University of Alberta

The effects of chronic AICAR administration on metabolic and contractile phenotype in rat skeletal muscle.

by

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Abstract

The AMP activated protein kinase is a ubiquitous signalling system finely tuned to respond to energetic stress. During exercise, cellular energetics are chronically depressed in skeletal muscle and this may be the signal which initiates transformation in metabolic and contractile phenotype. Skeletal muscle has been previously shown to possess the capacity to adapt to imposed environmental demands by dramatically remodelling contractile proteins and reference metabolic enzymes in order to meet changing activity patterns. Ten male Sprague-Dawley rats were included in this study. Five rats were subjected to chronic 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) while five control rats received only vehicle for 28 days. Gastrocnemius (Gastroc), soleus (Sol), plantaris (Plant) and extensor digitorum longus (EDL) muscles were extracted and used to determine changes in metabolic and contractile phenotype. Following the treatment period, there were no changes in the content of myosin heavy chain, a key contractile protein, in any of the muscles. However, MHC IIa mRNA transcripts were significantly decreased in Gastroc muscle. In addition, hexokinase (HXK), citrate synthase (CS) and 3-hydroxyacyl coenzyme A dehydrogenase (HADH) activities were significantly increased in Gastroc and Plant muscle. In addition, HXK content increased significantly in Gastroc and Plant. The ratio of GAPDH/HADH, a previously established marker of metabolic phenotype, was significantly decreased in Gastroc muscle. This suggests that chronic AMPK activation is capable of causing a fast-to-slow transformation of the metabolic phenotype.

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List of Abbreviations

Ad libitum	at one's pleasure
ADP	5'-adenosine diphosphate
AMP	5'-adenosine monophosphate
ATP	5'-adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CoA	Coenzyme A
Cr	creatine
ddH_2O	double distilled water
de novo	new, over again
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiotreitol
ECL	enhanced chemiluminesence
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
emb	embryonic
exoc	extraocular
In vivo	within a whole, living organism
kb	kilo-bases
kDa	kilo-Daltons
mRNA	messenger RNA
β-NADH	β-Nicotinamide adenine dinucleotide reduced
β-NADP	β-Nicotinamide adenine dinucleotide 3'-phosphate
neo	neonatal
PEG	polyethyleneglycol
PCA	perchloric acid
PCr	phospho-creatine
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ZMP	AICAR 5'-monophosphate
ZTP	AICAR 5'-triphosphate

Chapter I

Introduction

For many years the plasticity of skeletal muscle has been studied by researchers wishing to better understand skeletal muscle and its phenotypic transformation during training, inactivity and disease states. Currently the most accepted way to name fibre types is through myosin heavy chain (MHC) content. MHC is the most important myofibrillar protein and is highly correlated with a fibre's velocity of contraction and enzymatic profile. Fibre types are therefore delineated according to their MHC profiles as type I, IIA, IIB or IID. Although the concept of phenotypic transformation is not new, the mechanisms leading to transformation remain elusive. One possible mechanism relates to the presence of nucleotides within the cell. The cellular energy charge (EC) is defined as the ratio ATP/AMP. During fibre type transformations from the fast to the slow phenotype it has been observed that EC drops considerably in accordance with typical stimuli for a fast-to-slow transformation (i.e. increased activity, increases in tonic nerve stimulation). It is therefore possible that phenotypic transformation may occur in response to changes in cellular energetics of the muscle fibre. A likely candidate for the transduction of the EC signal is the AMP-activated protein kinase (AMPK). This pathway has been described as a cellular fuel gauge and has been shown to be highly sensitive to changes in EC. In addition it has been suggested that AMPK can modify downstream targets and cause transcriptional

increases in skeletal muscle. Thus it represents a likely avenue of regulation of muscle fibre phenotype.

Purpose

The purpose of the current study was to determine if AMPK is responsible for altering skeletal muscle phenotype *in vivo*. To this end, five rats were subcutaneously injected with 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) for 28 days while controls received an equal volume of saline solution. After euthanisation, muscle tissues were examined for activity of key enzymes of glycolysis, terminal substrate oxidation and myosin heavy chain (MHC) fibre types.

Significance

While other authors have suggested that AMPK can regulate gene expression in skeletal muscle, this is the first study to examine the extent to which the metabolic and contractile phenotypes are regulated by the AMPK-related pathway. The significance of the work undertaken lies in the potential demonstration of a novel mechanism whereby changes in the cellular energetic potential may regulate contractile changes in skeletal muscle fibres via the AMPK pathway. Knowledge of the factors that regulate phenotype represent an important step toward enhancing our understanding of muscle in health and disease, especially in which the cellular energetic charge is compromised.

Hypothesis

It was hypothesized that AMPK is responsible for detecting and transducing a drop in ATP/AMP and regulating phenotype in skeletal muscle. Specifically, it was hypothesized that chronic AMPK activation with AICAR administration would result in a fast-to-slow phenotypic transformation in rat skeletal muscle as evidenced by the increased expression of slow MHC isoforms (i.e. I, IIa) and/or repression of the fastest isoforms (i.e. IId, IIb). In addition a shift was predicted in the enzymatic profile of muscle fibre toward a more oxidative phenotype.

Limitations

The use of AICAR as a pharmacological activator of AMPK raises some possible concerns. In addition to accumulating ZMP, AICAR may increase intracellular adenosine and nucleotides such as ATP, AMP, GTP and ZTP (101,102). In addition, ZMP may act to mimic the stimulatory effects of AMP-responsive enzymes such as glycogen phosphorylase (136) and fructose-1,6-bisphosphatase (125).

Another possible limitation relates to sample size. With only 5 rats in both the control and treatment groups there is the possibility of a type II error. However, due to the nature of AMPK activation, previous effect sizes have been considerable. Power and sample size calculations following the current work suggest that in order to reach significance for the main outcome of this work (i.e. MHC isoform content), sample size would have to be raised approximately 6 fold.

Definitions

1. Energy charge

Originally defined by Daniel Atkinson in the 1960s, energy charge is the energetic status of the cell as measured by nucleotide concentrations. When referring to AMPK, ADP and Pi are dropped from the calculation of energy charge since they play no direct role in determining AMPK activity (45). Thus energy charge is now calculated as ATP/AMP.

2. Cellular fuel gauge

A theory of AMPK function which proposes that AMPK is responsible for detecting the energetic state of the cell much like the fuel gauge in a car.

3. Contractile phenotype

The biochemical characteristics of the fibre as they pertain to its contractile properties such as peak tension, unloaded velocity of shortening and ATPase activity. This is operationally defined by MHC content and distribution.

4. Metabolic phenotype

The biochemical characteristics of the fibre as they pertain to its preference for oxidative or glycolytic metabolism. This is operationally defined as the ratio of a key glycolytic enzyme to a key oxidative enzyme. A shift in this ratio indicates a shift in metabolic phenotype.

5. Plasticity

Relating to the intrinsic ability of muscle to alter its phenotype and therefore its functional properties in response to imposed environmental demands.

Chapter II

Literature Review

Myosin Heavy Chain Isoforms and Contractile Phenotype in Skeletal Muscle

Myosin, the most important contractile protein of skeletal muscle, is a 480 kDa hexameric protein consisting of two heavy chains and four light chains. While there are at least eight myosin heavy chain (MHC) isoforms, four types predominate in adult skeletal muscle (128), namely slow MHC-Iβ, and fast types MHC-IIa, -IId and -IIb. MHC isoforms are used to delineate the contractile phenotype of fibre types which are thus named type I, IIA, IID and IIB. Muscle fibre contractile phenotype is defined by MHC isoform content as it plays a predominate role in determining functional characteristics such as contractile velocity, maximal isometric tension and myofibrillar ATPase activity. Accordingly, velocity of unloaded shortening is slowest in type I fibres and fastest in type IIB (12). When hybrid fibres are considered, a continuum of fibre types is formed from slowest to fastest based upon ATPase activity and velocity of shortening (11). This continuum is I ↔ IC ↔ IIC ↔ IIA ↔ IIAD ↔ IIDA ↔ IID ↔ IIDB ↔ IIBD ↔ IIB. When viewing this continuum it is important to note that IC and IIC fibres do not correspond to another class of MHC isoform. The 'C' designation is retained from a previous fibre typing method (15) which erroneously identified a new class of fibre intermediate to pure types I and IIA. In reality these fibres co-

express MHC I and IIa in varying amounts however the mistaken nomenclature remains popular. The continuum also represents a shift in economy of contraction with slower fibres being more efficient. That is to say, slower fibres create more tension/mol ATP consumed (13,96). It is noteworthy that this continuum of economy also describes the order in which fibre transformations occur (90,114). Thus, the shift from fast-to-slow following increased activity may represent a compensatory mechanism governed by the need for a more energy efficient phenotype during stressful activity.

