present at each time was normalized to that at time 0 and plotted against time to compare degradation rates. * $P < 0.05$ vs. WU. # $P < 0.05$ vs. WT. ‡ $P < 0.05$ vs. WU. (n=5 per group).

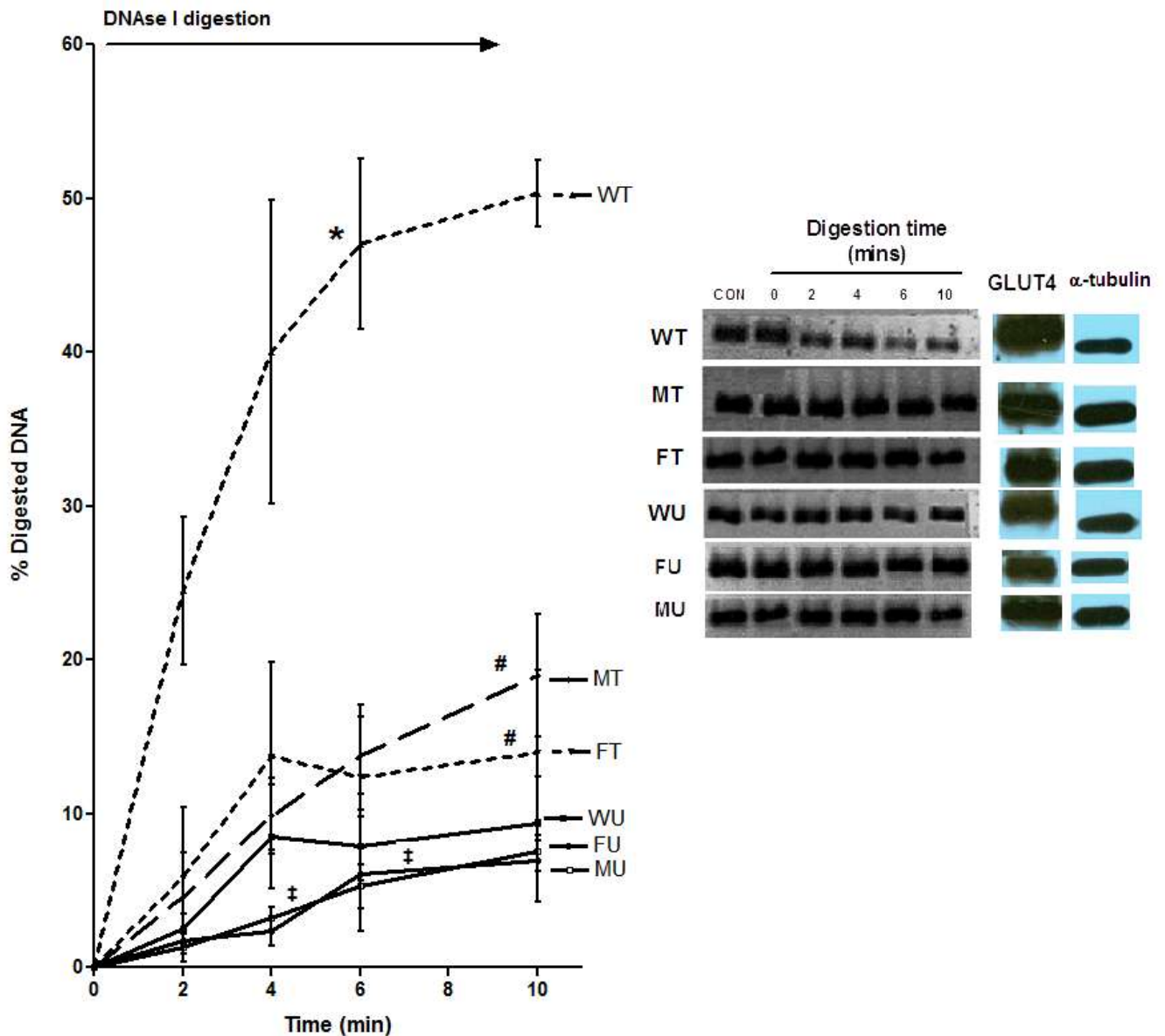


Figure 4.11: Accessibility of the 350bp region containing the MEF2A binding site on the *Glut4* promoter to DNase I in trained and untrained rats.

