Involvement of p300 in caffeine-induced hyper-acetylation of histones at the MEF2 binding domain on the *Glut4* gene.

By

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DECLARATION

I, Kovin Ashley Chetty, hereby declare that the work on which this dissertation is based on 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. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any matter whatsoever.

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ARTICLES IN INTERNATIONAL PEER-REVIEWD JOURNALS

1. James A. H. Smith, Tertius A. Kohn, **Kovin A. Chetty** and Edward O. Ojuka. CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the *Glut4* gene. *American Journal of Physiology Endocrinology & Metabolism*. **295:** 698-704, 2008

2. Emmanuel Mukwevho, **Kovin A Chetty**, Veeraj Goyaram, Dimakatso Gumede, Jerad Li, Edward O Ojuka. Regulation of GLUT4 expression by Nuclear Respiratory Factor-1: Evidence for a transcriptional cascade involving Myocyte Enhancer Factor-2A. In Review, submitted to *Biochimica et Biophysica Acta* in January 2012.

PRESENTATIONS AT CONFERENCES

1. Cell Biology Mini Symposium 2009. **Exercise & Type II Diabetes : How Chromatin Remodeling May Influence Glucose Transport**. June 2009, University of Cape Town

2. 39th Physiological Society of South Africa (PSSA) Annual Conference: **Caffeine Induces Histone Hyper-acetylation at the MEF2A binding site on the *Glut4* gene via p300 up-regulation**. August 2011, University of the Western Cape (UWC), Cape Town

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LIST OF ABBREVIATIONS

AICAR	5-Aminoimadazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
Ara	adenosine-9-β-D-arabino-furanoside
ATP	Adenosine triphosphate
bp	Base pair
bHLH	Basic-helix-loop-helix
BSA	Bovine serum albumin
C2C12	Mouse skeletal muscle cell line
C2C7	Mouse skeletal muscle cell line
Caf	5mM caffeine
Caf+Cur40	5mM caffeine+40μM curcumin
Caf+Cur25	5mM caffeine+25μM curcumin
CaMK	Calcium/calmodulin dependent kinase
CaMKK	CaMK Kinase
CAT	Chloramphenicol acetyltransferase

CBP	CREB binding protein
CH	Cysteine-Histidine
ChIP	Chromatin immunoprecipitation
Con	Control
COS 1	Monkey kidney cell line
Cur	Curcumin
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxy-Ribonucleic Acid
dNTP	Dinucleotide Triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDL	Extensor digitorium longus
EMSA	Electrophoretic mobility shift assay
FCS	Foetal calf serum
GEF	GLUT4 Enhancer Factor
GLUT4	Glucose Transporter 4
GST	Glutathione-S-transferase
GTM	General transcription machinery

HAT	Histone acetyltransferase
HCl	Hydrochloric acid
HDAC	Histone deacetylases
HeLa	Human breast cancer cell line
HIF-1	Hypoxia-induced factor-1
hSA	Human skeletal actin gene
HTLV-1	Human lymphotropic virus type 1
HS	Horse serum
IgG	Immunoglobulin G
INP	Input
IP	Immunoprecipitate
kDA	KiloDalton
KN-93	CaMK II inhibitor
Lys	Lysine
LSB	Laemmeli Sample Buffer
MCK	Muscle Creatine Kinase
MEF2	Myocyte Enhancer Factor 2
$MgCl_2$	Magnesium Chloride
MHC	Myosin Heavy Chain

mRNA	messenger Ribonucleic Acid
NaBu	Sodium Butyrate
NaCl	Sodium Chloride
NDFM	Non-Fat Dry Milk
NaF	Sodium Fluoride
NFAT	Nuclear Factor of Activated T-cells
$NaHCO_3$	Sodium Hydrogen Carbonate
$Na_2P_2O_7$	Sodium Pyrophosphate
Na_3VO_4	Sodium Ortho-Vanadate
PBS	Phosphate Buffered Saline
PC12	Rat adrenal medulla cell line
PC3	Human Prostrate Cancer cell line
PCR	Polymerase Chain Reaction
PIC	Pre-Initiation Complex
PVDF	Polyvinylidene Difluoride
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBP	Tata Binding Protein

TBST	Tris Buffered Saline containing 0.1% Tween-20
TR	Thyroid Hormone Receptor
TRE	Thyroid Hormone Response Element
wt	Wild Type
ZMP	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-51-monophosphate

ABSTRACT

The expression of glucose transporter 4 (GLUT4), the major glucose transporter in skeletal muscle is increased by caffeine and regulated by myocyte enhance factor-2A (MEF2A) and other transcription factors (12; 22; 38; 58; 91). Previous studies have shown that caffeine-induced GLUT4 expression is associated with hyperacetylation of histones surrounding the MEF2A binding site on the *Glut4* gene (101) but the histone acetyl-transferases (HATs) involved are unknown. The aim of this study was to determine whether p300, a HAT known to interact with MEF2 transcription factors, is involved in acetylating histones at the Glut4 gene.

To investigate this, differentiated C2C12 myotubes were incubated with 5mM caffeine ± 25μM or 40μM curcumin (a potent inhibitor of p300 HAT activity). Chromatin Immunoprecipitation (ChIP) assays were used to measure the levels of acetylated histone H3 (Lys $^{9/14}$) and the amount of MEF2A that bound to the *Glut4* promoter. Western Blotting was conducted to determine total acetyl H3, p300 and GLUT4 contents.

Caffeine treatment significantly increased total acetylated H3 and p300 contents by 33% and 12% respectively (P<0.01 vs. control). Addition of curcumin causes a dose-dependent decrease in caffeine-induced total acetylation and p300 content: 40μM curcumin significantly reduced the increase in total acetyl-H3 levels by 47% and reduced the rise in p300 by 43% (p<0.01 Vs Caffeine) whereas the 25μM curcumin treatment resulted in 27% and a 20% reduction in total acetyl histone H3 and p300 contents, respectively, compared to the caffeine-treated group (P<0.01). At the MEF2A binding site on the *Glut4* gene caffeine treatment caused a 30% increase in acetylation of histone H3 and a 48% increase in MEF2A binding (P <0.001 vs. Control) but curcumin attenuated both caffeine-induced hyperacetylation of histone H3 and MEF2A binding in a dose- dependent manner: The 40μM treatment significantly reduced caffeine-induced acetylation of histones by 41 % (P< 0.01vs. Caffeine) and MEF2A binding to the *Glut4* gene by 48%; p<0.01 Vs. Caffeine) whereas the 25μM curcumin treatment caused a 28% reduction in acetylated H3 (P< 0.01 vs. Caffeine) and a 30%

reduction in MEF2A binding at the *Glut4* gene. GLUT4 expression was 16% increased by caffeine treatment (vs. control) but Curcumin (25μM) decreased caffeine-induced up-regulation of GLUT4 by 22%. These results show that p300 serves an integral role in *Glut4* gene expression in C2C12 myotubes and is responsible for acetylating histones at the MEF2A binding on the Glut4 gene.

CHAPTER ONE

BACKGROUND, AIMS AND JUSTIFICATION OF THE STUDY

1.1 Introduction

Regular physical activity is recommended to patients with type II diabetes, or those predisposed to the disease because it increases glucose disposal capacity via increased transcription of the insulin-sensitive glucose transporter 4 (GLUT4) in skeletal muscle (54; 71). Exercise-induced increase in *Glut4* transcription is due to increased binding of transcription factors, including GLUT4 enhancer factor (GEF) and myocyte enhancer factor 2 (MEF2), to their binding domains on the *Glut4* gene (91). Although the signalling mechanisms that are responsible for increased interaction of these transcription factors with the *Glut4* gene have not been fully elucidated, several lines of evidence suggest that fluctuations in cytosolic Ca^{2+} levels during contraction, leading to activation of Calcium/Calmodulin dependent protein kinases (CaMKs), play an important role : For example, Smith et al. (140), showed that inhibition of CaMK II activity during

intermittent high-intensity swimming prevents MEF2A binding to the *Glut4* gene and prevents *Glut4* transcription. A similar conclusion was drawn by Mukwevho et al. (101) ; they showed that treatment of C2C12 myotubes with caffeine, an agent that increases cytosolic Ca^{2+}, caused a two-fold increase in the amount of MEF2A that bound to the *Glut4* gene when compared to controls. Moreover, when CaMK II activity was inhibited using KN-93, this increase was abolished. Therefore, CaMK II activity seems to be a major signaling effector in MEF2-mediated GLUT4 upregulation.

Increased binding of transcription factors to their respective domains on gene promoters depends heavily on acetylation of histones that neighbour these binding domains (10; 40; 42). Hyperacetylation of histones relaxes chromatin structure and results in improved accessibility of transcription factor binding sites. Histone acetyltransferases (HATs) are the enzymes that catalyse histone acetylation. Hypoacetylation of histones, on the other hand, results in chromatin condensation and inactivation of gene transcription. Hypoacetylation of histones is catylzed by enzymes called histone deacetylases (HDACs), which remove acetyl groups from histone tails.

The activities of these opposing enzymes regulate chromatin structure and are regulated dynamically according to transcriptional needs (40; 42; 47; 143; 144).

Scicchitano et al. (132) showed that when CaMK is inhibited, there is a reduction in the level of acetylation of histone H4 at the MEF2 binding site on Muscle Creatine Kinase (MCK) promoter in L6 myotubes. This hypoacetylation of histones resulted in lower levels of MEF2A that bound to the *MCK* promoter and lower MCK transcription. On the other hand, Smith et al. (140), showed that activation of CaMK II by exercise increased acetylation of histones in the vicinity of the MEF2 binding domain and MEF2A binding to the *Glut4* promoter; resulting in GLUT4 up-regulation. Hyperacetylation was also reported when CaMK II was activated by caffeine in C2C12 myotubes (101). Collectively, these observations indicate that there is a correlation between CaMK II activation, histone acetylation and gene expression.

There are many reports which show that MEF2 binding to genes and its transcriptional activity is controlled by chromatin remodeling enzymes. In the inactive state MEF2 is repressively bound to HDACs, which keep promoter regions condensed

and inactive (40; 42; 92; 93; 143). When CaMK II is activated, MEF2 dissociates from HDACs and can then associate with HATs. One such HAT is p300, a coactivator of transcription with multiple roles in eukaryotic transcription (42; 81; 93; 136). p300 is endowed with a potent histone acetyltransferase (HAT) domain that acetylates MEF2 on seven different lysines along the protein to enhance its DNA binding activity (4; 83; 115). Furthermore, being a large protein (300 KDa), it acts as a scaffold allowing many smaller proteins with discrete functions--such as the components of the general transcription machinery including TAF250, TATA-binding protein (TBP) and RNA polymerase II to bind and collectively induce transcription (69; 95; 154; 159).

1.2. Aims

Although previous studies have shown that CaMK II activation by caffeine or exercise increases the level of acetyl H3 in the region surrounding the MEF2A binding site on the *Glut4* gene (101; 140), the acetyltransferase(s) responsible for this effect has/have not been identified. As p300 is a potent HAT (9; 108) that influences MEF2 transcriptional activity in muscle, neurons and T-lymphocytes (4; 83; 87; 155; 160), this study was designed to investigate whether p300 plays a role in: a) acetylating histones that neighbour the MEF2 binding domain of the *Glut4* promoter and b) GLUT4 expression, in response to caffeine in C2C12 myotubes.

1.3. Justification of the study

Physical exercise serves as a therapeutic means of lowering blood glucose in people with type II diabetes or hyperglycaemia (57). The elucidation of molecular mechanisms and pathways by which exercise upregulates GLUT4 expression in skeletal muscle thus becomes vital as it might reveal the pharmaceutical targets that could be developed in

order to target these mechanisms and ultimately treat hyperglycaemia related sequelae. As chromatin remodeling enzymes have crucial roles in regulating gene expression, and have been found to be altered in disease states such as diabetes (121), the identification of the HAT(s) involved at the *Glut4* gene would contribute to a better understanding of how exercise regulates GLUT4 expression with respect to accessibility of the *Glut4* promoter. This may subsequently serve as a target for pharmaceutical therapies in treating Type II diabetes.

CHAPTER TWO

LITERATURE REVIEW

2.1. Synopsis

This chapter is devoted to providing the relevant background information for a understanding of the regulation of GLUT4 expression in skeletal muscle. It provides scientific evidence which show that upregulation of GLUT4 by exercise or other means improves glucose homeostasis. This chapter also describes the transcriptional factors and the major enzymes that regulate GLUT4 expression, emphasizing the roles of myocyte enhancer factors, the histone modifying enzyme p300, and Ca^{2+}/CaMK. The chapter is divided under the following topics:

- The anti-diabetic effects of exercise : evidence that GLUT4 upregulation is beneficial in hyperglycaemic conditions.
- Regulation of the *Glut4* gene by the myocyte enhancer factor 2 family of transcription factors.

- Regulation of MEF2 transcriptional activity via CaMKII and AMPK-dependent chromatin remodeling.

- The structure, function and regulation of p300.

- Evidence showing that p300 interacts with MEF2 for MEF2-dependent gene expression.

- Use of curcumin as an inhibitor of p300 .

2.2. The anti-diabetic effects of exercise.

Type II diabetes is defined as a lifestyle-related disease where there is elevated fasting blood glucose due to reduced tissue sensitivity to insulin (165). The 'lifestyle' connotation refers to a sedentary disposition or inadequate activity (113; 137) coupled with a high energy diet that contains excessive fat and sugar content (86; 125). Insulin is a hormone which mediates the rate-limiting step in the entry of D-glucose into skeletal muscle (72; 114). In the post-prandial state, when plasma insulin is elevated, glucose entry into muscle is primarily facilitated by the insulin-responsive GLUT4 (30; 31; 67).

GLUT4 serves as a vital component in the maintenance of euglycaemia as 70-80% of plasma glucose enters skeletal muscle (166), adipose tissue (56; 151; 152) and cardiac muscle (1) via this transporter. When circulating insulin levels are low, GLUT4 is found stored in intracellular vesicles and recycles very slowly between the vesicles and the sarcolemma (27). In fact, the steady state dynamics of GLUT4 trafficking between the vesicular and membrane fraction favours the intracellular localization of the transporter (18; 126; 152).