Clustering of MHC Genes

Both human and mouse MHC genes exist in clusters. Human and mouse Iβ (slow) and Iα (cardiac) MHC are located on chromosome 14, separated by 4.5 kb of genetic material (103,129). These genes are similarly located in both rat and rabbit (33,67). The rest of the MHC genes are found clustered on chromosome 17 in humans and chromosome 11 in mice (38). Due to the co-ordinated and overlapping manner in which skeletal MHC isoforms are expressed, the clustering of these genes is noteworthy. Using high-resolution techniques the clustering of skeletal MHC genes has been verified (127) (Figure 2-1). In addition to determining the precise clustering of MHC genes the same authors also found that both human and mouse MHC loci are highly conserved in terms of order, transcriptional direction and spacing, suggesting that these qualities may be important during expression. The fact that other multigene families and contractile proteins are not clustered suggests that the arrangement

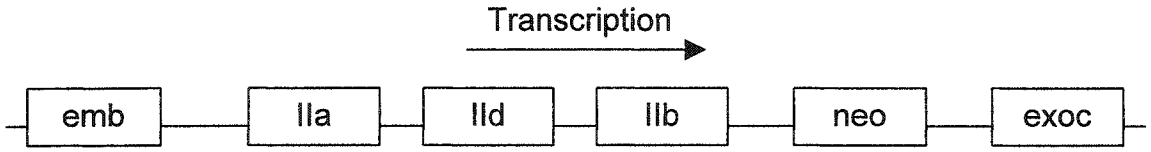

Figure 2-1 The clustering of MHC genes has been determined (24). While the transcriptional order does not correspond to the order of developmental expression it may play a role in a default genetic program in adult muscle.

of the MHC gene family is significant (127). However, the clustering of MHC genes does not relate to their temporal expression during development. It is possible that the ordering of the fast genes is related to their expression in a default genetic program (108). As seen in Figure 2-1, the adult fast genes are arranged in their order of ATPase activity from slow to fast. Additionally, the embryonic isoform which is sometimes recapitulated in situations of extreme fibre damage or stress is located upstream of the other genes (68). Further study should be devoted to determining if the ordering of these genes is indeed related to their expression in a default genetic program.

It would appear that the genes themselves are also highly conserved. Embryonic and extraocular genes share the least similarity with the other isoforms while the three fast skeletal muscle genes share between 92-95% of their base pairs. The 3′ untranslated regions are less conserved and are therefore used to differentiate between the various genes (124). The conservation of location and base pair sequence in these genes is not surprising in light of the convergent functions of their products. Thus, it is possible that the clustering of these genes is due to their similar function rather than some regulatory pattern. This is substantiated by the fact that MHC IIb and IId knockout mice compensate with an overexpression of MHC IIa (1), suggesting

that the expression of MHC is more dependent upon function than genetic localization.

Research Models Which Induce Transformation

A number of models have been used in order to induce contractile phenotype transformations. Models which represent decreased neuromuscular activity such as spaceflight (18), spinal cord transection (120) or hindlimb unloading (32) result in a marked slow to fast transformation (118). In contrast, models which induce increased neuromuscular activity either through endurance exercise or chronic low frequency stimulation induce a fast-to-slow transformation (91). In recent years there has been an attempt to understand the patterns of gene expression during fibre type transformations (87) and to determine which factors regulate gene expression in muscle (5,90,100,118).

Metabolic Phenotype in Skeletal Muscle

While muscle fibres may be organized according to MHC based contractile phenotype they may also be defined in terms of their metabolic phenotype as either fast glycolytic or slow oxidative (86). There is a great deal of overlap between these populations, however it is apparent that the slow population is characterized by increased oxidative capacity and decreased glycolytic capacity. Discrimination between populations can be made by the ratio of a glycolytic enzyme such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to an oxidative enzyme such as 3-hydroxyacyl coenzyme A

dehydrogenase (HADH) (89). Lactate dehydrogenase (LDH) isozyme pattern is another powerful tool for the metabolic classification of phenotype. Type I fibres contain a combination of all five LDH isozymes whereas type II fibres contain only the LDH5 isozyme. According to such measures there exists a continuum of metabolic phenotypes related to functional demands placed on the fibre (88). When metabolic heterogeneity is considered we see that a great deal of complexity is added to any scheme for the classification of fibre type. While this complexity may be undesirable from a reductionist research perspective, it allows for a large range of plasticity within the muscle and exquisite fine tuning of the response to imposed functional demands. Such exquisite regulation suggests intricate control mechanisms that are capable of altering the activity levels of key enzymes in glycolytic, citric acid cycle and oxidative pathways. Interestingly, the metabolic profile of a fibre is much quicker to change following alteration of functional demands than contractile phenotype (89-91).

Calcineurin/Calmodulin

Recently the Ca^{2+} and calmodulin sensitive serine/threonine phosphatase calcineurin has been suggested as a possible regulator of muscle phenotype (23,29). This theory is intriguing since Ca^{2+} is chronically elevated during increases in tonic nerve stimulation (21) and could potentially be linked to the expression of a slow type genetic program.

Calcineurin is a heterodimeric protein consisting of both catalytic (CNA) and regulatory (CNB) subunits. The CNA subunit contains a calmodulin binding

domain and can be activated constitutively (78). When activated by calmodulin or by increased Ca^{2+}, calcineurin can dephosphorylate and activate the nuclear factor of activated T cell (NFAT) proteins (26,95). Both calcineurin and NFAT proteins are ubiquitous, however calcineurin is found in muscle and brain at concentrations ten-fold higher than in other tissues. The NFATc1 and NFATc3 isoforms are also especially abundant in skeletal muscle (50). When dephosphorylated, the NFAT proteins are rapidly translocated to the nucleus. In order for the transcriptional signal to continue, repeated activation of NFAT proteins is required to counter their constant phosphorylation and export from the nucleus (6).

While calcineurin is strongly implicated in the skeletal muscle hypertrophic response (29) its role does not appear to be exclusive. The hypertrophic response in overloaded muscle (29) and with insulin-like growth factor 1 administration (75,109) has been shown to be at least partially mediated through calcineurin. However, a constitutively active form of calcineurin expressed in murine skeletal muscle does not cause hypertrophy (77) suggesting that other pathways must also be involved. In the same way, calcineurin appears to play only a partial role in determining muscle phenotype. A role in regulating muscle plasticity is suggested by Dunn (29) and Chin (23). However their results do not correspond to the overwhelming transformations that can occur in models such as chronic low-frequency stimulation (CLFS) or denervation (for review see (118) and (91)). Recent evidence shows that NFAT transcription factors are not required to promote the expression of a troponin T isoform found in

phenotypically slow fibres (19). This may be explained by the fact that calcineurin can also transmit its effects through the myocyte enhancer factor 2 (MEF2) (134), however further study suggests that calcineurin does not play a dominant role in skeletal muscle transformation (117).

Another pathway which may aid in the skeletal muscle response to increased Ca^{2+} is the calmodulin dependent protein kinase (CaMK). Both calcineurin and CaMK are responsive to increased Ca^{2+} and calmodulin activation. Furthermore they are both capable of activating the MEF2 transcription factors (134). Moreover, both CaMKI and CaMKIV are capable of inducing cardiac hypertrophy in cardiomyocytes and in transgenic mice (85). Further results have led to the belief that these pathways act synergistically through the activation of both NFAT and MEF2 transcription factors.

Cellular Energetics During Transformation and AMPK

A consistent observation during fast-to-slow phenotypic transformation is the considerable drop in ATP/ADP ratio (24). Any drop in the ATP/ADP ratio, such as commonly seen during an exercise bout, is amplified as the ATP/AMP ratio which varies approximately as the square of the ratio ATP/ADP (46). Therefore a 5 fold drop in the ATP/ADP ratio yields a 25 fold drop in the ratio ATP/AMP. The AMP activated protein kinase (AMPK) related pathway responds to situations of cellular stress and specifically to a drop in energy charge (for review see(43)) since it is especially responsive to the latter ratio. This makes it

extremely sensitive to cellular energetics and has prompted previous authors to label it a "cellular fuel gauge" (44).