Nuclei isolated from triceps muscle from different treatment groups were incubated with DNase I for the times indicated. Control represents a reaction without added DNase. The amount of DNA present at each time was determined by PCR. The amount of DNA present at each time was normalized to that at time 0 and plotted against time to compare degradation rates. * $P < 0.05$ vs. WU. # $P < 0.05$ vs. WT. ‡ $P < 0.05$ vs. WU. (n=5 per group).

CHAPTER FIVE

DISCUSSION

5.1. Summary of main findings

The present study was conducted to characterise the effect of fructose consumption on skeletal muscle GLUT4 expression in trained and untrained rats and to identify the mechanisms involved at the transcriptional level. The main findings of this present study were as follows:

(a) Consumption of a 10% fructose drink for 13 days did not decrease GLUT4 levels in the untrained state but significantly attenuated the GLUT4 increase (~66%) that occurred due to swimming. A 10% maltodextrin drink also blunted the GLUT4 increase.

(b) Exercise increased the accessibility of both the interlinker and nucleosomal regions of the *Glut4* promoter containing the MEF2 binding site. Fructose or maltodextrin consumption attenuates this effect.

(c) Exercise increased histone H3 acetylation in the region of the *Glut4* promoter containing the MEF2 binding site and increased MEF2A binding. Fructose or maltodextrin consumption attenuated this effect.

(d) The above effects occurred in the absence of any significant change in fasting plasma glucose and insulin.

5.2. GLUT4 expression is unaffected by fructose in the untrained state.

A significant finding of this study is that the 13-day 10% fructose feeding protocol did not decrease GLUT4 content in skeletal. This finding is in contrast with findings by Meeprom et al. (66) who showed that rats fed a 63% fructose diet for a period of 8 weeks showed a 56% decrease in GLUT4 expression. The study by Meeprom et al. (66) used an

extremely high fructose dose for a long period of time to induce features of the metabolic syndrome (e.g. insulin resistance) in the animals and therefore these features are what could have influenced the GLUT4 expression in these rats. Indeed, there is evidence that skeletal muscle GLUT4 expression is decreased in the metabolic syndrome (46) and is a mechanism by which glucose homeostasis is impaired. Inflammatory cytokines produced by the adipose tissue, such as TNF- α (tumour necrosis factor- α) and interleukin-6 (IL-6) have been found to reduce GLUT4 expression (85; 99) in skeletal muscle, thus providing a link between metabolic syndrome and decreased GLUT4. Our study assessed the effect of feeding rats a fructose solution that had a caloric density comparable to that found in popular fructose-containing beverages (~0.4 kcal/ ml) for a short duration (13 days) in order to examine the initial effects of fructose before the development of the metabolic syndrome. The absence of high fasting insulin, glucose levels and free fatty acids in all experimental groups in our experiment shows the absence of the metabolic syndrome (Table 4.2). The absence of a decrease in GLUT4 content in the present study is consistent with the idea that features of the metabolic syndrome may influence GLUT4 expression. However, it must also be noted that some long-term high fructose dose studies, with features of the metabolic syndrome (47), have also failed to show any effect on GLUT4 expression (45; 47). Clearly further research on the relationships between fructose consumption, the metabolic syndrome and GLUT4 expression is still warranted.

Health researchers are increasingly recognising the dose-dependency in the metabolic effects of fructose. For example, at doses lower than 100g/ day no evidence of impaired insulin sensitivity was seen in humans (48). However, an intake of >250g/ day was reported to cause insulin resistance and doses between 100-250g was without important effect (42). In light of these dose-dependent effects and because animal studies often use doses of fructose that are far in excess of what humans would realistically consume, conclusions drawn from such studies may be misleading about the health aspects of fructose consumption (48).