In individuals with Type II diabetes, there is diminished glucose transport into skeletal muscle due to insulin resistance (29). Regular physical activity in the form of exercise improves glucose transport into muscle by an insulin-independent mechanism and upregulation of GLUT4 content (26; 78; 107).

2.2.1. Exercise increases GLUT4 content in skeletal muscle.

Kraniou et al. (71) exercised untrained male and female subjects for 60 minutes on a cycle ergometer and analysed muscle biopsies. They reported a rapid upregulation of both *GLUT4* mRNA and protein which remained elevated 3 hours post-exercise (71).

On a similar note, Green et al. (45), noticed that 16 hours of intermittent cycling exercise which depletes muscle carbohydrate reserves also results in a rapid 28% upregulation of GLUT4 in vastus lateralis muscle (45). Ren et al. (123) had shown earlier that swimming caused a 2-fold increase in *GLUT4* mRNA and a 50% increase in total GLUT4 content in the epitrochlearis muscle 16 hours after a prolonged session of exercise. Interestingly, these researchers found that on the second day of exercise, GLUT4 protein increased a further two-fold but GLUT4 mRNA remained unchanged. They concluded that the rapid increase in GLUT4 content, which seems to be controlled by pre-translational mechanisms, serve as an early adaptive response of muscle to exercise (123).

Hughes et al. (54), investigated the effects of exercise on GLUT4 levels and glucose insulin action in 18 subjects with impaired glucose tolerance. In this study, subjects performed training at 50% and 75% of heart rate reserve for 12 weeks. Using a two-step hyperinsulinaemic-euglycaemic clamp, peripheral insulin action was determined as well as GLUT4 concentrations in skeletal muscle. They reported an

improved glucose tolerance in all trained subjects, along with a 60% increase in GLUT4 content and lower plasma glucose concentrations after the 12 weeks of training (54). A similar finding was made by O'Gorman et al. (107), where acute and short term (7 days) training also caused an increase in overall GLUT4 content in vastus lateralis muscle and improved glucose disposal in obese type 2 diabetic participants. Interestingly, this group observed that the improvement in glucose homeostasis was a result of increased GLUT4 content and not due to changes in insulin signalling; suggesting that exercise affects GLUT4 expression via mechanisms that are independent of insulin (107). Christ-Roberta et al. (26) explored this further in overweight non-diabetic and type II diabetic subjects and made a similar finding that exercise training did not improve insulin-stimulated glucose disposal via activation of the insulin signalling pathway.

These studies provide evidence that exercise increases glucose disposal in both non-diabetic and type II diabetic individuals through mechanisms that up-regulation *Glut4* gene transcription.

2.2.2. Exercise induces GLUT4 translocation in skeletal muscle.

Just a single bout of moderate exercise has been shown to augment glucose uptake within 15 min of exercise in normal human subjects (109) This effect is also true in the highly insulin-resistant obese Zucker rats. King et al. (66), noted a two-fold increase in membrane GLUT4 content and a four-fold increase in glucose transport in these rats after exercise when compared to non-exercised rats (66). This study shows that even when muscle is insensitive to insulin, as in the case of diabetes, there are other mechanisms that are sensitive to exercise that influence glucose homeostasis. Kennedy et al. (63) investigated whether exercise-induced translocation of GLUT4 in diabetics was any different to that of normal human subjects and found no statistical difference between groups: In diabetics, post-exercise sarcolemmal GLUT4 was increased by 74± 20% above resting levels whereas in normal subjects there was a 71±18% increase after a 45-60 minute of cycle exercise at 60-70% of VO_2 max. This finding shows that individuals with insulin resistance or diabetes respond similarly to exercise as normal subjects (63).

Experiments with transgenic mice that overexpress GLUT4 have conclusively demonstrated that by increasing the expression of this transporter in muscle, whole body glucose disposal can be improved (16; 122). Ren et al. (122) showed that in both fed and fasted rats, overexpression of the GLUT4 protein lowered plasma glucose. In *GLUT4* transgenic mice, plasma glucose levels were 172±7 mg/dl and 78±7 mg/dl in fed and fasted rats, respectively, whereas plasma glucose was 208± 5 mg/dl in the fed state and 102±5 mg/dl when fasted in non-transgenic counterparts. The rate of glucose disposal was 70% higher in transgenic mice than non-transgenic mice (122). Brozinick et al. (16) performed GLUT4 over-expression studies in the db/db diabetic mice model and examined the effects of differential GLUT4 over-expression on glucose utilization, *in-vitro* glucose transport and GLUT4 translocation in young (10-12 week old) and old (28-30 week old) mice. They showed an overall improvement in glucose tolerance in young mice that over-expressed GLUT4 when compared to age-matched non-transgenic counterparts. Older mice showed deterioration in glucose tolerance compared to younger mice but the decline was less in mice that over-expressed GLUT4

4-5-fold compared to mice that over-expressed GLUT4 only 2.3-fold. Glucose infusion rates were also increased in GLUT4 transgenic mice compared to non-transgenic controls (16). Ikemoto et al. (55) assessed the effect of a low-level increase in the tissue expression of GLUT4 on glycaemic control in mice that were fed a high fat diet. Expression of the GLUT4 transgene in these mice averted the impairment in glucose control that was seen in non-transgenic mice. This study showed that a two-fold increase in GLUT4 expression, although small, was enough to prevent a primary symptom of type II diabetes in a mouse model.

The studies mentioned in this section provide evidence to support the notion that increasing GLUT4 content in skeletal muscle by exercise serves as a therapeutic mechanism to help those with type II diabetes control hyperglycaemia and prevent diabetic-related sequelae. However, many individuals living diabetes, obesity or insulin resistance are unable to exercise adequately. This underscores the importance of developing pharmaceutical agents that may target signalling pathways that are activated by exercise to increase GLUT4 expression and translocation.

2.3. Transcriptional regulation of the *Glut4* gene.

The *Glut4* gene is subjected to complex hormonal and metabolic regulation as revealed by numerous studies in adipose tissue and muscle. This section of the literature review provides insight into the transcription factors that regulate the expression of the *Glut4* gene.

2.3.1. Identification of MEF2 and GEF domains on the Glut4 promoter.

Liu et al. (80) undertook the task of identifying the cis-DNA elements responsible for tissue-specific expression of GLUT4 by characterising and cloning the rat *Glut4* gene. Multiple *GLUT4* luciferase reporter deletion constructs of the gene were created and transiently transfected into differentiating C2C12 myotubes to determine which fragment of the gene conferred myotube-specific GLUT4 expression. Reporter assays revealed a 103 base pair fragment (located between -522 to -422 [Domain II] from the transcriptional initiation site which was necessary for GLUT4 expression. Further mutagenesis of this fragment showed that it contained a sequence analogous to the

myocyte enhancer factor 2 (MEF2) binding site on the *muscle creatine kinase* (MCK) promoter. Liu et al. (80) also conducted Electrophoretic mobility gel shift assays (EMSA) which showed that MEF2 isoforms bound this region. This study concluded that the 103 base pair fragment contained a MEF2 domain that was necessary but insufficient for transcriptional activation of the *Glut4* gene.

Knight et al. (68) identified another factor that was shown to co-operatively work with MEF2 via protein-protein interactions. This factor was named the GLUT4 enhancer factor (GEF) (68; 112) which was identified to bind upstream of domain II on the *Glut4* gene which they called Domain I. Using transfection assays, Knight et al. (68), showed that expression of GEF or MEF2 by itself could not significantly activate GLUT4 promoter activity. However, when expressed together, promoter activity increased 4- to 5-fold; suggesting that GEF and MEF2 function together to activate *Glut4* transcription in skeletal muscle.

Exercise has been shown to increase both GEF and MEF2 binding to the *Glut4* enhancer in human skeletal muscle (91). In this study, 7 subjects performed 1 hour of

cycling at ~70% of VO_2 peak. Muscle biopsies were then taken and assessed for DNA-binding activities by EMSA. The results of the EMSA showed that an acute single bout of exercise was enough to significantly increase binding of both the MEF2A/MEF2D heterodimer pair and GEF to the *Glut4* gene. Furthermore, exercise did not cause an increase in MEF2D or GEF nuclear content, however, MEF2A content was significantly increased as revealed by immunoblotting (91). These studies show us that the *Glut4* gene is regulated by these two transcription factors namely, GEF and MEF2, which respond to signals activated by exercise.

2.3.2 The myocyte enhancer factor-2 family of transcription factors.

The MEF2 group of transcription factors are a highly conserved group of DNA binding proteins in eukaryotes (43; 117). They belong to the MADS box (MCMI, Agamous and Deficiens and SRF) (116) family of transcription factors as they share a highly conserved 57- amino acid fragment on the N–terminal of the protein (161). MEF2 is highly expressed in T-lymphocytes (37; 160) neurons (37) and skeletal and cardiac muscle (98) where studies have shown MEF2 to be highly crucial for myogenic

differentiation (15). This conclusion came from studies where mutations in a single *Mef2* Drosophila gene prevented myoblast differentiation (79; 120). Further studies have also shown that dominant negative MEF2 mutants also prevent myoblast differentiation (111). Even though MEF2 proteins lack myogenic activity, their contribution to myogenic differentiation lies in its strength to exalt the activity of myogenic basic-helix-loop-helix (bHLH) proteins such as MyoD and myogenin (34) In addition to myogenesis, MEF2 proteins are involved in cell proliferation, apoptosis, survival and are key regulators of adaptive programs, stress responses, muscle fibre-type switch, cardiac hypertrophy and remodeling of neuronal circuitry (37; 65; 134; 156; 158).

There are four members of the MEF2 class of transcription factors namely MEF2A, MEF2B, MEF2C and MEF2D. These proteins are encoded by their respective genes: *Mef2a*, *Mef2b*, *Mef2c* and *Mef2d*, which are located on four different chromosomes (43; 53) These four isoforms share a high amino acid identity of more than 95% similarity (43). As mentioned above, MEF2 proteins have homologus DNA binding domains such as the N-terminal MADS box (aa 1-57), the highly conserved

MEF2 domain (aa 58-86) and the more divergent transactivation domain that aids in transcriptional activation. The MEF2 domain is of particular significance as it is this domain that localises DNA binding affinity and specificity as well as mediating homo- and heterodimerization between MEF2 members (3; 36; 116; 157). At the MEF2 cis-element [(CTA(AT)$_4$TAG] on the *Glut4* gene, MEF2A and MEF2D form a MEF2A-MEF2D heterodimer, which has been shown by Mora and Pessin, (99) to be essential for hormonal GLUT4 expression. McGee et al. (91) have shown that exercise enhances the DNA-binding activites of both MEF2A-MEF2D heterodimer and GEF in human skeletal muscle thus indicating that heterodimer binding is not only in response to hormonal regulation but also in response to exercise.

2.4. Regulation of MEF2 transcriptional activity

2.4.1 Interaction of MEF2 with HDACs and HATS.

As with all transcription factors, MEF2 binding to its cis-element is largely determined by the accessibility of the chromatin structure neighbouring the binding domain (47). In a transcriptionally inactive scenario, MEF2 is bound to class II histone deacetylases (HDACs) (93; 94) In addition to binding MEF2, HDACs have another function, which is the inhibition of transcription by chromatin remodeling (96; 97). For MEF2 to become an active transcription factor, HDACs have to disassociate from MEF2, then only can transcription of a MEF2-dependent gene occur (93; 94). Calcium signaling in the form of calcium/calmodulin dependent protein kinases (CaMKs), are activated by exercise (124; 139; 140) and are responsible for this disassociation (McGee et al. (91). MEF2 proteins are then free to associate with histone acetyltransferases (HATs), another chromatin remodeling enzyme that reverses the effect of HDACs (47; 118). These mechanisms are explained in this section as well as evidence, supporting the notion that this occurs at the *Glut4* gene and other genes, are given in this section.

2.4.2 CaMK II-dependent chromatin remodeling at MEF2-dependent gene promoters.

Accessibility of transcription factors to their binding sites on gene promoters is determined by numerous post-transcriptional modifications on proteins called histones surrounding the cis-elements. Structurally, eukaryotic DNA is wound around an octamer of highly conserved histone proteins (H2A, H2B, H3, and H4). When an octamer of histone proteins have 146 bp of DNA wrapped around it, it is referred to as a nucleosome. Nucleosomes are separated from each other via regions of linker DNA and serve as monomers of chromatin that function as building blocks to manage eukaryotic DNA into high-order chromatin fibres. The N-terminal tails of these histones, which protrude from the surface of the nucleosome, undergo many post-translational modifications such as methylation, phosphorylation, sumoylation and acetylation (10; 73). Acetylation and deacetylation of lysine residues on the N-terminal tails of histones have been the most appreciated and researched post-translational modification that has been linked to chromatin structure and transcriptional activity (46; 47; 106). Current understanding of MEF2-mediated transcription, suggests that acetylation state in the

vicinity of the transcription factor is reversed during transcription (93; 94). MEF2 is repressively bound by Class II Histone Deacetylases (HDACs) such as HDAC4 and HDAC5. HDACs inhibit transcription by removing acetyl groups from lysine residues present on N-terminal tails on histones. This results in chromatin condensation and repressed transcriptional activity (21; 70; 96; 97). In diseases such as cancer, diabetes, cardiac-hypertrophy and asthma, altered HAT and HDAC activites have been implicated as contributors to the development of these diseases thus revealing that epigenetic mechanisms such as acetylation have pertinant consequences in disease states (110).

Calcium dependent protein kinase II (CaMK II) has been identified to be a major activator of MEF2-dependent transcription in skeletal muscle. It is now clear that CaMK II activates MEF2 transcriptional activity by phosphorylation of HDAC5 on serines-259 and -498 (7; 48; 92). These phosphorylated residues then serve as docking sites for the molecular chaperone protein 14-3-3, which bind HDACs to export them out of the nucleus (7; 48; 92). This promotes interaction between MEF2 and histone acetyltransferases (HATs) such as p300 or p300/CBP associated factors (118; 130).

Histone acetyltransferases catalyse the reverse reaction to HDACs i.e. they transfer acetyl groups onto lysine residues present on nucleosomal histones. This relaxes and opens up chromatin structure to transcriptional coactivators which is why histone hyperacetylation is synonymous with transcriptional activation (47).