Known Effects of AMPK

AMPK has a number of important targets which it regulates through phosphorylation and inactivation including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (7) and acetyl-CoA carboxylase (ACC) (20). Recently, AMPK was shown to co-ordinately regulate a decrease in the activity of acetyl-CoA carboxylase and an increase in malonyl-CoA dehydrogenase activity in liver, muscle and adipose tissue following treadmill exercise (83). The outcome of this action is a decrease in malonyl-CoA concentration and subsequent increase in fatty acid oxidation and inhibition of *de novo* synthesis of triglycerides (83). AMPK has also been shown to increase the activity of a limited number of mitochondrial citric acid cycle enzymes following chronic stimulation of the kinase (131) such as citrate synthase, malate dehydrogenase and succinate dehydrogenase. Thus when energy supplies are low AMPK can inhibit key enzymes involved in sterol and fatty acid synthesis thereby deactivating ATP consuming processes. At the same time it seems able to increase the activity of key enzymes involved in oxidative metabolism and glucose transport, presumably to increase the supply of ATP. Previous authors have, therefore, labelled AMPK a "metabolic master switch" (130).

In addition to modifying metabolic enzymes via direct phosphorylation, AMPK appears to possess the ability to alter transcription in a number of tissues.

AMPK has been shown to increase the expression of glucose regulatory proteins (28) and repress certain glucose activated genes (133). Previous authors have shown that AMPK is capable of decreasing the transcription of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in liver (65). Also in liver, AMPK can inhibit transcription of the glucose responsive genes L-type pyruvate kinase and fatty acid synthase (62). Spot 14, a gene whose protein product may be responsible for the activation of lipogenic genes (27) is also inhibited.

Recently the focus of research into AMPK has shifted toward its ability to modify transcription in skeletal muscle. In muscle, glucose transporter 4 (GLUT4) transcription is upregulated by AMPK in possible conjunction with MEF2 (137). The same authors believe that AMPK is responsible for the exercise responsive increase in GLUT4 mRNA (66). Further research has suggested cooperative roles for AMPK and a signalling protein from the same kinase subfamily, CaMK, in the exercise induced increase in GLUT4 (80). A role for AMPK in the regulation of fibre phenotype is suggested since it has been shown that certain metabolic enzymes are co-ordinately regulated with the expression of contractile proteins, including MHC isoforms (30,41). Other authors have speculated that AMPK is a regulator of muscle metabolic (52) and contractile phenotype (82,90).

Mechanism of AMPK Activation

AMPK is a ubiquitous signalling intermediate found in numerous mammalian tissues including liver, heart and skeletal muscle. This enzyme is a heterotrimeric complex consisting of α, β, and γ subunits. Recently it was discovered that there are several isoforms of these subunits. To date, two α isoforms (α1, α2), two β isoforms (β1, β2) and three isoforms of the γ subunit (γ1, γ2, γ3) (22) have been identified. There is no significant kinase activity unless all three subunits are expressed simultaneously (31).

AMPK is activated by an increase in AMP within the cell (46) and inhibited by high ATP (48). Intracellular AMP activates AMPK via 4 mechanisms: 1) allosteric activation of AMPK; 2) increasing the affinity of AMPK to the upstream regulator AMPK kinase (AMPKK); 3) allosteric activation of AMPKK; 4)

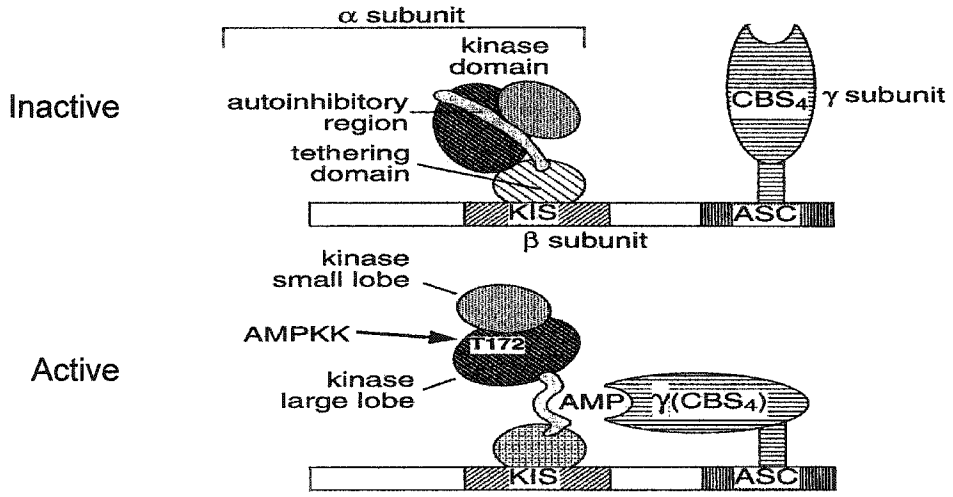

Figure 2-2 A model of AMPK activation adapted from Cheung et. al. 2000 (21). Increases in AMP result in a conformational change in the protein allowing the exposure of Thr 172 and thus its phosphorylation by the upstream kinase AMPKK. AMPKK is activated by the same rise in AMP.

decreasing the affinity of AMPK to inhibitory protein phosphatases (46). When activated, the upstream kinase, AMPKK will in turn activate AMPK by phosphorylating it at Threonine 172 (Figure 2-2). The increased affinity of AMPK for AMPKK continues until almost all of the AMPK has been phosphorylated. This phenomenon is referred to as "zero-order ultrasensitivity" (37) and provides a mechanism for the transient increase in AMP to be translated into a lasting effect. Recently it has been shown that this phosphorylation may increase the sensitivity of AMPK to AMP (115). The same authors suggested that there are other sites of phosphorylation in both the α and β subunits which could lead to fine-tuning of the AMPK response.

AMPK is also activated by increasing Cr and inhibited by high PCr (92). This may be the mechanism by which AMPK is activated during lower intensity exercise, or short term exercise in which large perturbations in the ATP/AMP ratio do not occur. The effects of an increase in the PCr:Cr ratio are thought to be allosteric and therefore, not mediated through the upstream kinase AMPKK (92). It should be noted that the method of empirical measurement of AMPK activation involves preparation of the enzyme which results in the loss of any allosteric modifiers such as PCr or Cr. Thus it is difficult to accurately assess the involvement of the PCr:Cr ratio in regulating AMPK activity *in vivo*.

Experimental methods of AMPK activation are often related to increases in cellular stress levels (e.g. heat shock, hypoxia, exercise, arsenite). It is difficult in these situations to differentiate the direct effects of AMPK from those of other stress activated protein kinases. Another method of AMPK activation is the use

of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR), a known activator of the AMPK pathway (25,49). AICAR is taken up into skeletal muscle and phosphorylated via adenylate kinase to the AMP analogue, ZMP. With this method it is possible to increase AMPK activity without altering nucleotide levels. The effects of ZMP are not completely specific however. It is known that ZMP also mimics the AMP mediated effects on glycogen phosphorylase (136) and fructose-1,6-bisphosphatase (125).

Localization of AMPK Isoforms

While the manner in which AMPK increases transcription is unknown, the $\alpha 2$ isoform is preferentially located within the nucleus of non-muscle (104) and muscle (3) tissues. In addition the $\alpha 2$ subunit is highly expressed in skeletal muscle tissue (113), especially white skeletal muscle (3). During exercise in humans Wojtaszewski et. al. showed that the $\alpha 2$ isoform is activated in skeletal muscle while $\alpha 1$ remains inactive, irrespective of exercise intensity (132). These authors found that the stable activation of the $\alpha 2$ isoform was due to phosphorylation by the upstream kinase while the $\alpha 1$ isoform seemed to be unresponsive to AMPKK. Not only is $\alpha 2$ activation more stable than the $\alpha 1$ isoform due to its increased affinity for AMPKK, but the $\alpha 2$ is more responsive to AMP (104). Along with the preferential localization of $\alpha 2$ in the nucleus these findings make the $\alpha 2$ isoform ideally suited to regulate gene expression in skeletal muscle. Indeed, the sucrose non-fermenting 1 (SNF1) α isoform, a

known homologue of the AMPK α isoform, is capable of regulating gene expression in the yeast *Saccharomyces cerevisiae* (36,99).

Summary

Muscle fibres are functionally defined by their metabolic and contractile phenotypes. During exercise the energetic charge in muscle is chronically depressed. Therefore, energetic charge may be the signal which regulates adaptations in metabolic and contractile phenotypes. The 5'-AMP-activated protein kinase is a potential candidate for regulating the fast-to-slow transformation since it is exquisitely responsive to energetic charge and may play a role in transcription in skeletal muscle. The purpose of this study was to determine if chronic AMPK activation would result in a fast-to-slow phenotypic transformation in rat skeletal muscle.