5.3. Fructose consumption attenuates exercise-induced GLUT4 expression: a phenomenon related to epigenetic changes

Another significant finding of our study is that while fructose consumption was unable to decrease GLUT4 expression in untrained rats, it attenuated the exercise-induced increase in GLUT4 protein. The 5-day swimming protocol used in our study was able to increase GLUT4 protein content in triceps muscle by ~66%. This increase in GLUT4 by exercise is consistent with other studies on rats (55; 59; 95). However, we report for the first time that this adaptive response to exercise is severely attenuated by fructose or maltodextrin consumption (Figure 4.3). To investigate the mechanisms that might be responsible for this effect of fructose and maltodextrin, we examined the major events that control GLUT4 transcription. As reviewed in section 2.3, the expression of GLUT4 is dependent on hyper-acetylation of histones at the MEF2 binding domain on the *Glut4* gene and increased MEF2A binding. Our data show that the exercise-induced increase in GLUT4 was accompanied by increases in MEF2A binding and histone H3 hyper-acetylation at the MEF2 binding site on the *Glut4* gene, consistent with earlier studies (55; 59; 95). However, both fructose and maltodextrin consumption markedly reduced MEF2A binding and histone H3 acetylation at the MEF2 binding site on the *Glut4* gene, suggesting that the effects of these carbohydrates on exercise-induced GLUT4 expression might take place at the level of transcription. The finding also provides an example of modulation of exercise-induced adaptations by nutrients. This is a form of gene regulation called epigenetic modulation which involves modifications in DNA (e.g. methylation) and histone proteins (e.g. acetylation and methylation) without any change in DNA sequence.

Indeed, it is recognised that several nutrients, including fructose, can exert regulatory effects on gene expression by affecting epigenetic modifications (77). The ability of fructose to induce epigenetic changes in certain genes is known. For instance, consumption of high amounts of fructose increases the expression of the intestinal fructose/ glucose transporter (GLUT5) by increasing trimethylation of histone H3 at Lysine 4 (H3 K4) at the *Glut5*

promoter (110). Additionally, high fructose consumption also increases the expression of *Cabp2* (Calcium binding protein 2) and *Gcgr* (Glucagon receptor) by inducing trimethylation of H3K4 at their promoter.

At this stage it is not known what exercise signal upstream of histone H3 acetylation and MEF2A binding is modulated by fructose and maltodextrin feeding. A possible mechanism by which high fructose consumption may attenuate exercise-induced GLUT4 expression is given in Figure 5.1. Muscle contraction during exercise is known to increase the activity of the enzyme endothelial nitric oxide synthase (eNOS) thereby increasing the production of nitric oxide (NO) which is an important activator of AMPK in skeletal muscle (46). It has been shown that eNOS activity is required for the AMPK-induced increase in GLUT4 in exercise (28). The importance of eNOS comes from the observation that AICAR, an AMPK activator and exercise-mimetic is unable to increase GLUT4 mRNA in both L6 myotubes and *in vivo plantaris* muscle when eNOS is inhibited. NO activates AMPK either directly or indirectly by activating AMPK kinase (46). High fructose consumption is known to stimulate the production of uric acid (Figure 2.1) which increase serum uric acid level (75). Uric acid is an important inhibitor of eNOS (113). Based on this information, it is possible that high fructose consumption attenuates the exercise-induced activation AMPK and its downstream effects on GLUT4 expression through uric-acid. Further studies are needed to test this hypothesis. It is interesting to note that fructose and maltodextrin consumption did not completely block exercise-induced increases in GLUT4 protein, histone H3 acetylation and MEF2A binding to the *Glut4* promoter. Indeed, modest increases in all these variables were seen in response to exercise, suggesting that other pathways may be involved.