2.4.3. CaMK II activation causes histone 3(Lys $^{9/14}$) hyperacetylation at the MEF2A binding site on the Glut4 gene.

Smith et al. (140), investigated the role that CaMK II activation played in MEF2A binding to and acetylation of histone 3 (Lys $^{9/14}$) on the *Glut4* promoter and GLUT4 expression in rats in response to intermittent exercise. After receiving 5mg/kg of the CaMK II inhibitor, KN-93, rats were subjected to 5 X 17-minute bouts of swimming. Thereafter triceps muscles were harvested and assayed for the aforementioned parameters. A 2.2-fold increase in both MEF2A binding to the *Glut4* promoter and acetylation of histone H3 surrounding the MEF2A binding site on the *Glut4* gene were noticed in this study, as well as a 1.8-fold increase in GLUT4 expression. More importantly, rats that were injected with KN-93 showed attenuation in MEF2A binding, histone hyperacetylation of

the *Glut4* promoter and GLUT4 expression. This study concluded that CaMK II activation was necessary for histone hyperacetylation and MEF2A binding to the *Glut4* promoter *in vivo* (140). Mukwevho et al. (101), also explored the mechanism by which caffeine increased GLUT4 expression in C2C12 myotubes and tested whether these mechanisms were also CaMK II dependent. They used KN-93 to inhibit CaMK II and dantrolene to prevent Ca^{2+}release from the sarcoplasmic reticulum. Caffeine is thought to activate CaMK II activity by triggering release of calcium from the sarcoplasmic reticulum (101; 110). The use of caffeine in these experiments were based on an earlier study by Ojuka et al. (110) who exposed L6 myotubes to 5mM caffeine for 3 hours daily for 5 days and found an increase in GLUT4 protein as well as increases in MEF2A and MEF2D in rat epitrochlearis muscle. The activation of CaMK II by caffeine is thought to lead to a sequence of cellular events in skeletal muscle that mimics exercise.

Mukwevho et al. (101) showed a 1.8-fold rise in GLUT4 mRNA and a 2.2-fold increase in MEF2A binding to the *Glut4* gene when compared to controls. All aforementioned caffeine-induced changes were abolished by both KN-93 and

dantrolene treatment. They went further to show that when C2C12 myotubes were treated with caffeine, nuclear abundance of HDAC5 decreased. However, when KN-93- or dantrolene were used, myotubes showed no change in nuclear HDAC5 content when compared to controls suggesting that it was CaMK II activity that caused this nuclear exodus. This study provided evidence that CaMK II activation in C2C12 myotubes due to caffeine administration might increase GLUT4 content via an increase in histone hyperacetylation and MEF2A binding to the *Glut4* gene (110).

These studies, conducted in our lab, show that CaMK II activation leads to an increase in *Glut4* gene activity. The histones surrounding the MEF2A binding site are found to be hyper-acetylated and MEF2A is found to be more bound to the *Glut4* gene in response to CaMK II activation. These changes in response to CaMK II activation correlate well with the current understanding of MEF2 mediated transcription (93) which suggests that following HDAC5 dissociation and nuclear export, transcriptional co-activators are recruited to MEF2 to reverse the acetylation state of histones surrounding the vicinity of the transcription factor (118). Conversely, Scicchitano et al. (132) have

shown that when CaMK is inhibited, there is a reduction in the level of acetylation of histone H4 at the MEF2 binding site on *muscle creatine kinase* (*MCK*) and myogenin promoters in Arg^8-vasopression induced myogenic differentiation in L6 myotubes. Hypoacetylation of H4 in L6 myotubes also resulted in lower levels of MEF2A bound to the *MCK* promoter and lower MCK and myogenin content as revealed by western blotting (132). These studies show a positive correlation between acetylation of histones and binding of MEF2 to its cis-element on promoter regions and would therefore suggest that histone acetylation is necessary for MEF2 mediated transcription to occur. The identification of the HATs involved in acetylating histones at MEF2-regulated genes, such as the *Glut4* gene, thus becomes necessary. This thesis therefore, aims to explore the potent HAT p300, which interacts with MEF2 at various levels in transcription as a possible HAT at the MEF2A binding domain on the *Glut4* gene.

2.4.4. AMP-activated protein kinase also regulates GLUT4 expression by phosphorylating histone deacetylase 5.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved, fuel-sensing master switch that is present in the most primitive unicellular organisms right through higher evolved mammalian cells (49). It is activated by stresses that induce an increase in cellular concentrations of AMP relative to ATP which includes glucose deprivation, hypoxia, and more importantly, muscular contraction (14; 33; 50; 51). The principle function of APMK appears to be the generation of ATP through the stimulation of fatty acid oxidation and glucose transport (GLUT4 translocation) with concurrent inhibition of ATP-consuming pathways such as lipid/protein synthesis, cell growth and proliferation (17; 52; 74; 128; 133).

AMPK is activated in skeletal muscle during exercise and can be induced in cultured muscle cell lines by treatment with 5-Aminoimadazole-4-carboxamide ribonucleoside (AICAR), an analog of adenosine, which leads to an accumulation of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-51-monophosphate (ZMP). As ZMP mimics AMP,

AMPK is activated allosterically by the binding of ZMP to the β-subunit of AMPK thus making it a better substrate for upstream kinases (32; 76).

The following studies have made use of AICAR stimulation of AMPK activity in muscle with particular emphasis in measuring the response in GLUT4 expression :

Zheng et al., (164), showed that a single subcutaneous injection of AICAR into the quadriceps of mice and rats resulted in an increase of GLUT4-mRNA 13 hours post – administration. To determine which region of the *Glut4* promoter was induced in response to AICAR treatment, transgenic animals mice were generated, carrying a CAT reporter gene driven by 1.154 bp of the human *Glut4* gene. Reporter assays showed that AICAR induced either 1,154bp or 895 bp of the *Glut4* gene but did not activate transcription within 730bp of the promoter. It was thus concluded that all elements required for AICAR-induced transcription of the *Glut4* gene were located within 895bp of promoter. As this region of the gene contains two important regulatory domains, namely Domain I which binds GLUT4 enhancer factor (GEF) and a MEF2 binding site, electromobility shift assays (EMSA) was conducted on nuclear extracts prepared from

gastroceminus muscles. The results of these EMSA experiments showed an increase binding activity of MEF2A and/or MEF2D to oligonucleotides corresponding to MEF2 binding site on the *Glut4* gene, furthermore, an increase in GLUT4 protein in rats that were treated with AICAR for two weeks was also noted in this study (164). This study shows that there is an increase in *Glut4* promoter activity in response to AICAR treatment and more importantly that this upregulation is related to the binding activities of MEF2 protein to the *Glut4* gene (164).

Ojuka et al. (110), showed that 1.0mM AICAR treatment of L6 myotubes resulted in a significant upregulation of GLUT4 protein ($p<0.001$) when compared to untreated controls. In this study, the addition of adenosine-9-β-D-arabino-furanoside (AraA) to AICAR treatments attenuated the upregulation of GLUT4. Ojuka et al. (110) also showed that AICAR treatment also upregulated both MEF2A and MEF2D protein in L6 myotubes. This study demonstrates that AMPK activation in muscle increases GLUT4 expression via a mechanism that is probable increases in MEF2 content. (110). Thus far, the studies reviewed above show that in addition to an increased binding activty of

MEF2 protein to the *Glut4* promoter as shown by Zheng et al. (164), Ojuka et al. (110) demonstrates that there in an increased concentration in transcription factor MEF2 to bind the *Glut4* gene on response to AICAR treatment (110).

As HDAC5 phosphorylation is only partially reduced when CaMK pathways are blocked (90), McGee et al. (90) aimed to determine whether AMPK could be the other kinase that phosphorylates HDAC5 thus promoting GLUT4 expression. Using Scriptaid, a low toxic HDAC inhibitor, they showed that HDAC inhibition resulted in a seven-fold increase ($p<0.05$) in GLUT-mRNA and a nine-fold increase ($p<0.05$) in H3 acetylation without any change in nuclear abundance in HDAC5 content thus showing that the inhibitor does not alter HDAC localization. McGee et al. (90) also showed that HDAC5 was indeed a direct substrate for AMPK by creating HDAC mutants that expressed a serine 259/498 to alanine mutation. By performing enzyme assays they demonstrated that AMPK was unable to phosphylate mutant HDAC5 at the aforementioned alanine residues but did phosphorylate wild type HDAC5 at serines 259 and 498. This was affirmed endogenously when myotubes were treated with AICAR for 60 min; which

caused a 40% increase in phospho-AMPK and a 65% and 100% increase in HDAC phosphorylation at serines 259 and 498 respectively. Nuclear abundance of HDAC5 decreased significantly ($p<0.05$) as revealed by immunoblotting, whereas ChIP assays showed that a substantial decrease in HDAC5 associated with the MEF2 binding site on the *Glut4* promoter while RT-PCR showed that AICAR induced transcription as GLUT-mRNA was increased significantly (90).

2.5. p300: a Potent Histone Acetyltransferase (HAT).

2.5.1 Overview

This section looks at p300, the protein that we hypothesise to have a role in acetylation of histones at the MEF2A binding site on the *Glut4* gene. The transcriptional co-activator, p300 was first identifed as a protein that interacted with the E1 adenovirus (82) The MADS/MEF2 domains of MEF2 transcription factors have been identified to interact directly with p300 (130). p300 also acetylates MEF2 on seven conserved lysine

residues along the MADS box and transactivation domains of (4; 83). Most significantly, p300 possesses a potent HAT domain at the core of the protein and has the ability to acetylate all core histones at multiple lysine residues along protruding N-terminal tails (9; 108). Given these abilities of p300 and the numerous interactions it has with MEF2 transcription factors, we hypothesise that p300 is involved in regulating chromatin structure at the MEF2A binding site on the *Glut4* gene.

2.5.2. Structure of p300.

p300 is a large (300kDa), global metazoan, specific transcriptional co-activator with potent HAT activity and plays multi-decisive roles in proliferation, differentiation and apoptosis. Its central role is to integrate and co-ordinate multiple signals received from diverse extra-cellular cues so that an appropriate level of transcriptional events occur in response to various physiological stimuli. It shares a strikingly similar homology both functionally and structurally to that of CREB-binding protein (CBP), thus they are often referred to as p300/CBP family of transcriptional co-activators (42; 59; 136). p300 coactivates transcription with a multitude of transcription factors, through various

mechanisms that aim to form a stable and efficient transcriptional complexes (20). As this study only aims to investigate whether the HAT domain of p300 is attributable to acetylating histones on the *Glut4* gene, a review of studies showing the importance of the HAT domain of p300 is given especially with respect to MEF2-mediated transcription.

Structurally (Fig.1), p300 consists of roughly 2400 amino acids and harbours typical structural motifs synonymous of transcriptional machinery (42). These include a centrally located bromodomain, three cysteine-histidine (CH) rich domains, and a centrally located HAT domain (20). The CH domains are thought to be important in mediating protein-protein interactions, however, recent work has revealed that within the CH domains lies three zinc fingers enabling these domains to mediate both protein-protein and protein-DNA interactions. These interactions facilitate stabilisation of the Pre-Initiation Complex (PIC) which consists of obligatory factors such as RNA polymerase II, TAFII250 and TATA-Binding Protein (TBP); commonly referred to as the general transcription machinery (GTM) (13; 21; 89). Bromodomains are considered to

have dual roles at promoter regions (89). Firstly, they aid in identification of nucleosomal substrates for HATs thereby promoting interactions between enzymes and histones. Secondly, once specific histone sites are acetylated, bromodomains are recruited to acetylated residues on both histone and factors of the transcription apparatus thereby promoting stable interactions for further acetylation, resulting in an acetylation cascade (21; 42; 89).

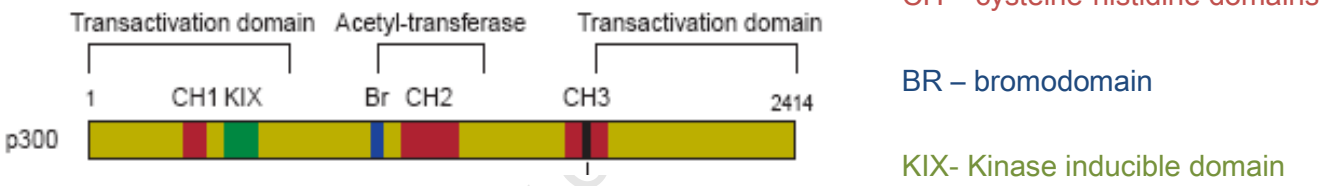

Figure 1. : Structure of p300/CBP indicating its major domains(20)

Structural diagram showing the main functional domains in p300 consisiting of the CH1, CH2 and CH3 domains, the KIX domain and the bromodomains. This diagram also shows that both the N- and C-terminis can act as transcactivation domains with the HAT domain located centrally.

2.5.3 Functions of p300.

p300 is a transcriptional integrator that possess intrinsic HAT activity and is capable of acetylating both histones as well as other cellular factors such as transcription factors (9; 83) Ogryzko et al. (109) first identified p300 to possess intrinsic histone acetyltransferase activity by purifying p300 that bound the E1 12S protein and then subjecting p300 to histone acetyltransferase assays using calf thymus histones and radiolabelled acetyl-CoA. These assays showed that p300 did posses HAT capabilites which were mapped to amino acids 1135-1810 of p300. Furthermore, they showed that p300 acetylated all core histones in mononucleosomes but preferentially acetylates H3 and H4.

Studies show that acetylation of these factors affects their activities by enhancing their DNA binding and transcriptional activities, interactions with other proteins, turnover and nuclear export (83; 89). Considering transcriptional regulation, p300/CBP proteins are respected as highly resourceful general transcriptional integrators. According to recent work they perform their duties through a cohort of mechanisms (refer to Figure

1.2): Firstly they serve as bridging molecules that connect the sequence-specific promoter binding transcription factors to the general transcription machinery (42; 136). This is the scenario in transcription factors that do not possess the binding domains to directly bind RNA polymerase II and associated factors. p300 also directs the congregation of other co-factor proteins into multi co-activator complexes (13). Secondly, p300 acts as a scaffold for such proteins; enabling an increase of the presence of these co-factors at local promoter regions through protein–protein and protein-DNA interactions (69; 154; 159). Thirdly, p300 is a potent histone acetyltransferase and also has the ability to acetylate transcription factors thus resulting in enhanced binding capabilities and ultimately transcriptional activation.