Chapter III

Methods

Animal Treatment and Care

All animal procedures were completed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of Alberta Animal Welfare Committee. Male Sprague-Dawley rats were housed individually in an environment with 50-55% humidity, at 20 °C, with a light:dark cycle of 12:12 hours. Animals received food and water *ad libitum*. The drug AICAR, a potent activator of the AMPK pathway (49) was chronically administered for 28 days. A previous study involving chronic AMPK activation showed that AMPK was downregulated by chronic AICAR administration (131), therefore AICAR was administered in a stepwise manner (i.e. 0.1 g/kg/day for 14 days followed by 0.5 g/kg/day for 14 days). AICAR was purchased from Sigma-Aldrich Co. (St. Louis, MO) and delivered subcutaneously in sterile phosphate buffered saline (PBS) to 5 rats while 5 controls received an equal volume of PBS. At the completion of the experiment, rats were euthanised via sodium pentobarbital overdose and samples collected. Euthanisation took place 1.5 hours after the final AICAR injection. Under heavy anaesthesia muscles of one hindlimb were freeze-clamped and excised under liquid nitrogen while the contralateral hindlimb muscles were frozen in melting isopentane (-156°C). All muscles were subsequently stored in liquid nitrogen until analysis. The muscles

used in this study were extensor digitorum longus (EDL), gastrocnemius (Gastroc), plantaris (Plant), and soleus (Sol).

Enzyme Assays

Spectrophotometric assays were carried out on Gastroc, Plant and Sol for the enzymes citrate synthase (CS), HADH, hexokinase (HXK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

i) Sample Preparation

Muscles were pulverized under liquid nitrogen into a fine powder. The homogenization procedure for HXK and CS assays was followed according to Suarez et. al. 1986 (116). Pulverized tissue was homogenized on ice in a glass homogeniser in buffer containing 50 mM Tris, 1 mM EDTA and 0.1% Triton X-100 at pH 7.6. Extracts were subsequently freeze fractured three times and centrifuged at 12,000 x g at 4°C for 5 minutes. The resulting supernatant was extracted and used immediately for HXK and CS enzyme activity assays.

Homogenization for HADH and GAPDH was performed according to the method of Reichmann et. al. 1983 (97) in a high salt buffer containing 78 mM Na_2HPO_4, 22 mM KH_2PO_4 and 5 mM EDTA at pH 7.2. Pulverized tissues were homogenized with a glass homogeniser on ice. Samples were centrifuged at 21,000 x g for 30 min at 4°C and assayed immediately.

ii) Hexokinase Assay (EC 2.7.1.1)

The hexokinase assay was performed with the above mentioned homogenate according to an established method (123) measuring the production of NADPH at 340 nm and 30°C. Briefly, 20 μl of homogenate was added to 930 μl of assay buffer containing 100 mM Tris, 5 mM $MgCl_2$, 5 mM ATP, 0.4 mM β-NADP and 0.3 units of glucose-6-phosphate dehydrogenase (Sigma-Aldrich, St. Louis, MO, EC 1.1.1.49). The cuvette was allowed to warm to temperature before the reaction was initiated with 50 μl of 20 mM D-glucose.

iii) Citrate Synthase Assay (EC 4.1.3.7)

The CS assay was performed according to the method of Srere 1969 (112). Briefly, 5 μl of homogenate was added to 945 μl of assay buffer containing 100mM Tris, 0.1 mM DTNB and 0.3 mM acetyl-CoA. The cuvette was allowed to warm up to temperature before addition of 50 μl of oxaloacetate to begin the reaction. The reaction was run at 30°C with the production of mercaptide ion measured at 412 nm.

iv) 3-Hydroxyacyl CoA Dehydrogenase (EC 1.1.1.35)

HADH assays were performed according to the method of Reichmann (97). Cuvette contents included 455 μl of ddH_2O, 500 μl of assay buffer (200 mM triethanolamine-HCl, 10 mM EDTA at pH 7.0), 20 μl of 14 mM β-NADH and 10 μl of homogenate. The cuvette was warmed to a temperature of 30°C before the

addition of 15 μl of 12.5 mM acetoacetyl-CoA to initiate the reaction. Absorbance was measured as the disappearance of β-NADH at 340 nm.

v) Glyceraldehyde 3-Phosphate Dehydrogenase (EC 1.2.1.12)

GAPDH assays were performed according to a previously established method (97). Cuvette contents included 400 μl of ddH_2O, 500 μl of assay buffer (100 mM triethanolamine-HCl, 10 mM EDTA, 6.6 mM $MgSO_4$), 5 μl of homogenate, 20 μl of 14.4 mM β-NADH, 10 μl of 150 mM ATP, 20 μl 120 mM glutathione-SH and 10 μl (65 U) 3-phosphoglycerate kinase (Sigma-Aldrich, St. Louis, MO, EC 2.7.2.3). The cuvette was warmed to 30°C and the reaction initiated with the addition of 35 μl of 200 mM 3-phosphoglycerate. Absorbance was measured as the disappearance of β-NADH at 340 nm.

Hexokinase Western Blots

Western blots for HXK expression were performed in Gastroc, Plant and Sol. Muscle samples were pulverized under liquid N_2 into a fine powder and then homogenized in glass grinders on ice in buffer (50 mM Tris, 1 mM EDTA, 0.1% Triton X-100). Samples were subsequently centrifuged at 12,000 x g and 4°C for 10 min and assayed for protein content with Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples were diluted with standard sample buffer containing 0.4 M Tris pH 7.0, 1.84% sodium-dodecyl sulfate (w/v), 4% 2-mercaptoethanol (v/v) and 6.4% glycerol (v/v)

and loaded onto polyacrylamide gels in the amount of 50 μg/lane. Electrophoresis was performed with the Bio-Rad Protean III apparatus (Bio-Rad) using discontinuous gels and run at 175 V for 1 hour. Gel composition was 5% and 8% acrylamide for stacking and separating gels, respectively. Proteins were transferred onto nitrocellulose membranes for 4 hours at 400 mAmps using the Bio-Rad Mini-Trans Blot Cell apparatus (Bio-Rad).

Membranes were blocked overnight with 5% skim milk powder in Tris-buffered saline (TBS) at 4°C. Membranes were subsequently washed with TBS-tween and TBS and the primary polyclonal anti-type II hexokinase antibody (Chemicon International, Temecula, CA) was applied in blocking solution at a concentration of 1:1000 for 1 hour at room temperature with gentle shaking. Membranes were washed as before and secondary antibody (peroxidase labelled anti-rabbit IgG, Vector Laboratories, Burlingame, CA) was applied at a concentration of 1:1000 in blocking solution for 30 min at room temperature. Visualization was performed with the ECL Western Blotting Detection Reagents kit (Amersham Biosciences, Sweden). All western blots were documented and analyzed with the Syngene Chemigenius bio imaging system using Genesnap and Genetools analysis software (Syngene, U.K.).

Anti-Phospho-Threonine Western Blots

Hexokinase II was immunoprecipitated from Gastroc by incubating 5 μl of polyclonal anti-type II hexokinase antibody (Chemicon) with 30 μl of protein-A

sepharose CL-4B beads (Sigma) in immunoprecipitation (IP) buffer containing 50 mM Tris, 50 NaF, 5 mM $Na_4P_2O_7$, 1 mM EDTA and 1 mM EGTA overnight at 4°C. The beads were then centrifuged at 16000 x g and 4°C for 10 seconds and the supernatant was discarded. Then 1 ml of IP buffer was added and the centrifugation and rinse steps were repeated twice. Beads were incubated for 4 hours at 4°C with 1 ml IP buffer, 10 μl of 10% BSA and 500 μl of 30 μg/ml tissue homogenate diluted in IP buffer. The freshly prepared tissue homogenate used was the same as was used for the previously described hexokinase western blots. After incubation the samples were centrifuged for 10 s at 4°C and 16000 x g and the supernatant was aspirated. 1 ml of IP buffer was added and the rinse procedure was repeated once more. Following IP procedure the beads were resuspended in sample buffer containing 0.4 M Tris pH 7.0, 1.84% sodium-dodecyl sulfate (w/v), 4% 2-mercaptoethanol (v/v) and 6.4% glycerol (v/v).

Electrophoresis and blotting were performed as per the hexokinase western blot procedure above. Membranes were blocked overnight at 4°C in casein blocking solution (Vector Laboratories) followed by washing twice in TBS for 5 minutes. Membranes were then incubated for 10 minutes in 5 ml of casein including 50 μl of avidin D blocking solution added (Vector Laboratories) followed by washing once with TBS-tween for 5 minutes. Membranes were blocked in 5 ml of casein solution including 50 μl of biotin blocking solution (Vector Laboratories) for 10 minutes and rinsed again for 5 minutes in TBS-tween and then twice for 5 minutes in TBS. Monoclonal anti-phospho-threonine (Clone

PTR-8, Sigma) primary antibody was diluted 1:1000 in casein blocking solution and the membranes were incubated for 1 hour followed by three 5 minute washes with TBS-tween and two 5 minute washes with TBS. Biotinylated anti-mouse IgG secondary antibody (Vector Laboratories) was diluted 1:400 in casein and the membranes were incubated for 30 minutes, with gentle shaking. Membranes were rinsed twice for 5 minutes in TBS-tween and then twice for 5 minutes in TBS before incubation with 5 ml of casein, 7.5 μl of reagent A and 7.5 μl of reagent B biotinylated alkaline phosphatase conjugate (Duolux Chemiluminescent/Fluorescent substrate kit, Vector Laboratories). Membranes were rinsed twice for 5 minutes with TBS-tween, twice for 5 minutes with TBS and once for 3 minutes in 0.1 M Tris at a pH of 9.5. The membranes were blotted to remove excess Tris solution and Duolux substrate was added for 5 minutes in the dark. All western blots were documented and analyzed with the Syngene Chemigenius bio imaging system using Genesnap and Genetools analysis software (Syngene).