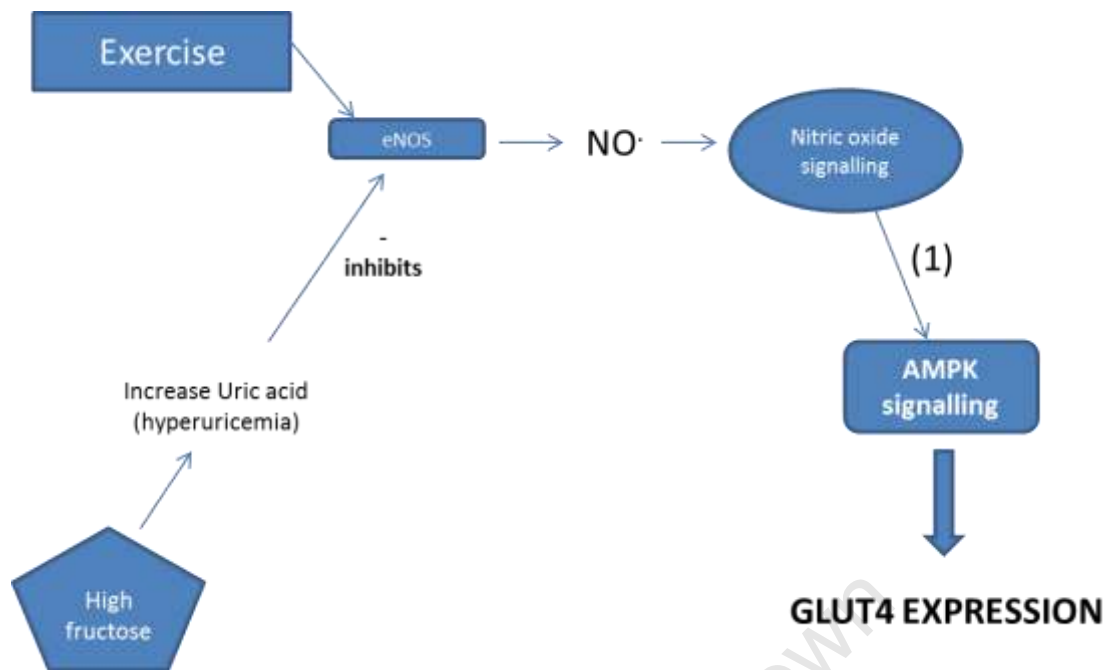


Figure 5.1: Proposed mechanism for the attenuating effect of fructose on exercise-induced GLUT4 expression

There is evidence that nutritional status, particularly carbohydrate availability has a regulatory effect on the post-exercise increase in GLUT4 (26; 83). For instance, there is evidence that muscle glycogen concentration, determined by availability of dietary carbohydrate, regulates the induction of GLUT4 expression after exercise. Exercising with low muscle glycogen level leads to a greater transcriptional activation of GLUT4 compared to exercising when muscle glycogen content is normal (26; 83). In our study, caloric intake in both carbohydrate-fed groups was higher than controls. Maltodextrin and fructose-fed trained rats consumed similar amounts of 10% carbohydrate solution and chow (Figures 4.1 and 4.2) and therefore had a similar nutritional status in terms of caloric intake.

To the best of our knowledge, there has been no study that investigated the nutritional modulation of the GLUT4 adaptive response to exercise at the level of epigenetic regulation and, furthermore, data on the effect of fructose consumption on exercise-responsive genes is lacking (12; 33). Our finding that fructose and maltodextrin modulate the GLUT4 adaptive response to exercise is important starting point in these investigations.

5.4. Nuclease accessibility of the *Glut4* promoter region.

This thesis describes for the first time the use of a nuclease digestion assay to measure the accessibility of a 350bp segment of the *Glut4* gene promoter containing the MEF2 binding site. The assay has been described previously for the analysis of a number of plant genes where it was found that increased accessibility of chromatin to MNase and DNase I correlate with chromatin regions with highly acetylated histones and high transcript abundance (8; 73; 94; 95). These observations indicate increased accessibility of transcription factors to their binding domains in these regions of chromatin and therefore validate the nuclease digestion assay as a measurement of accessibility to transcription factors. Indeed, probing chromatin structure with nucleases in intact nuclei is a well-established method for measuring the accessibility of DNA to transcription factors and overall competency for transcription (82).