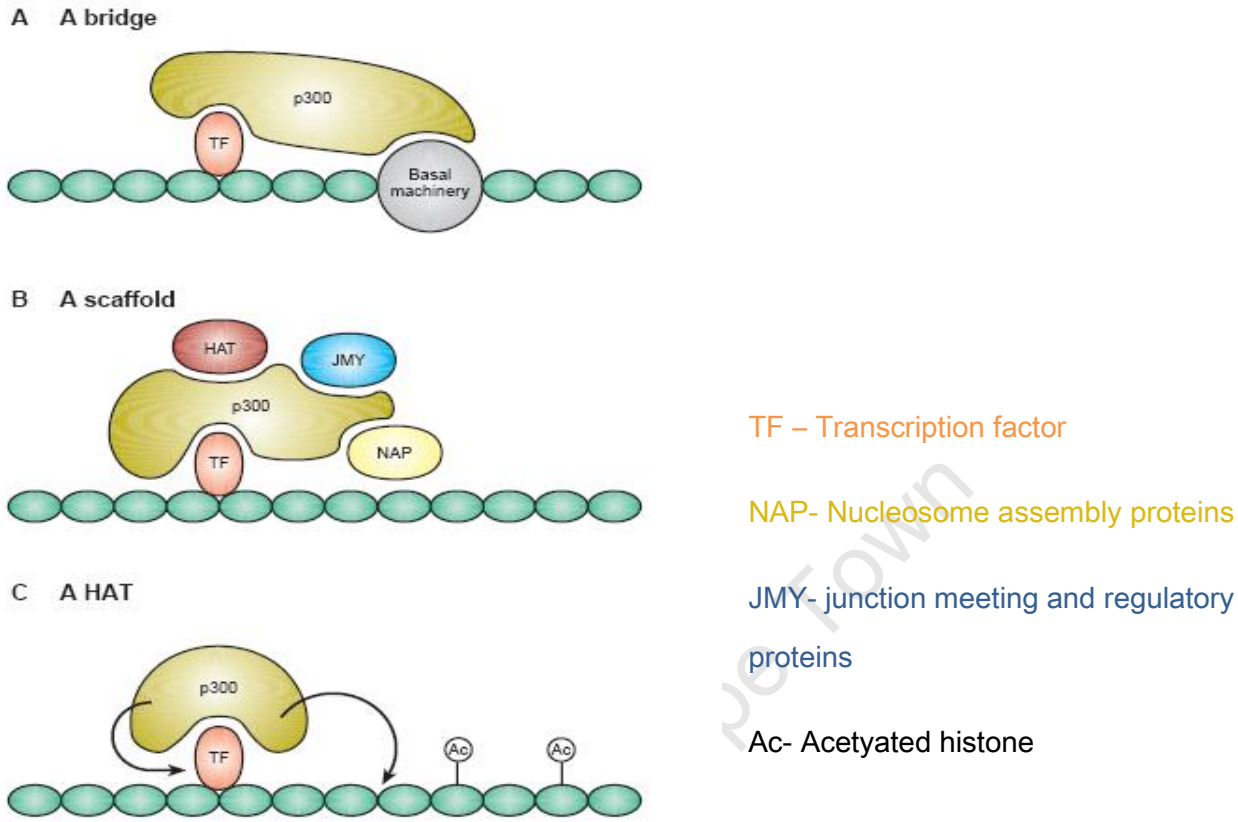

Figure 2. : Mechanisms by which p300 facilitates transcriptional activation (20).

Various mechanisms of transcriptional activation by p300. Firstly, p300 can act as a brdge connecting sequence-specific transcriptional activators to the general transcriptional machinery. Secondly, being a large protein, p300 acts as a structural scaffold for the assembly of multiprotein complexes. Lastly, p300 is endowed with a potent HAT that is capable of acetylating histones as well as transcription factors (18).

2.5.4. Is p300 activity regulated by phosphorylation?

Chawla et al. (23) have shown that CaMK IV phosphorylation is necessary for CBP to mediate its transcriptional functions in neuronal cells. More recently and significantly, Yaun et al. (162) have shown that in response intermittent hypoxia in PC12 cells, p300 is phosphorylated *in vitro* by CaMK II and that this phosphorylation is absolutely necessary for the transcriptional activity of the hypoxia-inducible factor 1(HIF-1). This was determined by transfecting PC12 cells with pGAL4p300, a GAL4 fusion protein containing full length p300 protein and a luciferase reporter construct pG5E1bLuc. These cells were then exposed to 120 cycles of intermittent hypoxia (IH), which increased p300 transcriptional activity. Further experiments showed that IH was mimicked when PC12 cells were co-transfected with CaMK II-290 and pGAL4p300. However, when KN-93 was administered, a CaMK II inhibitor, neither p300 nor CaMK II was able to activate the IH response. It was also revealed that p300 was phosphorylated directly by CaMK II in an *in vitro* assay (162). These last two studies

provide evidence to show that there are CaMK dependent effects on p300 however, research into the effects of CaMK activity on p300 is still limited at this stage.

2.5.5. Regulation of p300 by autoacetylation.

There exists a highly basic autoacetylation loop within the HAT domain within p300 that is found to be hyperacetylayed when p300 is most active. Studies have shown that the catalytic activity of p300 can increase 4-10-fold when autoacetylated (149). p300 autoacetylation occurs via an intermolecular manner of 17 lysine residues within the HAT domain of p300 (60; 61). This serves as a switch to regulate the HAT activity and ultimately the ability of p300 to fulfil its transcriptional co-activator duties.

2.4.6. Interaction between p300 with MEF2 at various MEF2-dependent genes.

Using the 3T3 cell line and a reporter construct consisting of a MEF2-binding site derived from rat embryonic *myosin heavy chain* (*MHC*) promoter, Sartorelli et al. (130) showed that co-transfection of p300 expression vector increased MEF2C trans-activation 3-fold more than when there was no expression vector. Furthermore, using an

in vitro protein-protein interaction assay they showed that p300 protein interacted with various segments of GST-MEF2C fusion protein and concluded that the MADS box region of MEF2 factors was necessary and sufficient for the interaction between these proteins (130). Using EMSA, Slepak et al. (138) showed that p300 formed a complex with MEF2D but not MEF2A or MEF2C on the *human skeletal actin* (hSA) promoter in cardiac myocyte, however when these experiments were repeated using the HeLa cell line, p300 and MEF2D failed to induce hSA activity. Slepak et al. (138) concluded that there are additional specific cell-type factors that contribute to the expression of the promoter and that in cardiac myocytes and that MEF2D acts as a selective channel for p300-dependent activation of transcription.

MEF2C has been shown to acetylated by p300 both *in vitro* and *in vivo* in the C2C12 myogenic cell line (83). This was achieved via conducting *in vitro* acetylation assays with immunoprecipitated p300 and His-thioredoxin-MEF2C fusion proteins. The outcomes of this experiment showed that MEF2 was indeed acetylated *in vitro*. To determine whether MEF2C was acetylated *in vivo*, embryonic kidney stem cells (293T)

cells were transfected with overexpressed Flag-MEF2C in the presence of labelled sodium [3H]-acetate and acetylation assays were conducted. MEF2C was found to be acetylated *in vivo* as well when overexpressed (83).

In human MEF2C there are two lysine residues between amino acids 115 and 210 (K116 and K119) and four lysines between amino acids 211 and 302 (K234, K239, K252 and K264). Furthermore, multiple sequence alignment revealed that the 4 lysines between 221 and 302 are fully conserved in MEF2A, MEF2B and MEF2D in vertebrates suggesting that these are general acetylation sites for p300 (83).

The MADS box of MEF2 has also been found to acetylated at Lys 4 by p300 as revealed by mass spectrometry coupled with acetylation assays (4). This modification by p300 on MEF2 has been shown to augment MEF2 binding to the *muscle creatine kinase* (*MCK*) enhancer as revealed by EMSA. As the MADS box of MEF2C is highly conserved domain in vertebrates these results suggest, in accordance with sequence alignment studies of MEF2, that p300 acetylation of the MADS box of MEF2 proteins serve as a general mechanism in MEF2 mediated transcription.

To determine the effect of Lys4 acetylation on the co-activation MEF2C activity and p300, COS1 cells were co-transfected with wild type (wt) or mutant MEF2C, p300 and a plasmid containing luciferase reporter activity. Results showed that the mutant form of MEF2 caused a reproducible 35% reduction in luciferase reporter activity suggesting that the Lys4 acetylation serves an integral part of the co-activation mechanism. To determine if p300 influences DNA binding, EMSA was carried out again with ^{32}P-radiolabelled probe containing the *MCK* and nuclear extracts of myogenic C2C7 cells alone and cells that contained purified p300. The results showed that p300 presence caused an increase in endogenous binding activity and that the HAT domain was necessary for this effect since LysCoA, administration abolished binding (4).

Suggesting that Lys 4 acetylation by p300 enhances MEF2C binding capabilities

Wei et al. (153) have showed that p300 overexpression induces cardiac hypertrophy in Sprague-Dawley mice. The consequences of increased p300 expression with respect to cardiac hypertrophy are the acetylation of p300 substrates histone H3 and MEF2. Using genetically manipulated mice that over-expressed p300, this study showed that p300

selectively targeted MEF2-containing elements as revealed by microarray analysis. Furthermore, p300 overexpression induced p300 HAT activity 2.4-fold and induced hyperacetylation of MEF2 without any changes in HDAC activity (153). A 47% increased in acetyl-H3 was also observed in response to p300 overexpression when compared to wild type mice. This study revealed that small increases in p300 expression are enough to induced hyperacetylation of histone H3 and hyperacetylation of MEF2 possibly at the seven lysine residues mapped by Ma et al. (83) and Angelelli et al. (4) .

De Luca et al. (28) investigated the possible role that p300 plays in regulating the thyroid hormone receptor (TRs) and MEF2A. In addition to mapping the regions of TR and MEF2A that physically interacted with p300, they investigated whether these proteins formed a complex at the *α-myosin heavy chain* promoter in human osteosarcoma (U2OS) cells. The findings of this study were that TR, MEF2 and p300 form a ternary complex *in vivo* and that the N-terminus of MEF2A (MADS box and MEF2 domain) binds the C-terminus of p300 (aa 1572 -1868) (28). The findings of De Luca et al. (28) are relevant to our study as the *Glut4* gene possesses a thyroid

hormone response element (TRE) in close proximity to the MEF2A binding site; it would be important to determine if the ternary complex exists on the *Glut4* gene.

The previous studies reveal that p300 is capable of interacting with both MEF2A and MEF2D. p300 also hyperacetylates MEF2C on lysine residues that are conserved throughout all members of the MEF2 family, which is thought to be a general mechanism to enhance the binding and transactivation functions of MEF2 and ultimately promote transcription (93) . As we have already established that MEF2A and MEF2D bind the *Glut4* gene as a heterodimer (99), we now can see there is evidence supporting our hypothesis that p300 may be involved in *GLUT4* transcription. To determine if p300 is involved in acetylating histones at the MEF2 binding site on the Glut4 gene, we are going to use a highly specific inhibitor of p300 HAT activity namely curcumin.

2.6. Curcumin: a p300 inhibitor.

2.6.1. Overview

Curcumin, which gives the yellow colour to the common Indian spice turmeric, is isolated from the rhizome of widely cultivated *Curcuma longa* plant (2). It has been used as a traditional medicine for thousands of years, in the treatment of various ailments such as inflammation, fever, the common cold, and wound-healing (5; 39; 77; 141; 141). It also acts as a beauty care agent and a blood purifier (62; 129). In 2005, two hundred and fifty-six papers were published on the various biological effects of curcumin. Researchers claim that curcumin serves as a 'near-perfect starting material for drug discovery as curcumin was found to have antioxidant, anti-inflammatory, anti-viral, anti-bacterial, anti-fungal, hepatoprotective, hypolipidaemic and anti-cancer activities (6; 8; 75; 104; 105; 131; 135; 141; 150). Biochemically, curcumin has been shown to be an active oxygen scavenger, an inhibitor of lipid peroxidation, lipooxygenase (LOX), cyclooxygenase (COX) and protease inhibitor effects (11; 19; 41; 84). In summary, curcumin alters cellular expression profiles by modulating the activity of transcription

factors, kinases and enzymes. However, for the purpose of this thesis, its inhibitory effect on the HAT domain of p300 will be reviewed.

2.6.2. Curcumin inhibits p300 HAT activity.

Curcumin is a potent inhibitor of p300 *in vitro and in vivo* as revealed first by Balasubramanyan et al. (8). In this study curcumin was shown to be cell permeable and absolutely specific for the p300 HAT inhibition but not for p300/CBP associated factor (PCAF). They also revealed that curcumin binds directly to the p300 and not to the active site of either histone or acetyl-CoA, which may denotes that curcumin binding to p300 leads to a conformation change which results in a loss of binding efficiency for the active sites for both core histone and acetyl group. This also reveals that curcumin specifically and selectively targets the HAT domain of p300 rendering it incapable of enzymatic activity (8).

Diketone

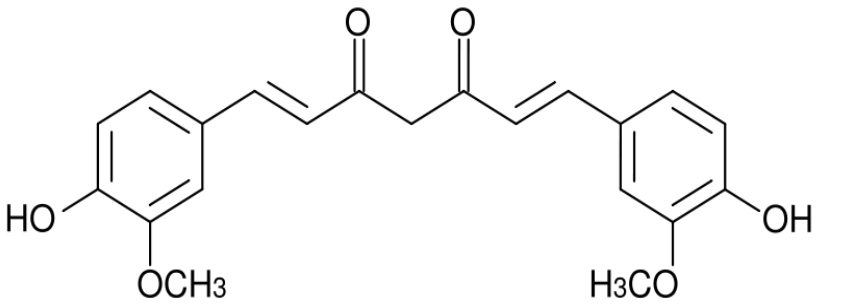

Figure 3. : Chemical Structure of Curcumin(142).