High Energy Nucleotides and AMPK Activity

Total AMPK and the active fraction of AMPK activity was assayed, as described previously (59), in freeze-clamped Gastroc and Sol after PEG precipitation. The assay buffer contained 40 mM HEPES, 80 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 0.2 mM ^{32}P-ATP, 5.0 mM $MgCl_2$, 8% glycerol, 0.01% Triton X-100, 0.2 mM synthetic AMARA peptide (AMARAASAAALARRR) and 2 μg of the 6% PEG precipitated homogenate. For the measurements of total AMPK, 200

mM 5'-AMP was also added to ensure full activation of the kinase. ^{32}P incorporation into the synthetic peptide was measured for 5 min at 30°C. Final measurements were expressed as pmol of ^{32}P incorporated/mg/min. Portions of the freeze clamped Gastroc and Sol muscle were also used to prepare 6% PCA extracts which were then neutralized with 0.5 M K_2CO_3. The samples were then analysed on the same day for nucleotide concentrations using ion-pair chromatographic analysis according to the method of Ally and Park 1992 (4). Nucleotides are resolved with 35 mM K_2HPO_4, 6 mM tetrabutyl ammonium hydrogensulfate buffer at pH 6.0 using octadecyl columns at a flow rate of 1.5 ml/min.

MHC Isoform Quantification

i) Immunohistochemistry

Melting isopentane frozen Plant and EDL muscles were used for immunohistochemical detection of MHC isoforms via specific monoclonal antibodies and light microscopy (94,107). Muscle sections were cut and applied to poly-L-lysine slides purchased from Cedarlane Laboratories (Hornby, ON, Canada). Sections were washed in PBS and PBS-tween and incubated with 3% H_2O_2 in methanol for 30 min. Blocking solutions were prepared in PBS-tween with 1% BSA and 10% horse (BS-1) or goat serum (BS-2) and sections were incubated overnight at 4°C . Slides were washed as above and antibodies were diluted in either BS-1: NOQ7.5.4D (47) (MHC I, 1:4000); SC-71 (MHC IIa, culture

supernatant, 1:50); BF-45 (MHC embryonic, culture supernatant, 1:1000); F88 (MHC Iα, 1:20); IgG control (non-selective IgG, 1:2000) or in BS-2: BF-F3 (MHC IIb, culture supernatant, 1:50) and applied for 90 minutes at room temperature (107). Slides were washed again before application of biotinylated secondary antibodies diluted 1:400 in appropriate blocking solution. Anti-mouse-IgM/BS-2 was applied to slides which had received the BF-F3 antibody while all others were incubated with anti-mouse-IgG/BS-1. Biotinylated secondary antibodies (Vector Laboratories) were applied in BS-1 or BS-2 for 60 minutes at room temperature. Sections were incubated with Vectastain ABC reagent (Vector Laboratories) for 30 minutes at room temperature and immunoreactivity was visualized with a solution of diaminobenzidine/$NiCl_2$ applied for 6 minutes (Vector Laboratories). This array of monoclonal antibodies allowed direct identification of fibres expressing Type I, IIa and IIb MHC and any associated hybrid fibre combinations. MHC IId was considered present in all fibres not staining for at least one of the other adult isoforms or for the developmental isoform MHC embryonic.

ii) SDS-PAGE

MHC isoforms were also assessed quantitatively via electrophoresis (SDS-PAGE) (119) in Gastroc, Plant and Sol. Briefly, samples were pulverized under liquid nitrogen and prepared according to the method of Laemmli (61) in a 0.4 M Tris solution also containing 1.84% sodium-dodecyl sulfate (w/v), 4% 2-mercaptoethanol (v/v) and 6.4% glycerol (v/v). After heating, the samples were

alkylated via the addition of 10 μl of 20% iodoacetamide. Total protein loaded into each lane was 1.0 μg. Stacking gels of 25% glycerol and 4% acrylamide were used to load samples. Samples were run on Hoefer SE 600 electrophoresis systems (Amersham Biosciences) at a constant voltage of 275 volts for 24 hours at 10°C on separating gels of 30% glycerol and 7% acrylamide. Silver staining was performed according to the method of Oakley et. al. 1980 (79). All gels were documented and analyzed densitometrically with the Syngene Chemigenius bio imaging system using Genesnap and Genetools analysis software (Syngene).

iii) RT-PCR

Portions of the freeze clamped MG muscle were analysed for the expression of skeletal muscle MHC isoforms (I, IIa, IId, IIb) at the mRNA level using reverse transcription-polymerase chain reaction (RT-PCR) according to the method of Jaschinski et. al. 1998 (54). α-Actin primers were selected and checked for binding sites and cross-reactivity against MHC sequences. Samples were extracted with Tri-Reagent according to manufacturer's instructions (Invitrogen, Burlington, ON). RNA concentration was determined by measuring the absorbance at 260 nm using a microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) using flat bottom UV-transparent 96-well plates (Corning Inc. Costar) and samples were diluted to 1 μg/μl. Samples were also examined at 280 nm to judge purity. Reverse transcription of mRNA was

achieved using 1 μl of sample, 0.5 μl (20 U) of RNAse Out (Invitrogen), 0.75 μl (150 U) Murine Moloney Leukemia Virus (Invitrogen), 1 μl of 10 mM DNTP mix (Sigma), 1 μl of 10 μM Oligo dT (Sigma), 1 μl of 0.1 M DTT (Invitrogen), 1 μl DEPC H_20, 1.5 μl of 5X first strand buffer (Invitrogen). The final volume in each tube was 7 μl. Samples were heated for 60 minutes at 37°C. 0.4 μl of the final cDNA product was added to a mix of 4.76 μl of 10X Accutaq buffer (Sigma), 0.95 μl of 10mM DNTP mix (Sigma), 1μl DMSO (Sigma), 21.68 μl DEPC H_20, 5.67 μl of 5μM mixed MHC primers (Sigma, see Table 3-1), 2.48 μl of 5 μM mixed Actin primers (Sigma, see Table 3-1) and 0.236 μl of (1.18 U) Accutaq (Sigma). Annealing temperatures were taken from Jaschinski et. al. (54) (i.e. MHC IIb & IIa: 59°C, MHC IId: 64°C, MHC I: 55°C). The number of cycles varied between 18 and 25.

Table 3-1 Primer sequences for MHC isoforms and α-Actin

Protein Product		Primer Sequence
MHC Iβ	Antisense	GGGCTTCACAGGCATCCTTAG
	Sense	ACAGAGGAAGACAGGAAGAACCTAC
MHC IIa	Antisense	TAAATAGAATCACATGGGGACA
	Sense	TATCCTCAGGCTTCAAGATTTG
MHC IId/x	Antisense	TCCCAAAGTCGTAAGTACAAAATGG
	Sense	CGCGAGGTTCACACCAAA
MHC IIb	Antisense	TTGTGTGATTTCTTCTGTCACCT
	Sense	CTGAGGAACAATCCAACGTC
α-Actin	Antisense	CGGTGAGGATTTTCATCAGG
	Sense	GCGGTGCTGTCTCTCTATGC

MHC isoform primer sequences were taken from Jaschinski et. al. 1998 (50). α-Actin sequence determination has been described in the text.

PCR products were run on 2% agarose gels for 3-4 hours at 100 mAmps and subsequently stained in ethidium bromide solution for 20-30 min. All gels were visualized under UV light and analysed densitometrically via the Chemigenius bio imaging system using Genesnap and Genetools analysis software (Syngene).

Statistical Analysis

All statistics were performed with a computerized software statistics package (SPSS 11.0, SPSS Inc, Chicago, IL). Differences between group means were determined via independent samples t-tests with significance determined at $p < 0.05$. All pre-planned comparisons were undertaken with one-tailed analysis whereas unplanned comparisons required two-tailed significance. One-tailed analysis was used when analysing ZMP, AMPK activities. Enzyme activities of CS, HXK, HADH, GAPH, GAPDH/HADH ratios and HXK expression and phosphorylation were also analysed with one-tailed tests. Finally, MHC content as determined by SDS-PAGE and immunohistochemistry was analysed with a one-tailed test. All data is presented as mean ± standard error of the mean.