The validity of nuclease digestion data is highly dependent upon the nuclear preparation and therefore requires the availability of intact nuclei (112). Therefore, a nuclei integrity assay was also performed as described in Section 3.9 in order to validate our nuclei extraction protocol in producing intact nuclei. The integrity assay measured the release of nucleic acids from isolated nuclei incubated in increasing NaCl concentrations. In the absence of NaCl, only a small amount of nuclei acid was released from the nucleus (13.2%) which is within acceptable limits (11). Our results show that our nuclei extraction protocol did not compromise the integrity of isolated nuclei.

Both MNase and DNase I were used in our study and, therefore, the digestion data reflects accessibility of DNA at both the inter-linker and nucleosomal regions of the segment of DNA that house the MEF2 binding domain on the *Glut4* gene promoter (92). In untrained rats, the DNase I data show that accessibility of the nucleosomal region is decreased by both fructose and maltodextrin while the MNase data show that accessibility of inter-linker region is decreased by fructose and not by maltodextrin; indicating that effects of

Fructose and Maltodextrin are not identical under this condition. As discussed earlier, GLUT4 protein levels, histone H3 acetylation and MEF2A binding were not changed by Fructose or Maltodextrin in untrained rats. This divergence between GLUT4 expression and epigenetic changes may be due to the fact that the epigenetic changes were not of sufficient magnitude to alter gene expression. This suggests that small changes in chromatin structure around the MEF2A binding domain do not affect basal GLUT4 expression. A similar phenomenon was recently demonstrated by Miller-Jensen et al. (67) in HIV genes regulated by the transcription factor nuclear factor- κ B RelA/p65, whereby transcription occurred in spite of small decreases in chromatin accessibility. The low levels of accessibility, histone H3 acetylation and MEF2A binding noted in untrained rats correlates with the presence of basal transcription of GLUT4.

Exercise training led to a significant increase in the rate of digestion by both MNase (~2.75-fold) and DNase I (~5.75-fold) in rats that received water compared to untrained controls. However, a significant level of attenuation in both parameters was noted when rats were fed fructose and maltodextrin. Our results thus show that the exercise-mediated increase in histone H3 acetylation and MEF2A binding are accompanied by increased accessibility to both MNase and DNase I digestion. Therefore, exercise remodels chromatin at both the nucleosomal and interlinker region such that chromatin adopts a more open conformation, explaining these increase nuclease digestion rates. Accessibility correlates well with changes in histone H3 acetylation, MEF2A binding and GLUT4 expression when there are clear differences.

Thus far, assessment of chromatin structure had been made indirectly using the ChIP assay to detect MEF2A binding to its binding domain and accompanying changes in histone H3 acetylation (8; 73; 94; 95). The nuclease digestion assay that we have described provides a more direct measure of chromatin accessibility. Its use, together with ChIP is highly recommended.

5.5. Fasting Serum biochemistry parameters

An analysis of fasting serum glucose and insulin was carried out because these are measures of insulin resistance. No change in serum glucose and insulin parameters was seen because the animals were apparently healthy. However, changes were already taking place at the molecular level as shown by our results. For example, there was a decrease in accessibility of the *Glut4* gene promoter in response to fructose feeding. Le et al. (42) have also shown that 4 weeks of fructose feeding in humans did not decrease insulin sensitivity but reported decreases in the level of key transcription factors like PGC-1 α . Collectively, these findings suggest that changes at the molecular level precede changes at the systemic level. We recommend that more effort be put in developing assays that provide accurate early warning signs at the molecular level for fructose-induced insulin resistance.

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