Curcumin is a bis-α,β-unsaturated β-diketone and undergoes keto-enol tautomerisation in equilibrium according to certain pH environments (85). The diketone is of particular significance as it has been revealed by Marcu et al. (88), to covalently bind the HAT domain of p300 in a sequence of addition reactions, known as the Michael reaction. When these carbonyls become saturated as in the case of tetrahydrocurcumin (THC), a hydrated form of curcumin, there are no p300 inhibitory effects. Furthermore, curcumin is highly selective for p300 and promotes proteosome dependent degradation of p300 without affecting PCAF or GCN5 (8). Curcumin is also capable of inhibitng the HAT activity of p300 in *in vitro* acetylation assays when either p53 or histone 3 are used

as substrates Marcu et al. (88). This study has also shown that curcumin inhibition of p300 activity can effectively blocked histone hyperacetylation induced by the HDAC inhibitor MS275 in PC3-M prostate cancer cells and in peripheral blood lymphocytes (88).

2.6.3. Studies that have used curcumin as a specific inhibitor of p300

To determine the effect of curcumin on histone acetylation and p300 with respect to nuclear factor κB (NF-κB) induced proinflammatory secretion under hyperglyaemic conditions in human monocytic THP-1 cells. Yun et al. (163), used the Enzyme-Linked Immunosorbent Assay (ELISA), to show that curcumin treatment significantly decreased total acetylation levels in a dose dependent manner. Furthermore, western blotting for p300 content on 1.5μM curcumin-treated nuclear lysates from THP-1 cells showed that curcumin treatment also decreased total nuclear p300 content (163). Similarly, curcumin was found to prevent diabetes-associated abnormalities in kidneys of STZ-induced Sprague-Dawley rats by inhibiting (NF-κB) association with p300 (25). In this study rats

were treated first injected with STZ to induces diabetes and subsequently treated with curcumin for one month. Using real-time reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical analyses of rat kidneys, the results of this study showed that curcumin treated rats had significantly lower p300 mRNA and protein expression levels ($p < 0.05$) (25).

Curcumin was shown to repress the activity of NF-κB by inhibiting p300 in lymphoblastic Raji cells, however in this study Chen et al. (24), showed that co-treatment of these cells with curcumin and the proteosome degradation inhibitor, MG-132, prevented curcumin dependent degradation of p300 as shown by western blotting. This study reinforces that the mechanism by which curcumin inhibits p300 is via a proteosome dependent degradation pathway and that p300 inhibition by curcumin can be reversed by the administeration of MG-132 (24).

In human umbilical vein endothelial cells (HUVECs), Chen et al., (23) provided evidence that curcumin treatment significantly reduced glucose induced upregulation of p300 protein, mRNA expression and total acetylated H3 via RT-PCR and westen

blotting (23). Teiten et al. (148) have also used curcumin as an inhibitor to determine whether it would affect p300 dependent proliferation of prostate cancer cells through the Wingless (Wnt/β-catenin) signaling pathway. Results from these experiments displayed that 50μM curcumin treatment significantly ($p<0.01$) abolished both p300 and CBP content in 22rv1 prostate cancer cells (148). Ryu et al. (127) also determined the inhibitory effects of curcumin on p300 on Wnt/β-catenin signaling pathway but more importantly investigated the differential effects of curcumin analogs demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and tetrahydroxycurcumin (THC) on inhibition of p300, an inducer of the (Wnt/β-catenin) signaling pathway in HEK293 cells. The results of these experiments showed that DMC and BDMC substantially reduced p300 levels in a dose dependent manner while THC did not affect p300 expression as revealed by western blotting. These results suggest that the centrally located carbonyls of curcuminoids are essential in reducing p300 levels, which is a finding that is in accordance with previous studies (88; 127).

As Wei et al. (153) showed that p300 overexpression induced cardiac hypertrophy, Morimoto et al. (100), have shown that curcumin supresses p300-induced hypertrophic responses in cardiomyocytes such as β-MHC promoter activity and acetylated histone 3 and 4 and p300 content. Experiments by Sun et al. (146) showed that p300 interacts with Mef2c, GATA4 and Nkx2.5 genes and hyper-acetylated histones at the promoter region of these genes in neonatal mouse cardiac myocytes. However when 30μM curcumin was administered to the myocytes for 24 hours, global acetylation of histone H3 was reduced 0.3983-fold compared to untreated controls ($P < 0.05$). A significant decrease in the acetylation of H3 at promoter regions of GATA4, mef2C and Nkx2.5 genes were observed along with subsequent significant reductions in their expression (146). A similar finding was observed by Sunagawa et al. (147); they showed that curcumin (50 $mg.kg^{-1}.day^{-1}$) inhibited p300 expression in male Sprague-Dawley rats.

On a similar theme, Feng et al. (35) investigated the role that p300 plays in diabetes-induced cardiomyocte hypertrophy. Cardiomyocytes isolated from newborn Harlan Sprague-Dawley rats were exposed to 25mM glucose and curcumin for 48hours

to determine the effects of p300 inhibition of MEF2A, MEF2C and several other physiological markers of maladaptive hypertrophy. They showed that curcumin, in addition to significantly decreasing p300 levels also prevented glucose-induced up-regulation of MEF2A and MEF2C mRNA and protein levels. This study also showed that p300 inhibition also affects total MEF2A expression which is a novel finding. A possible explanation for this could be due to the fact that MEF2 can autoregulate its own expression (119) and as MEF2 interacts with p300 (130; 138) for its transcription of many genes in skeletal, it is highly possible that MEF2 recruits p300 for its own expression.

These studies have collectively shown that curcumin is a specific inhibitor of p300 HAT activity in multiple cell lines and subsequently affects p300 mediated gene expression. It is for this reason that curcumin was chosen to investigate the potential role p300 might play at the *Glut4* gene.

CHAPTER THREE

DESCRIPTION OF EXPERIMENTAL PROCEDURES

3.1. Introduction

This study aimed to determine whether p300, a highly expressed histone acetyltransferase (HAT) in muscle, is involved in acetylating histones at the MEF2 binding site on the *Glut4* gene in response to caffeine in C2C12 myotubes. The C2C12 myogenic cell line was chosen for this study as it serves as an appropriate model for the investigation of biochemical and molecular events that occur with respect to CaMK II activation and *Glut4* transcription when treated with 5mM caffeine (44; 101; 110). Our approach to answer this question involved the use of Curcumin, a highly specific inhibitor of p300 HAT activity.

3.2. General tissue culture protocol

C2C12 myoblasts were cultured in Dulbecco's minimum essential medium DMEM containing 5mM glucose and maintained at 37ºC in 5% CO_2. DMEM was supplemented with 10mM creatine, 100µU/ml streptomycin, 100µMU/ml penicillin, 0.25µg/ml *penstrep fungizone* (Highveld) and 10% heat-inactivated foetal calf serum (FCS) (Highveld). Culture medium was changed every second day and cells were washed with 10ml phosphate buffered saline (PBS). Sub-confluent cultures were passaged via trypsinisation with 0.25% trypsin-EDTA (Sigma) when myoblasts reached 60% confluency and reseeded in 10cm^3 Petri dishes (Greiner Bio-one). For the experiments, cultures were grown to 80% confluency and then differentiated for 8-13 days by replacing FBS with 2% horse serum (HS) (Sigma). These cells were also washed every second day with 10ml PBS. After cells were fully differentiated, i.e. when nearly all myoblasts were fused to form myotubes, treatments with caffeine and curcumin commenced.

3.3. Treatment groups

Both caffeine (Sigma) and curcumin (Sigma) were used in this study to address the aims of the study, which was to determine whether p300 was involved in acetylating histones at the MEF2A binding site on the *Glut4* gene. Previously, Mukwevho et al. (101) had shown that caffeine treatment of C2C12 myotubes upregulated total GLUT4 expression via hyperacetylation of H3 (Lys $^{9/14}$) and increased MEF2A binding to the *Glut4* promoter via CaMK II-dependent mechanisms. To determine whether p300 was involved in these effects curcumin was added to the medium to inhibit p300 activity. Two doses of curcumin i.e., 40μM (**Higher dose**) that has been shown to effectively inhibit p300 HAT activity, (8) and 25μM curcumin (**Lower dose**) were administered to C2C12 myotubes to determine if there were dose-dependent effects. In total there were four different treatment groups:

a) Control (**Con**) group that received neither caffeine nor curcumin.

b) Caffeine (**Caf**) group that received 5mM caffeine only.

c) Caffeine plus high dose curcumin (**Caf+Cur40**) that received 5mM caffeine and 40μM curcumin and

d) Caffeine plus low dose curcumin (**Caf+Cur 25**) that received 5 mM caffeine and 25μM curcumin.

Curcumin was dissolved in dimethyl sulphoxide (DSMO), due to its poor solubility in water, to a stock concentration of 100mM. The appropriate concentrations of curcumin needed, i.e. 40μM and 25μM, were prepared by serial dilution and added to the respective culture dishes containing fully differentiated C2C12 myotubes. The final concentration of DMSO (Highveld) was less than 0.01% in culture dishes. Caffeine was prepared by dissoultion in deionized water to a stock concentration of 100mM. The parameters measured in this study were: global (total) acetylated H3 (Lys $^{9/14}$) and p300 contents, GLUT4 content by western blot; acetylated and total histone H3 in the vicinity of the MEF2 binding site on the glut4 gene, and glut4-bound MEF2A by the Chromatin Immunprecipitation (ChIP) Assay.

3.4.Description of treatments :

For determination of global (total) acetylated H3 (Lys $^{9/14}$) and p300 contents, acetylated and total histone H3 in the vicinity of the MEF2 binding site on the *Glut4* gene, and glut4-bound MEF2A cells were treated with caffeine ± curcumin once whereas for assessment of GLUT4 expression, cells were treated with caffeine ± curcumin for 5 days. These treatments are described below:

3.4.1. Acute treatments :

a) Control Group : Myotubes in the control group were treated with DMSO to a final concentration of 0.01%. This was achieved by addition of 10μL of DMSO directly to 10 ml of culture medium and incubation for 7 hours before harvesting.

b) *5mM Caffeine Group* : Caffeine treated cells were also treated with DMSO to a final concentration of 0.01%. However, after one hour 500μL of 100mM caffeine was added to 9500μL of culture medium in 15ml tubes and added to petri dishes containing myotubes and incubated for two hours at 37°C. Cells were then washed with sterile

PBS and replenished with media containing 0.01% DMSO until harvest four hours later. This was done because hyperacetylation of histones at the *Glut4* promoter had been shown to occur ~4 hours after caffeine administration (101).

c) *Caffeine + Curcumin Groups :* In these groups, myotubes were first incubated in media containing 40μM or 25μM curcumin for one hour. Medium was removed and fresh curcumin and 500 μl of 100mM caffeine stock solution added to culture dishes. Myotubes were exposed to the fresh medium containing 5mM caffeine for two hours before they were washed with sterile PBS. Media containing 40μM or 25μM curcumin was re-introduced to the culture dishes and incubated for a further 4 hours. Note: Repetative administration of fresh curcumin was needed because curcumin is metabolized very quickly to metabolites that are ineffective inhibitors of p300 (142). Therefore, in order to achieve an effective concentration within cells, repetitive doses of curcumin are necessary.

3.4.2. Chronic treatment for assessment of GLUT4 content.

Chronic treatment of myotubes with 5 mM caffeine does not increase GLUT4 content substantially but intermittent treatments over multiple days do (110). Therefore, for assessment of the effects of caffeine ± curcumin on GLUT4 expression treatments were carried out for 3 hours per day for 5 days.

3.5. Measurement of protein contents.

After treatments, the cells were harvested, homogenized and analysed by Western blot. GLUT4, α tubulin, p300, total acetyl H3 and total H3 contents were assayed.

3.5.1. Harvesting of crude protein extracts and determination of protein concentrations.

After cell treatments were correctly performed, myotubes were washed twice with cold PBS containing complete protease inhibitors (Roche) at a 1X concentration. Myotubes were then scraped loose from the plates in 300µL of homogenising buffer (10mM Tris-HCl, 1mM EDTA, 5mM $MgCl_2$1.4M sucrose, 10mM $Na_4P_2O_7$, 20mM NaF, 0.15µM

Okadiac acid, 8mM Na_3VO_4, 1X complete protease inhibitors, 10mM NaBu), and aliquoted into tubes. Cells were then lysed on ice for 10 minutes and then sonicated for 10s at 33% output.The protein concentrations of the crude extracts were determined using the Bradford Assay: Five microlitres of protein sample were aliquoted into 1ml of Bradford reagent (0.02% Coomassie brilliant blue G250; 4.75% ethanol; 8.5% phosphoric acid) for 5 minutes and the absorbance was then read at 595nm using a spectrophotometer. Concentrations of the unknown protein samples were determined from a standard curve generated using a series of standard solutions of bovine serum albumen (BSA) (Sigma).

3.*5.2. Western blotting.*

The crude protein extracts described above were solubilised in Laemmli sample buffer (LSB) (250mM Tris-HCl, pH 6.8; 2% SDS, 10% glycerol; 0.01% bromophenol blue, 50mM dithiothreitol [DTT]), incubated at 95°C for 10 minutes and used immediately for western blotting. Protein samples were not heated for GLUT4 western blots, as per specifications from manufacturers (Abcam). Approximately 30µg of each protein sample

as well as 5µL of standard protein ladder (Colourburst, Sigma) was resolved by sodium dodecyl sulphate–poly acrylamide gel electrophoresis (SDS-PAGE). The appropriate acrylamide (Sigma) gel concentrations were used to resolve proteins were performed in accordance with their molecular weight i.e. a 15% gel for Acetyl H3 (17 KDa) blots, a 6.25% gel for p300 (300 KDa) blots and a 10% gel for GLUT4 blots (45 KDa). A quantity of sample protein was loaded into each gel as determined by the Bradford Assay. Electrophoresis was conducted using the Biorad SDS PAGE apparatus at 100V for 1.5 hours in buffer (25mM Tris, 192mM Glycine, 0.1% SDS). Proteins were then transferred to a polyvinylidene diflouride (PVDF) membrane (Hybond) using a BioRad transfer apparatus overnight at 30V at 4ºC in 1 x Transfer Buffer (25mM Tris-HCl; 192mM glycine; and 15% methanol). Following overnight transfer (8- 12 hours), membranes were briefly rinsed in 1 x Tris-buffered saline containing 0.1% Tween 20 (TBST) and then blocked with TBST containing 5% non-fat dry milk (NFDM) for 1 hour at room temperature. Membranes were rinsed in TBST again for incubation with the appropriate primary antibody. Antibodies that were included : Anti-Acetyl H3 Lys $^{9/14}$ (Cat.No.9677,

Cell Signalling), Anti-p300 (Santa Cruz Cat. No. sc-585), anti- GLUT4 (Abcam Cat.No. ab35826), alpha tubulin (Abcam Cat.No. ab4094), anti MEF2a (Abcam Cat.No. 32866) and Histone H3 (Cat.No.9715, Cell Signaling). All antibody titres were diluted 1:1000 in TBST and all incubations with primary antibodies were carried out overnight at 4º C with rotation.