Chapter IV

Results

Chronic AICAR Administration Raises ZMP Levels but not AMPK Activity in Skeletal Muscle

The effects of AICAR administration on metabolite concentrations and AMPK activity measured in tissues collected 1.5 hours after final injection can be seen in Table 4-1. ZMP was not detectable in control muscle and significantly increased in treatment muscles to a concentration comparable to similar chronic treatment protocols (16,131). However, this did not lead to detectable changes in AMPK activity after 28 days of treatment. ATP levels were significantly increased in Gastroc muscle and an increasing trend was also seen in Sol after chronic AICAR treatment. Despite an increase in ATP, the ATP/AMP and ATP/ADP ratios, key to the activity status of AMPK, were not significantly altered.

Table 4-1 Nucleotides and AMPK activity

	Gastroc		Soleus	
	Control	AICAR	Control	AICAR
AMP μmol/g	0.12 ± 0.02	0.17 ± 0.02 (4)	0.13 ± 0.03	0.20 ± 0.04
ADP μmol/g	2.35 ± 0.25 (4)	2.74 ± 0.23 (4)	1.32 ± 0.36	1.63 ± 0.13
ATP μmol/g	10.49 ± 0.31	12.84 ± 0.75*	5.64 ± 1.35	7.65 ± 0.08
ATP/AMP μmol/g	94.04 ± 13.32	75.42 ± 6.92 (4)	42.55 ± 4.71	40.36 ± 7.23
ATP/ADP μmol/g	4.60 ± 0.40 (4)	4.67 ± 0.12 (4)	4.36 ± 0.16	4.76 ± 0.37
ZMP μmol/g	ND	1.08 ± 0.15*	ND	0.82 ± 0.34*
Total AMPK pmol/mg/min	596.6 ± 58.3	594.0 ± 94.3	465.1 ± 48.2	598.3 ± 55.5
Active AMPK pmol/mg/min	358.6 ± 45.6	376.4 ± 22.5	305.2 ± 32.6	360.6 ± 26.3
AMPK (% Active)	63.4 ± 13.1	69.6 ± 11.1	66.0 ± 3.9	61.4 ± 4.8

Data are expressed as mean ± SEM. n = 3 for Sol muscle. For Gastroc muscle n = 5 except where indicated in brackets. All concentrations are expressed as grams of wet weight. ZMP levels in control muscles were not detectable (ND). The % Active AMPK represents the active proportion of AMPK in each group. (* = $p<0.05$)

Chronic AICAR Administration Alters Enzyme Activity and Content

Reference metabolic enzymes were assayed in skeletal muscle to determine the effects of AMPK and to determine changes in metabolic phenotype. There were significant increases in HXK activity and expression in Gastroc and Plant but not in Sol (Figure 4-1). In addition HXK II phosphorylation in Gastroc muscle increased significantly from $4.1 \times 10^5 \pm 1.2 \times 10^5$ to $8.9 \times 10^5 \pm 1.3 \times 10^5$ raw densitometric units as determined by western blot (Figure 4-1B). This represented a 2.15 fold increase in phosphorylation. The increases in HXK content appeared very similar to the increases in activity and phosphorylation (~ 2.0 fold increases in all measures). To the author's knowledge, this is the first *in vivo* study to measure changes in HXK content and phosphorylation following AICAR administration.

Similar to HXK activity, CS activity was significantly increased in Gastroc and Plant but not Sol following chronic AICAR treatment (Figure 4-2). In addition, there were significant increases in HADH activity in Gastroc and Plant muscle but not in Sol (Figure 4-2). GAPDH activity was not significantly altered in any of the muscles analysed (Figure 4-2). The GAPDH/HADH ratio decreased significantly from 127.7 ± 13.3 to 61.2 ± 10.8 in Gastroc muscle, however neither the decrease from 30.5 ± 5.4 to 20.4 ± 1.15 in Plant nor the decrease from 7.1 ± 0.2 to 4.9 ± 1.1 in Sol was significant.

Chronic AICAR Administration does not Affect MHC Content

Adult MHC isoform content was quantitatively analysed via SDS-PAGE in Gastroc Plant and Sol muscles (Figure 4-3). There were no significant changes in the relative content of each MHC isoform in any of the muscles analysed (Table 4-2). These results were confirmed via immunohistochemical analysis with specific monoclonal antibodies as illustrated with representative photographs in Figure 4-4 and Figure 4-5. There were no differences detected in the proportion of fibres expressing a MHC isoform type or in the mean fibre area of the various fibre types in either Plant or EDL muscle (Table 4-3). The mean number of fibres analysed in each sample was 462.6 ± 20.0 and 361.5 ± 21.5 in Plant and EDL respectively.

As a final check for changes in contractile genotype, mRNA transcripts were quantified for each adult MHC isoform using semi-quantitative RT-PCR in

Gastroc muscle (Figure 4-6 & 4-7). There was a significant decrease in MHC IIa transcripts in this muscle. In addition, there were decreases for MHC Iβ and MHC IId, although these differences did not reach statistical significance. Only Gastroc muscle was analysed since it showed the greatest changes in parameters which reflect the metabolic phenotype.

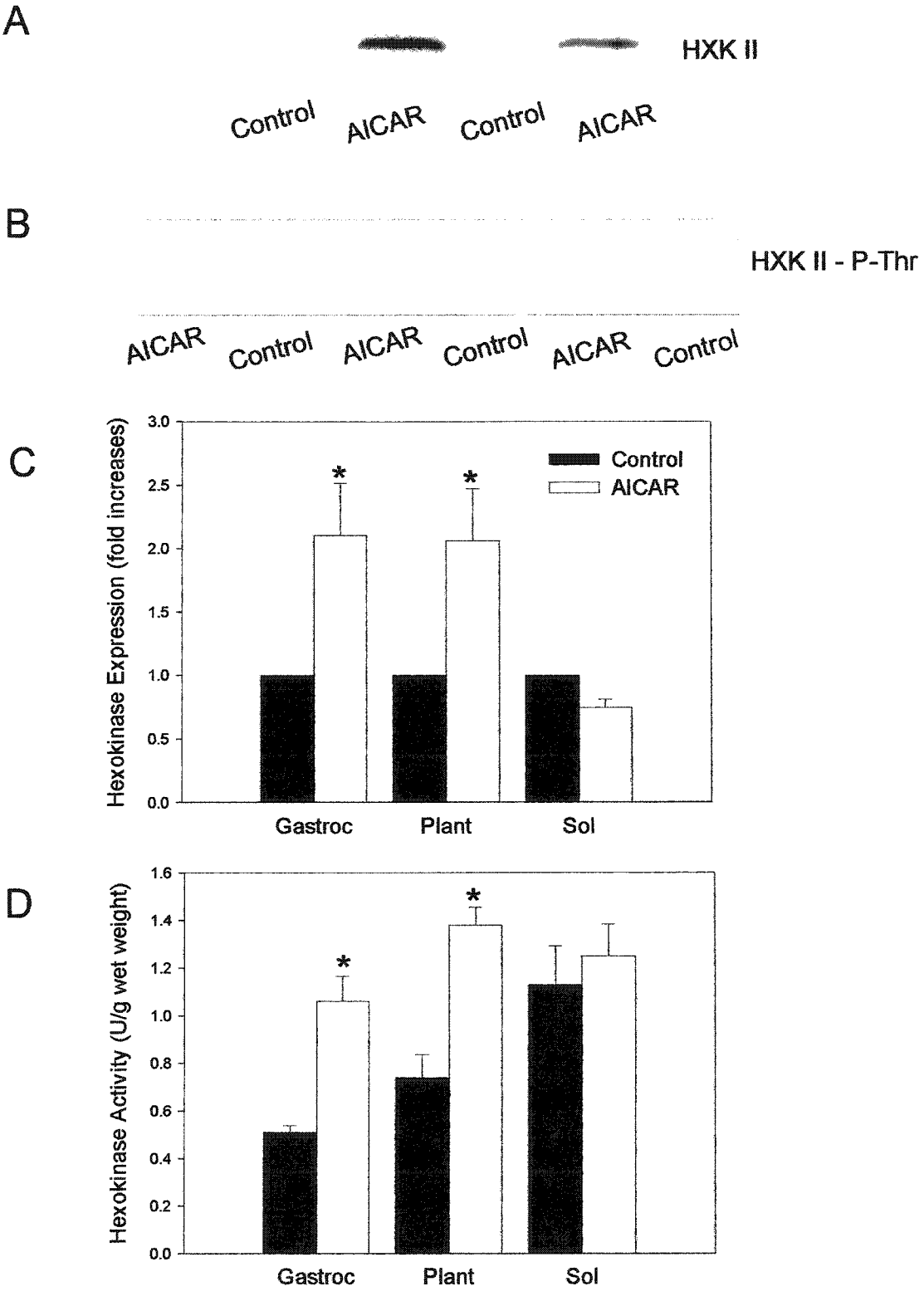

Figure 4-1 Hexokinase expression, phosphorylation and activity. (A) Type II hexokinas western blots (Gastroc). (B) Phosphorylation of HXK II at Thr (Gastroc). (C) Fold increases in HXK expression; (D) Hexokinase activity. All data are mean ± SEM. (* = $p<0.05$)