Following primary incubation, membranes were washed three times for 3 x 5 minutes in TBST and then incubated with a HRP-conjugated secondary antibody (anti-Rabbit polyclonal, Thermo-Scientific), also diluted 1 :1000 in TBST for one hour at room temperature with rotation. Membranes were then washed a second time (3 x 5 minutes) and then incubated in enhanced chemiluminescence (ECL) detection solution (Amersham) for 5 minutes. These membranes were then exposed to radiographic film for 1-5 minutes and the films developed using photographic developing and fixing solutions. Films containing developed bands were subsequently scanned using a scanner. Band intensities were quantified using UN-SCAN-IT gel 6.1 software (Silk Scientific). These intensities which were indicative of respective protein levels between

treatments, and were normalised to α-tubulin or histone H3 expressed relative to the control treatment in each blot.

3.5.3. Stripping of PVDF membranes.

Membranes that had been used previously were stripped in order to normalise data. These membranes were washed in TBST briefly and then incubated in Stripping Buffer (0.5M Tris HCl, pH 6.8, 10% SDS, 0.8% β-mercaptoethanol) at 55°C for 35 minutes. Membranes were then rinsed in deionised water, then TBST, blocked with TBST containing 5% non fat dry milk (NFDM) and re-probed for α-tubulin or histone H3 according to the western blotting protocol given above.

3.6. Chromatin immunoprecipitation assay.

The Chromatin Immunoprecipitation assay (ChIP) was used to assess the binding of MEF2A to the GLUT4 promoter and the degree of acetylation of histone 3 (Lys $^{9/14}$) surrounding the MEF2A binding site.

3.6.1. Formaldehyde fixing and sonication.

Following treatments C2C12 myotubes were cross-linked for 10 minutes by adding 270μl of 37% formaldehyde to the culture medium to give a final concentration of 1%. Cross-linking was inhibited by adding glycine (Sigma) to a concentration of 0.125M for 5 minutes. Cells were then washed twice with cold PBS containing 1 X complete protease inhibitors and 10mM NaBu, and then scraped loose from culture dishes and aliquoted into polypropylene tubes (Eppendorf). Samples were the centrifuged at 5000 x g for 5 minutes at 4°C which allowed the cells to pellet. The supernatant was then removed, and the pellet was reconstituted in 270μl SDS lysis buffer (1 % SDS, 10mM EDTA, 50mM Tris, pH 8.1 and 1 x complete protease cocktail) and subsequently lysed on ice for 5 minutes.

Sonication was then carried out to shear chromatin to fragments ~ 300-1,000bp using a Virsonic 60 sonicator (Virtis) set at 33% of maximal power. To achieve these fragment lengths, samples were sonicated for 12 bursts for 15 seconds with 60 seconds of rest between bursts to prevent overheating. Figure 3.1A shows that 12 bursts of sonication was indeed effective to produce shearing needed for immunoprecipitation

whereas 15 bursts produced shearing lengths of mostly 500kb and less. Samples were then centrifuged at 13 000 x g for 10 minutes at 4°C to pellet debris. The protein concentration of the supernatants were determined by the Bradford Assay (Section 3.2.2).

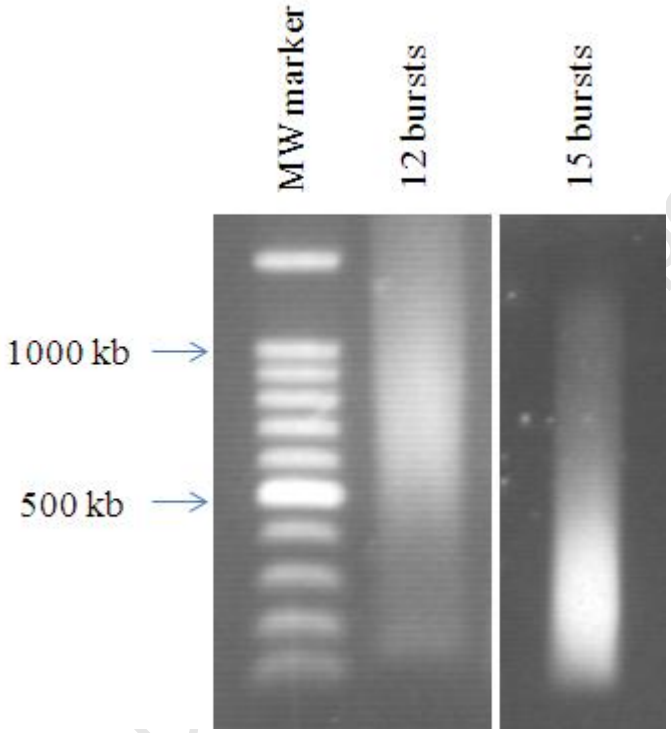

Figure 4 : Agarose gels showing chromatin shearing for the ChIP assay.

C2C12 myotubes were cross-linked for 10 minutes with 37% formaldehyde, then lysed in SDS lysis buffer and sonicated for 12 bursts for 15s seconds (A) or 15 bursts (B) with 33% maximun sonication intensity. Chromatin was then de-crosslinked and electrophoresed 100V for 45 minutes on a 1% agarose gel and stained using SYBR® Gold

3.6.2. Immunoprecipitation, de-crosslinking and DNA purification.

The ChIP kit (Millipore) was used for this procedure. After determining the protein concentrations of the sonicated cell lysates, it was calculated that 200µg of crosslinked protein/DNA sample would be sufficient for immunoprecipitation. This 200µg sample 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) and pre-cleared of any endogenous immunoglobulins by addition of 40µl (~80µg) of salmon sperm DNA/ protein A agarose beads (Millipore) for 1 hour at 4ºC. After this pre-clearing step, agarose was pelleted by centrifugation at 1000 x g for 1 minute. The supernatant, known as “input sample” (IN) was saved. Forty microliters of input sample was subjected to immunoprecipitation by incubation with an antibody against MEF2A (Abcam) (6µl) or acetylated histone H3 antibody (Cell Signalling) (6µl), that targets Lys 9 and Lys 14, for 36 hours at 4ºC. To control for non specific binding, parallel ChIP experiments were conducted using an anti-rabbit immunoglobulin G (IgG), agarose beads only (no antibody).

Following the antibody incubation period, immune complexes were precipitated with 40µl (80µg) of salmon sperm DNA/ protein A agarose beads for 2 hours at 4 ºC. The complexes were then allowed to pellet by centrifugation (1000 x g for 1 minute) and subsequently washed with a range of kit buffers for 2 minutes at 4ºC. These buffers included low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 500mM NaCl), lithium chloride wash buffer (250mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.0) and two washes with TE buffer (10mM Tris-HCl pH 8.1, 1mM EDTA). The washing involved the addition of 1 ml of each buffer to the agarose pellet, a brief incubation in the buffer (1 -2 minutes) with rotation at 4ºC followed by centrifugation (1000 x g for 1 minute at) in preparation for incubation with the next buffer. Washes with TE buffer were done at room temperature prior to elution of the immune complexes from the agarose beads with 150µl elution buffer (1% SDS, 100mM $NaHCO_3$) for 15 minutes also at room

temperature. This elution procedure was done twice to make a final “immunoprecipitate’ (IP) volume of 300µl.

Both Inputs (INs) and IPs were then de-crosslinked by incubation with 0.3M NaCl at 65ºC in a heating block for 6 hours. The protein portion was of the DNA/protein complex was then digested with proteinase K, (Sigma) (10µ/µl) for 1 hour at 45ºC. DNA was then purified using a DNA purification kit (Qiagen). Either 3500µl or 250µl of Binding Buffer was added to the IP or IN respectively. Ten µl of 0.3M sodium acetate was then added to the sample to adjust the pH (7.4) so that it would be most effective for DNA binding. Samples were then added to a spin column (Qiagen), that contained a filter that binds DNA (via a process of adsorption) and centrifuged at 13 000 rpm for 1 minute at room temperature. The column was then washed with 750µL of Wash Buffer (70% ethanol) and centrifuged at 13 000 rpm for 1 minute. DNA was eluted by addition of 30µl of deionised water to the column, and allowed to incubate for 5 minute at room temperature. DNA was collected via centrifugation of the column at 13 000 rpm for 1

minute. DNA concentrations and purity was calculated by spectrophotometry at 260nm using a quartz cuvette.

3.6.3. Polymerase Chain Reaction and agarose gel electrophoresis.

Five µl of DNA from both Input and corresponding Immunoprecipitate sample was amplified using the polymerase chain reaction (PCR) in 30µl reactions which contained: 1 x New England Biopeptide (NEB) reaction buffer, 0.2mM dNTPs, 2.5mM $MgCl_2$ (NEB), 0.2µM forward and reverse primers (Iqaba) (Table 2) and thermally cycled in an XP PCR machine (Bioer) using the following reaction conditions: 1 cycle at 94ºC for 10 minutes, 32 cycles at 94ºC for 30 seconds, 52 -63.9ºC for 30 seconds and 72ºC for 5 minutes. The cell cycle number and $MgCl_2$ concentrations were optimised for each PCR.

Figure 3.2 are representative gels for control ChIP experiments : Figure 3.2 A & B shows that when either no antibody or an anti-IgG was used, no PCR products were found which denoted that little or no non-specific precipitation of chromatin occurred. Figure 3.2C shows that when primers that extend a 5kb region downstream from the Glut4 promoter that does not incorporate the MEF2 binding site were used in MEF2A

ChIPs, no PCR products for formed. This showed us that our protocol was sensitive to the MEF2A binding region on the *Glut4* gene. Figure3.2D shows that our ChIP assay could also detect acetyl H3 at the surrounding the MEF2A binding site on the *Glut4* gene.

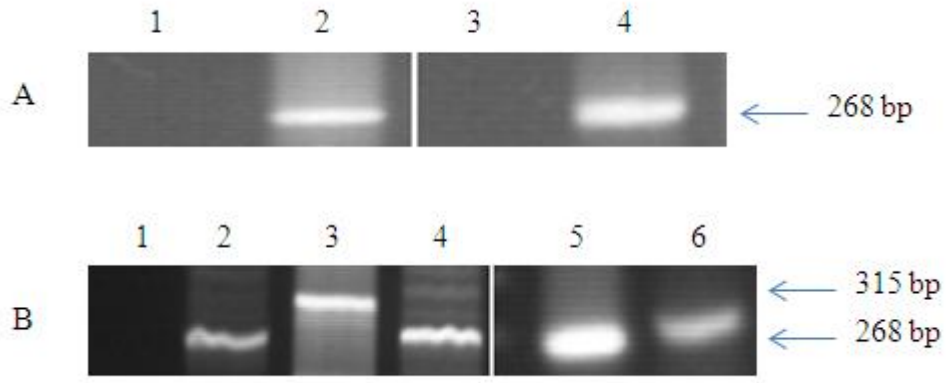

Figure 5 : Representative gels for control ChIP experiments

A: ChIP assays performed on input sample using +ve primers without antibody (with beads only) (lane 1) with IgG (lane 3) and with MEF2A antibody (lanes 2 and 4). **B**: ChIP results from IP samples using + primers and IgG (Lane 1) or MEF2A antibody (Lane 2). ChIP results from IN samples using MEF2A antibody and -ve or +ve primers are shown in lanes 3 and 4, respectively. Lanes 5 and 6 are blots obtained from ChIP assays using acetyl-H3 antibody and +ve primers. The -ve primers amplify a 315 bp region that does not contain the MEF2 binding element. It is located 5kb downstream.

3.7. Statistical analysis of data.

Data generated from these experiments were presented as means ± SD. Statistical differences between all treatments were determined using a one-way repeated measure ANOVA, followed by a Turkey-Kramer post Test, using the GraphPad Instat software. Significance was accepted when $p < 0.05$.

CHAPTER FOUR

RESULTS

Previously, Mukwevho et al. (101) had shown that caffeine treatment of C2C12 myotubes upregulated total GLUT4 expression via hyperacetylation of H3 (Lys $^{9/14}$) in the region surrounding the MEF2 binding domain on the glut4 gene and increased MEF2A binding to the *Glut4* promoter via CaMK II-dependent mechanisms. The purpose of the present study was to determine whether p300 is involved in this hyperacetylation.

4.1. Curcumin inhibits caffeine-induced acetylation of histone 3 (Lys 9/14) at the MEF2 binding site on the Glut4 gene in a dose-dependent manner.

To determine if p300 is involved in acetylating H3 in the vicinity of the MEF2 binding site on the *Glut4* gene, we co-treated C2C12 myotubes with 5mM caffeine for two hours and two doses of curcumin for seven hours and measured the level of H3 acetylation by

ChIP. Figure 4.1 shows that caffeine treatment (Caf) caused a 30 % increase (P<0.001 vs. Control) in acetylated H3 in the region surrounding the MEF2A binding site on the *Glut4* gene. This observation is consistent with the report by Mukwevho et al. (101). Addition of 25μM curcumin (Caf+Cur25) in the culture medium reduced acetyl H3 level by 28% (p <0.001; vs. Caf) whereas 40μM Curcumin (Caf+Cur40) caused a 41% (P< 0.001; vs. Caf) reduction. As curcumin is a potent inhibitor of p300 HAT activity (8), these results indirectly show that p300 plays an important role in the caffeine-induced hyperacetylation of H3 (Lys $^{9/14}$) at the region surrounding the MEF2 site on the *Glut4* gene.