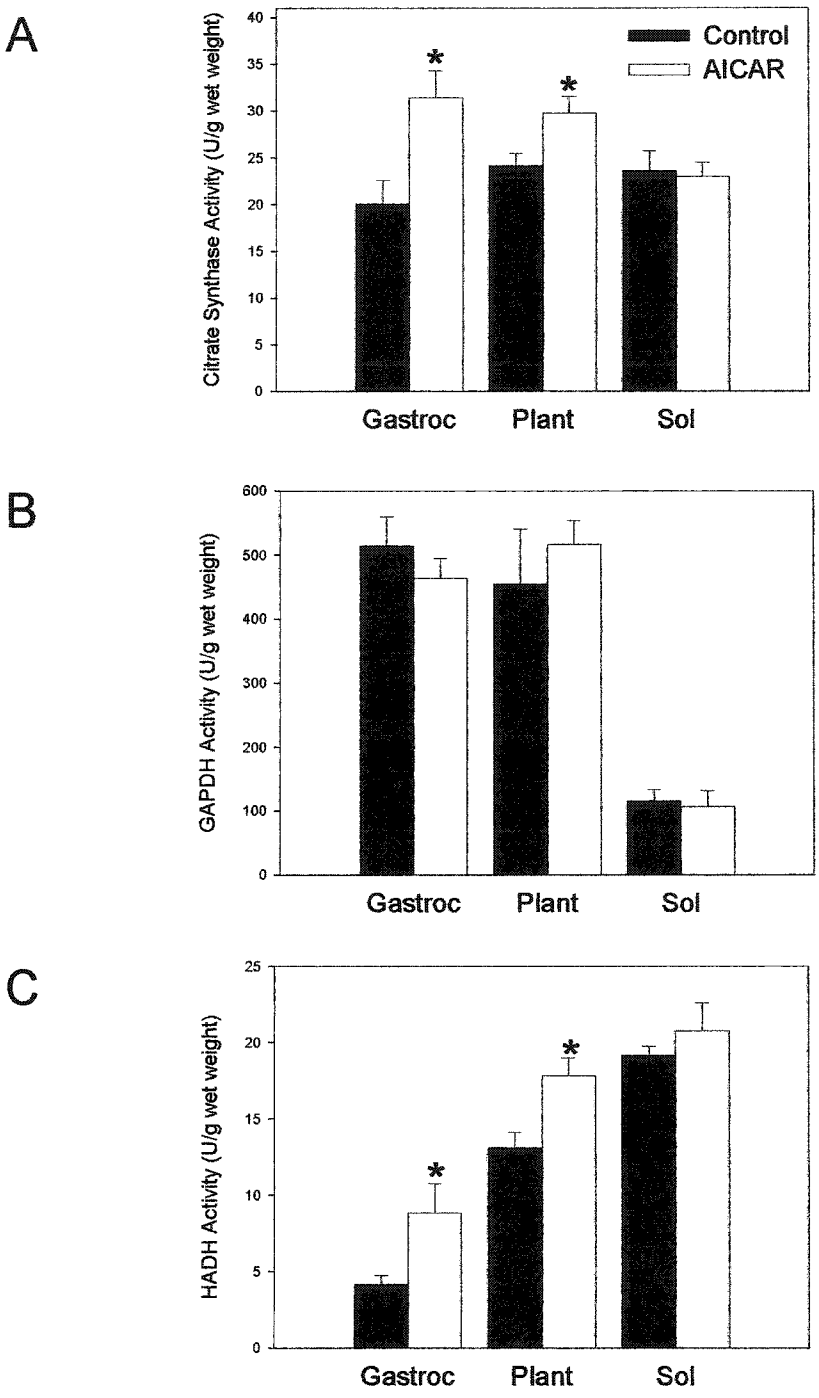

Figure 4-2 Metabolic enzyme activity in Gastroc, Plant and Sol muscles: (A) citrate synthase; (B) glyceraldehyde 3-phosphate dehydrogenase; (C) 3-hydroxyacyl Coenzyme A dehydrogenase. Control and AICAR treated muscles are expressed as mean ± SEM. (* = $p<0.05$)

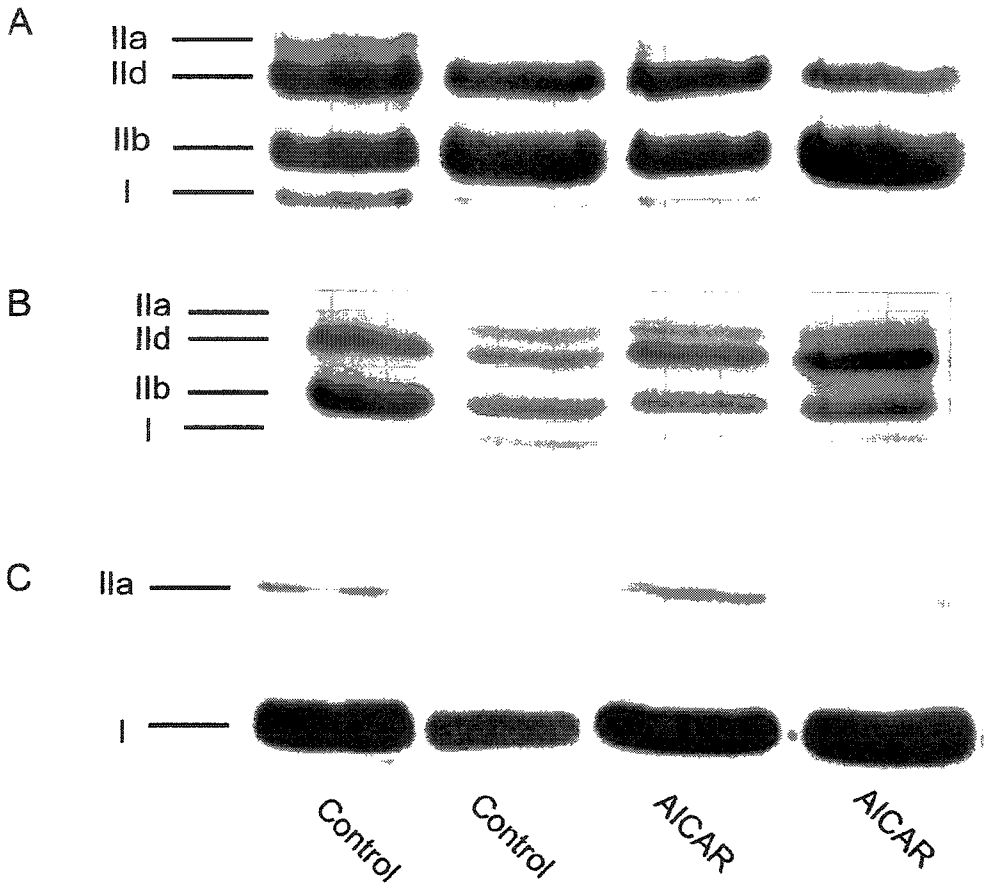

Figure 4-3 Representative MHC separation via SDS-PAGE. Samples shown arefrom: (A) Gastroc muscle; (B) Plant muscle; (C) Sol muscle.