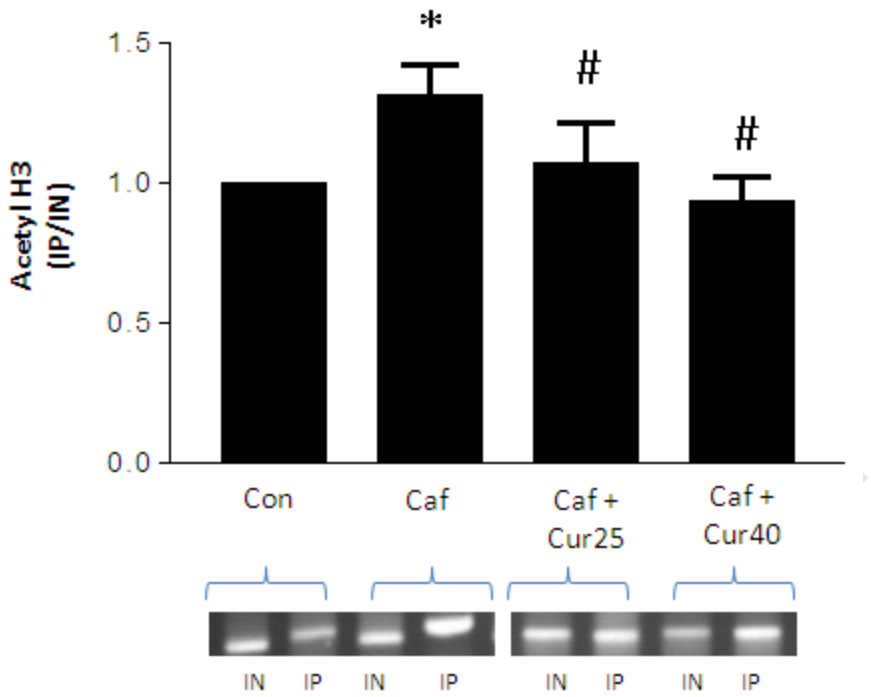

Figure 6 : Curcumin treatment attenuates caffeine-induced hyper-acetylation of histone H3 at the MEF2 binding site in the Glut4 gene in a dose-dependent manner.

C2C12 myotubes were treated with 5mM caffeine for two hours in the presence of 40μM or 25μM Curcumin. Cells were then crosslinked and subjected to the ChIP assay using an anti-Acetyl H3 (Lys 9/14) antibody and appropriate primers (see section 3.3.2). The bar graph represents the ratio of IP (Immunopreciptate)/ In (Input) normalised to control. Sample size n=5, *P <0.001 vs. CON, # P <0.001 vs. Caffeine. Data is presented as means ± SD.

4.2. Caffeine treatment up-regulates p300 content in C2C12 myotubes.

The mechanism by which caffeine and curcumin alter p300 action on the *Glut4* gene has not been studied. To begin these studies, we assessed the total content of p300 in myotubes that had been treated with caffeine ± the two doses of curcumin, as before, using western blot. We found that 5mM caffeine caused a significant up-regulation in p300 content in C2C12 myotubes ($p<0.001$ vs. Con). To our knowledge this represents a novel finding that may provide a mechanism to explain the increase in acetylation of histones in the neighbourhood of the MEF2A binding site on the *Glut4* gene. Inclusion of curcumin in the medium significantly decreased the caffeine-induced up-regulation of p300 in a dose dependent manner: Caf+Cur25 caused a 22% decrease in p300 content whereas Caf+Cur25 reduced p300 by 43% ($p <0.001$; vs. Caffeine; n=5)

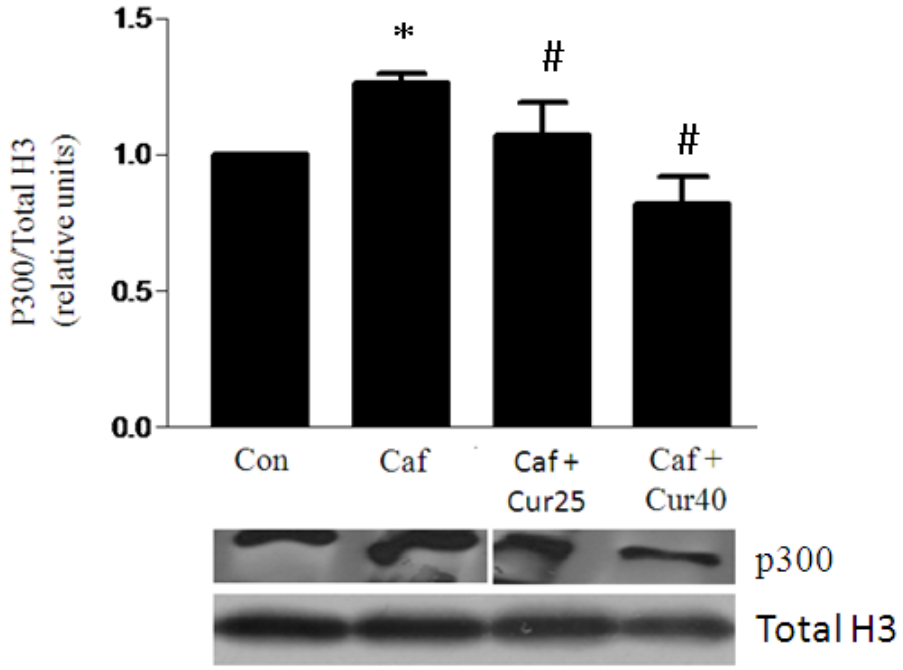

Figure 7 : Curcumin inhibits caffeine-induced increase in total p300 in a dose-dependent manner.

C2C12 myotubes were treated with 5mM caffeine for two hours in the presence of 40μM or 25μM curcumin for seven hours and analysed post treatment for p300 and total histone H3 by western blot. Results are normalised to untreated controls. * vs. Control (P < 0,001, n=5); # vs. Caffeine (P < 0.001, n=5). Data is presented as means ± SD.

4.3. Caffeine treatment increases total lysine 9 and 14 acetylation of histone 3 in C2C12 myotubes.

As the previous experiment showed that caffeine significantly increased total p300 content in C2C12 myotubes, we were interested in whether this correlated with a global increase in acetylated H3. Figure 4.3 shows that caffeine treatment induced a 34 % increase in total acetylated H3 (Lys $^{9/14}$) when compared to control myotubes ($P < 0.001$; vs. Control; n=5). Curcumin treatments decreased caffeine-induced up-regulation of global H3 acetylation (Lys $^{9/14}$) in a dose-dependent manner as shown in Figure 4.3: Caf+Cur 25 decreased total acetylation H3 by 27% ($p < 0.001$; vs. Caffeine; n=5) and Caf+Cur40 by 52% ($p<0.01$; vs. Caffeine; n=5).

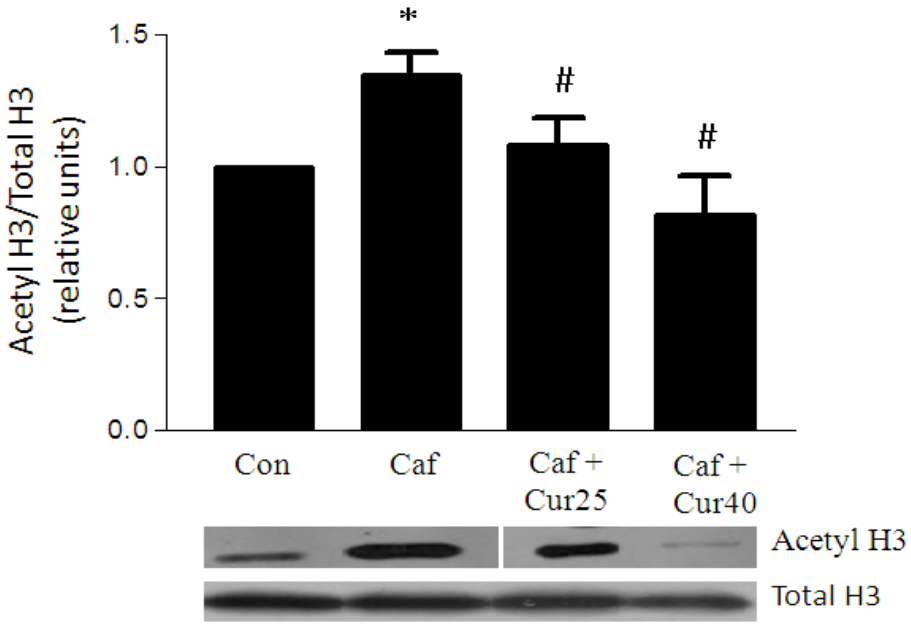

Figure 8 : Curcumin abolishes caffeine-induced hyperacetylation of histone H3 in a dose dependent manner.

C2C12 myotubes were treated with 5mM caffeine ± 25μM or 40μM curcumin for two hours and analysed post-treatment (7 hours) for total acetyl H3 and H3 by western blot. Representative blots and a bar graph are shown. In the graph Acetyl H3/ total H3 are normalized to untreated controls.* P < 0.001 vs. Con; # P <0.001 vs. Caffeine; , n=5. Data are presented as means ± SD.

4.4. Curcumin treatment abolishes caffeine-induced increase in MEF2A binding to the Glut4 gene.

To determine whether the acetylation level H3 in the region surrounding the MEF2 site on the *Glut4* gene correlated with MEF2A binding at the MEF2 site, we performed ChIP assays using appropriate primers and antibodies on myotubes treated with caffeine ± the two doses of curcumin. Figure 4.3 shows that caffeine treatment significantly increased MEF2A binding to the Glut4 gene by 48% when compared to control cells ($p<0.001$, n=5). This result is in accordance with Mukwevho et al. (101). The increase in bound-MEF2A caused by caffeine was completely abolished by Caf+Cur25. Caf+Cur40 further reduced *Glut4*-bound MEF2A to below control levels. As curcumin degrades

p300 and inhibits the HAT domain, the decreased binding observed in these experiments may be attributed to diminished acetylation of surrounding histones or MEF2 protein directly.

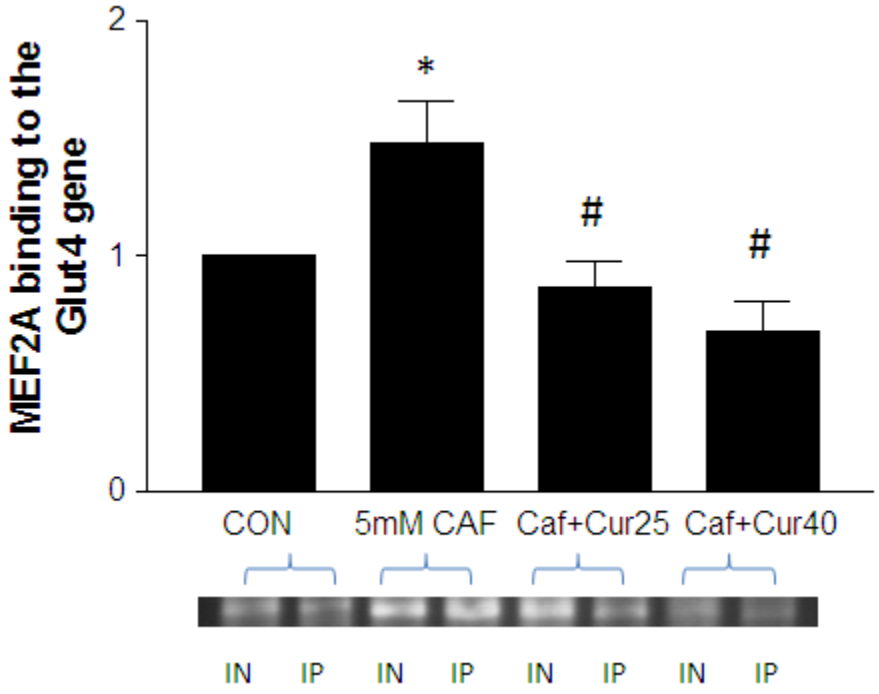

Figure 9: Curcumin treatment decreases caffeine-induced MEF2A binding to the Glut4 gene.

C2C12 myotubes were treated with 5mM caffeine for two hours in the presence of 25μM or 40μM curcumin. Cells were then cross-linked with formaldehyde 4hours after treatments and analysed by ChIP assays using appropriate primers and antibodies. The bar graph represents the ratio of Immunoprecipitated DNA (IP))/ Input DNA (IN) normalised to control. n=5, *P <0.001 vs. Con, # P <0.001 vs. Caf. Data is presented as means ± SD.

4.5. Curcumin decreases the caffeine-induced increase in GLUT4 content.

To assess the effects of caffeine ± curcumin on GLUT4 content in C2C12 myotubes western blots were performed. Myotubes were treated with 5mM caffeine ± 25μM curcumin for 3 hours per day for 5 days. Figure 4.5 shows that caffeine treatment resulted in a significant 16% increase in GLUT4 content after 5 days of treatment ($P<0.05$, $n=6$). This is consistent with Ojuka et al. (110) and Mukwevho et al. (101) who showed that caffeine also increased GLUT4 in L6 and C2C12 myotubes, respectively. Inclusion of 25 mM Curcumin in the medium abolished the effects of caffeine.

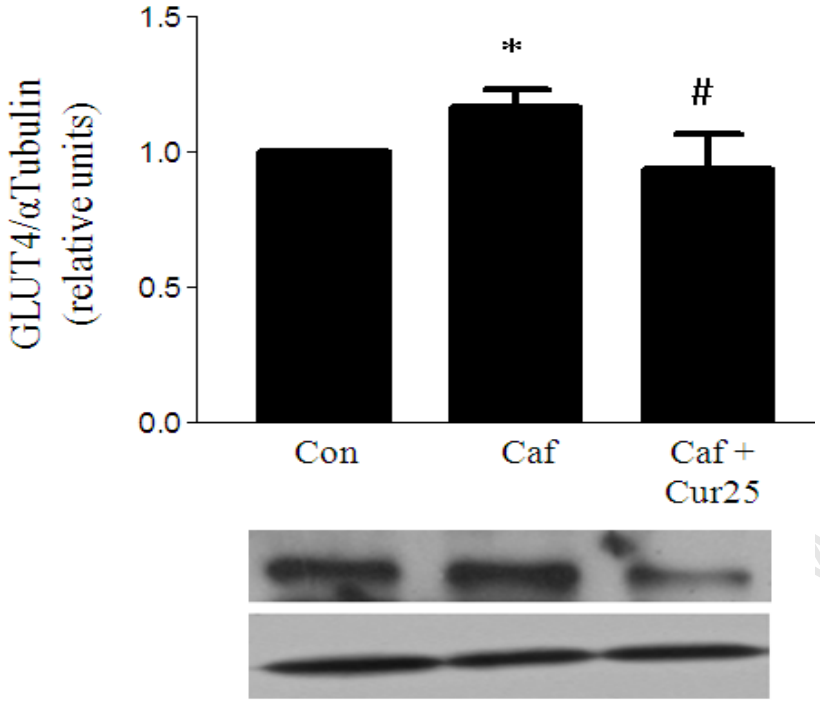

Figure 10 : Curcumin decreases caffeine induced upregulation of GLUT4 content in C2C12 myotubes.

C2C12 myotubes were treated with 5mM caffeine for three hours for five days with or without 25μM Curcumin and analysed post treatment for GLUT4 levels and α-tubulin by western blot described in section 3.2.3. Results are represented normalised to untreated controls with sample size, n=6. Represented are respective GLUT4 and alpha tubulin blots and a bar graph depicting GLUT4 levels normalised to alpha tubulin levels. * $P < 0.05$; # $P < 0.001$ vs Caffeine. Data are presented as means ± SD.

CHAPTER FIVE

DISCUSSION OF RESEARCH FINDINGS AND FUTURE RESEARCH

The aim of this thesis was to determine whether the histone acetyltransferase, p300, is involved in acetylating histones in the region surrounding the MEF2A binding site on the *Glut4* gene in response to caffeine treatment. The *Glut4* gene was of particular interest in the study because upregulation of its expression might serve as a potential therapeutic mechanism for patients suffering from type II diabetes.

5.1. Summary of main findings.

Previously, studies by Mukwevho et al. (101), showed that caffeine induces hyperacetylation of histones in the region surrounding the MEF2 binding domain on the *Glut4* promoter, with subsequent increases in glut4-bound MEF2A and GLUT4 expression in C2C12 myotubes. In this present study, we also found that treating C2C12 myotubes with 5 mM caffeine for 2 hours increased acetyl H3 in this region of

the glut4 gene by ~30%, Glut4-bound MEF2A by ~50% and GLUT4 content by ~ 22%, compared to controls. Furthermore, we have shown that caffeine treatment increased total contents of p300 and acetyl histone H3 by 30% and 40%, respectively. To our knowledge, this is the first study to show these effects of caffeine in skeletal muscle cells. We have also shown for the first time that curcumin decreases all these effects of caffeine in a dose-dependent manner (Figures 9-10). As curcumin is reported to be a highly specific inhibitor of p300 HAT activity (8; 88), these results imply that p300 is involved in acetylating histone H3 globally and also specifically in the region surrounding the *Glut4* gene, in response to caffeine treatment. These results also imply that the content of p300 in C2C12 myotubes influences the binding of MEF2A to the *Glut4* gene and the expression of GLUT4.

5.2. Increase in p300 content by caffeine.

As mentioned above, caffeine treatment increased p300 content rapidly. We observed ~ 30% increase in p300 4 hours after caffeine treatment. The mechanism by which caffeine increases p300 content is not known and requires further research. Nair and

co-workers (102; 103) have previously shown that p300 expression may be induced by calcium-dependent mechanisms. In one study they infected T cells with the human T-lymphotropic virus type 1 (HTLV-1), which localised to the endoplasmic reticulum and Golgi apparatus and caused a sustained release of calcium; they observed increased levels of p300 mRNA and several calcium-regulated genes (102). In a subsequent study they showed that treatment of T Jurkat cells for 36 hours with various concentrations of ionomycin, a calcium ionophore which increases intracellular Ca^{2+}, caused dose-dependent increases in p300 mRNA and protein (103). These studies reveal that the p300 gene responds to changes in intracellular calcium concentration. Previous studies from our group have shown that caffeine, in the concentration used in this study, also increases intracellular calcium concentration (110). Results from the studies by Nair et al. (102; 103) suggest that caffeine might have upregulated p300 via a calcium-dependent mechanism and provide a good starting point for future studies into the role of calcium in p300 expression by caffeine.

5.3. Global and *Glut4* gene histone hyperacetylation by caffeine.

Previous studies have suggested that increased hyperacetylation of histones by caffeine might be due to CaMK mediated effects on HDACs (101). Caffeine activates CaMK which acts on histone deacetylases (HDACs) to induce HDAC nuclear export and increased HAT binding transcriptional factors. However the increase in p300 content also induces hyperacetylation. Wei et al., (153) showed that over-expression of p300 in transgenic mice increased HAT activity without changing nuclear HDAC content. The hypothesis is also supported by findings from other laboratories who showed that total acetyl H3 increases when p300 is over-expressed in the hearts of transgenic mice, and targets MEF-dependent genes (146; 153). In this study, the elimination of global and *Glut4* histone hyperacetylation when p300 content was reduced by curcumin also supports this view point.

Hyperacetylation of histones could also have been due to CaMK-dependent increase in p300 activity since caffeine activates CaMK II. Yaun et al. (162), have

shown that p300 is phosphorylated *in vitro* by CaMK II and this phosphorylation is absolutely necessary for the transcriptional activity of hypoxia-inducible factor 1(HIF-1) in PC12 cells. Co-transfection of these cells with CaMK II -290 and pGAL4p300 phosphorylated p300 and activated HIF-1 but administration of KN-93 to inhibit CaMK II activity abolished these effects. These results show that p300 phosphorylation by CaMK is also important for HIF-1 activity *in vivo*. Chawla et al. (23) have shown that in neronal cells, p300 phosphorylation by CaMK IV is necessary for p300/CBP to mediate its transcriptional functions. These studies provide evidence that there are CaMK–dependent effects on p300 which enhances its transcriptional activity. As caffeine activates CaMK via a release of calcium from the sarcoplasmic reticulum (110), is its highly possible that there were positive CaMK-dependent effects that are enhancing the activity of p300 in our study. Futher experiments using CaMK inhibitors should also be performed in conjunction with caffeine to reveal whether CaMK activity contributes to p300 function in C2C12 mytotubes. Studies of the effects of CaMK activation on p300 HAT activity at the MEF2A binding site on the *Glut4* gene are needed to give further

explanation to the results of this study. In addition to the activation of CaMK II activity in C2C12 myotubes, caffeine has also been shown to activate AMP-activated protein kinase (AMPK) activity in cells (110). Studies have shown that AMPK can also phosphorylate HDAC5 on serine residues that result in nuclear export of HDAC5 and promote interactions between MEF2 and p300 (90). Therefore, the results obtained in this study are indicative of the sum total of CaMK II and AMPK activity. As the aim of this thesis focussed on the HAT functions of p300 and not on the relative contributions of each pathway on GLUT4 expression we did not inhibit either pathway. However, it is accepted and appreciated that these results are the consequences of the activation of both pathways, Further experiments will be conducted to determine the effects of each pathway on the expression of GLUT4 through p300 associations with MEF2. These experiments were not performed for this thesis, but will be perfomed in upcoming publications associated with this thesis.

5.4. Increase in Glut4-bound MEF2A.

Mukwevho et al. (101) showed that caffeine treatment also resulted in a significant increase in *Glut4*-bound MEF2A and a concomitant increase in GLUT4 content in C2C12 myotubes. Hyperacetylation of histones at gene promoters results in a neutralisation of attraction between positively charged histone tails and the negatively charged phosphate molecules of DNA; resulting in chromatin relaxation that increases accessibility to transcription factors (10; 40; 42). Thus, it is reasonable to argue that the increases in acetylation of H3 in the vicinity of the MEF2A binding site on the *Glut4* gene augmented MEF2A binding to its cis-element in this region which subsequently resulted in an up-regulation of GLUT4 expression. Previous studies have also found increased binding of MEF2A to the *Glut4* gene and increased mRNA after caffeine treatment (101).

Increased binding of MEF2 transcription factors to gene promoters may also be due to acetylation of MEF2 proteins. p300 acetylates MEF2C on seven highly conserved lysine residues present on the MADS box and transactivation domains along the protein and results in enhanced binding of the transcription factor to its cis element

on *muscle creatine kinase* (MCK) promoter (4; 83). MEF2A acetylation was not measured in our study, thus we are unable to say whether the increase in *Glut4*-bound MEF2A observed in caffeine treatment was also due to increased MEF2A acetylation. Further experiments would need to be performed to determine whether there was enhanced acetylation of MEF2A in response to caffeine.

5.5. Use of curcumin as a p300 HAT inhibitor.

To determine if p300 was involved in acetylating histones at the MEF2A binding site on the *Glut4* gene we used a selective inhibitor of p300 namely curucmin. Curcumin is highly specific for p300 HAT activity. Studies by Balasubramanyan et al. (8) and Marcu et al. (88) have shown that curcumin bind p300 but not PCAF or GCN5. Curcumin binds directly to p300 and results in a conformational change in the protein that results in a loss of efficiency for the active sites for both histone and acetyl groups. Curcumin inhibits p300 HAT activity by specifically binding the HAT domain of p300 in a sequence of reactions called Michael addition reactions (88). Once curcumin is covalently bound to p300, it renders the HAT domain incapable of acetylation which subsequently tags

p300 for protesome-dependent degradation. Figure 4.2 shows that p300 content was indeed decreased in a dose-dependent manner by curcumin and demonstrates that the curcumin doses chosen in our study was effective in inhibiting p300 content. The changes in p300 content correlates well with changes in global and *Glut4* promoter acetyl H3 and total acetyl H3 levels; suggesting that alterations in acetylation state was mediated by cucurmin effects on p300. Specifically, with regard to GLUT4 expression, the data suggests that inhibition of p300 by curcumin decreases the acetylation state of histone H3 in the region surrounding the MEF2 binding domain on the *Glut4* gene (Figure 4.1) to reduce MEF2A binding to its cis element (Figure 4.4) and subsequently decreases GLUT4 expression (Figure 4.5).

As discussed earlier in this thesis, p300 plays diverse roles in eukaryotic gene transcription. In addition to its HAT functions, p300 is also a bridging molecule that connects transcription factors to the general transcription machinery and acts a scaffold that promotes the formation of an enhanceosome at local promoter regions through

protein–protein and protein-DNA interactions. All these functions are essential for gene expression.

When curcumin binds the HAT domian of p300 it tags the protein for proteosome dependent degradation (88). Thus the binding is sequential in that the effects of curcumin on p300 is thought to be two-fold : binding and subsequently degradtion of the protein. Thus the question of can curcumin bound p300 still perform its transcriptional duties or does curcumin immediately tag it for proteosome dependent degradtion arises. Does this mean that the curcumin molecule serves as a impetus or biomarker for the proteosome degradation pathway? Or does it means that the cell no longer considers p300 a functional protein and therefore recycles its amino acids to produce functional, curcumin free p300? An experiment using myotubes treated with both MG-132, a proteosome dependant degradation inhibitor, and curcumin may indicate to us whether p300 can still perform its function if we assay for total acetylation levels and assays such as ChIP, which may reveal whether p300 can still bind its respective promoters. Since p300 is a potent HAT, and is vital protein for eukaryotic

transcription through its cohort of scaffolding and bridging fuctions to the transcriptional apparatus due to its apparent size, the level of p300 in cells are kept at a concentration that ensures survival (13; 20; 59). Small discrepancies in its content are thought to be deleterious to cells (40), therefore the mechanism by which curcumin induces degradation in cells may be a motive by the cell to degrade the protein to its monomeric constituents so that biosynthesis of p300 occurs, that is p300 that is unbound to curcumin which is fully functional. Thus, curcumin-binding to p300 may be inducing degradation indirectly, and even though curcumin is thought to tag the protein for degradation, this response may actually be a cell-based initiative to promote survival. Therefore the measurement in the responses of acetylation and transcriptional activity of cells that are treated with both MG-132 and curcumin will reveal whether curcumin bound p300 can still perform its functions when the proteosome degradation pathway is blocked which will also demonstrate whether degradation is actually a side effect of curcumin. Or it will reveal whether the use of MG-132 does a disservice to already innate cellular mechanism to keep p300 at a fully functional level so that survival is

uncompromised.From this we can also determine whether the use of MG-132 serves as a curcumin antagonist. These experiments were not in the scope of this thesis but will be performed for upcoming publications associated with this thesis.

Feng et al. (35) have shown that curcumin inhibition of p300 decreases MEF2A expression in cardiac myocytes. The decrease in MEF2A may be attributed to the fact that *Mef2a* expression may be regulated by p300 (119) and is also autoregulated. We did not measure MEF2 levels in response to curcumin in this study. However, if curcumin decreases MEF2A expression in C2C12 myotubes as it does in cardiomyocytes (35), this would also have contributed to the decreased binding of MEF2A to the *Glut4* gene that was seen in the present study. This possibility further underscores the need for exploring more effective inhibitors of p300 HAT activity. Finally, as curcumin has diverse effects in cells, there could be other mechanisms by which curcumin may influence GLUT4 expression in C2C12 myotubes which we have not addressed. Of particular relevance to this study would be curcumin's effects on kinases that regulate GLUT4 expression such as CaMK and AMPK. Interestingly,

curcumin has been shown to inhibit CaMK II expression in mouse hippocampus (145). Whether or not this occurs in myotubes is unknown. With respect to AMPK activation, curcumin has been shown to activate AMPK potently in hepatoma cells which subsequently inhibits gluconeogenesis (64). Although previous studies have shown that 5 mM caffeine does not activate AMPK (110), the combined use of 5 mM caffeine and curcumin on AMPK has not been studied.

5.6. Summary and conclusion.

In summary, this study provides evidence that p300 is involved in acetylating histone H3 at the MEF2A binding site on the *Glut4* gene. p300 interacts directly with the MADS/MEF2 domain of MEF2 transcription factors (130) and possesses a potent HAT domain that is capable of acetylating all core histones at multiple lysine residues along protruding N-terminal tails (9; 108). It also acetylates MEF2 on seven conserved lysine residues along the MADS box and transactivation domains (4; 83). Given these properties of p300 and the many interactions it has with MEF2 transcription factors; it is quite feasible that p300 is also involved in regulating chromatin structure at the MEF2A

binding site on the *Glut4* gene in response to other agents that increase GLUT4 such as exercise or AMPK. Future studies need to look into all these possibilities. Such studies will require genetically modified animals or the identification and development of highly specific inhibitors and activators of p300.

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