Table 4-2 Relative proportion (%) of each MHC isoform as determined by SDS-PAGE

		I	IIa	IId	IIb
Gastroc	Control	7.7 ± 1.5	9.7 ± 1.7	28.6 ± 2.7	54.0 ± 5.8
	AICAR	10.9 ± 1.6	11.3 ± 1.4	27.5 ± 2.0	50.3 ± 2.5
Plant	Control	12.4 ± 2.6	17.1 ± 2.9	33.0 ± 1.9	37.4 ± 5.0
	AICAR	9.3 ± 0.8	15.1 ± 0.8	33.8 ± 1.7	41.8 ± 2.0
Sol	Control	89.4 ± 6.3	10.6 ± 6.3	ND	ND
	AICAR	87.2 ± 3.7	12.8 ± 3.7	ND	ND

Data are expressed in % as mean ± SEM. n=5 muscles for each group. Some isoforms were not detectable (ND) in certain muscles. (* = $p<0.05$)

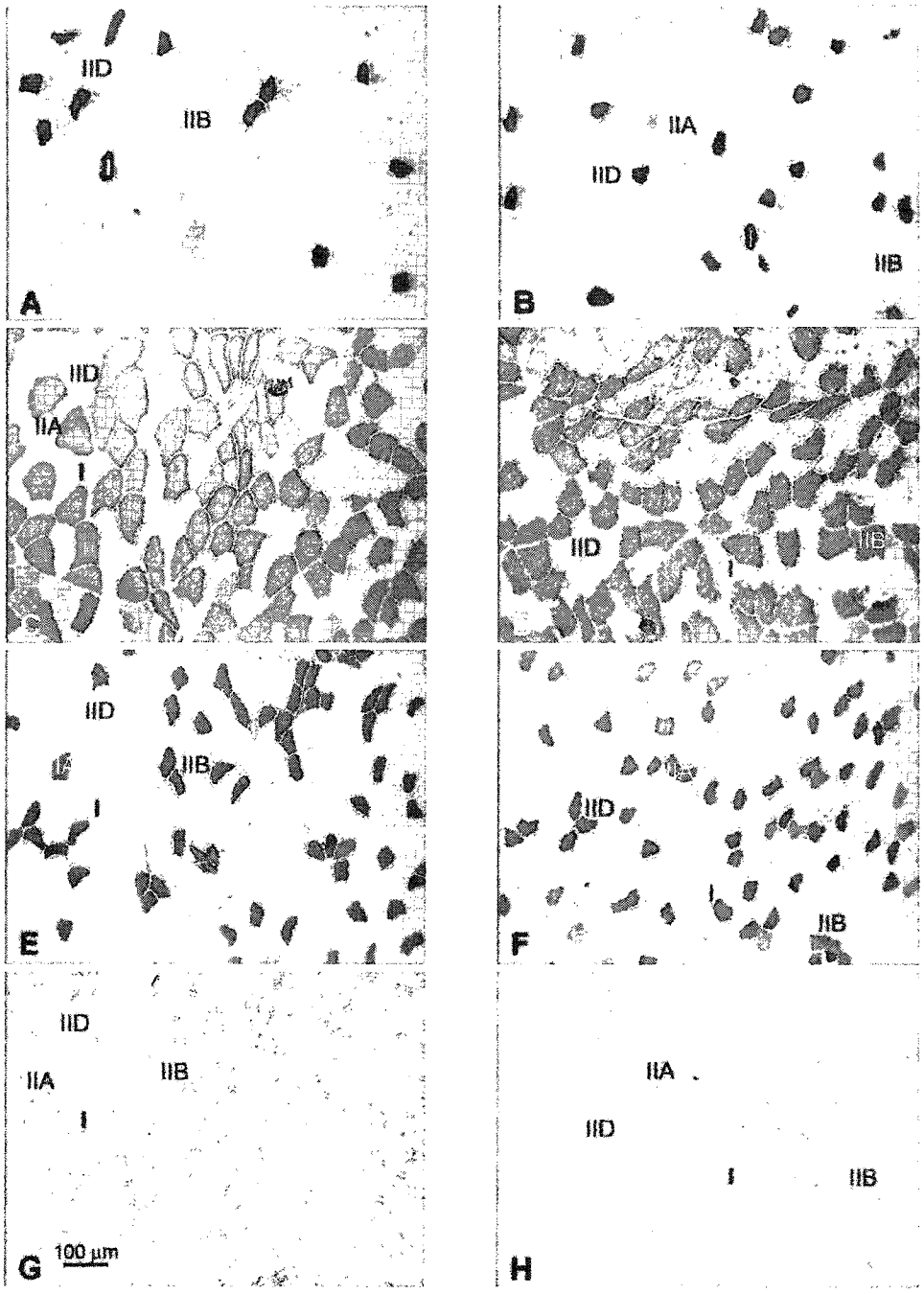

Figure 4-4 Immunohistochemical staining of Control (A, C, E, D) and AICAR (B, D, F, H) EDL muscles. Sections are stained with: NOQ (MHC I - A,B); SC71 (MHC IIA - E,F);BFF3 (MHC IIB - C,D); nonspecific IgG (IgG control - G,H) antibodies.

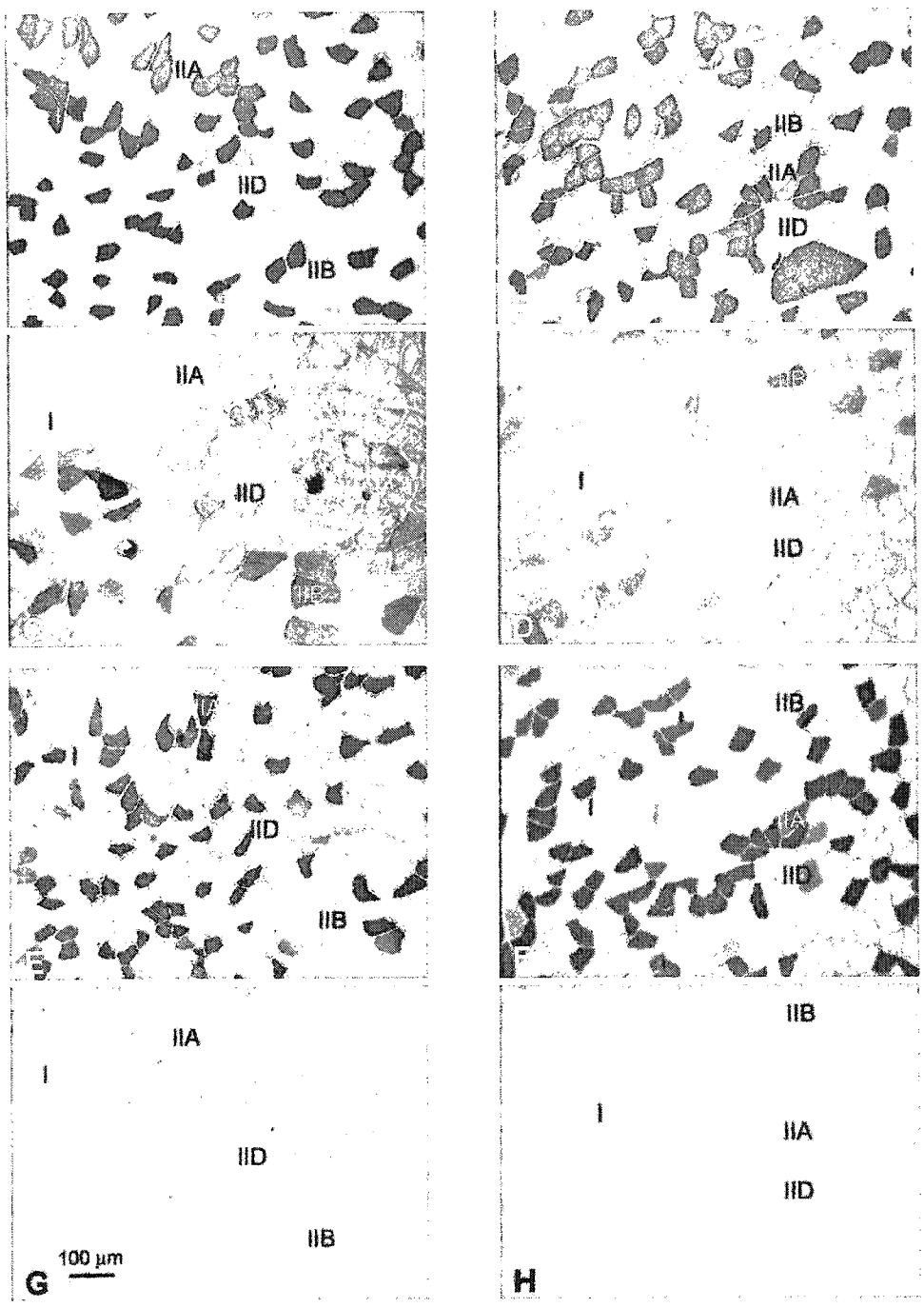

Figure 4-5 Immunohistochemical staining of Control (A, C, E, D) and AICAR (B, D, F, H) Plant muscles. Sections are stained with: NOQ (MHC I - A,B); SC71 (MHC IIA - E,F);BFF3 (MHC IIB - C,D); nonspecific IgG (IgG control - G,H) antibodies.

Table 4-3 Relative proportion (A) and area (B) of MHC content determined by immunohistochemistry

A

		I	IIA	IID	IIB
Plant	Control	26.5 ± 1.9	36.2 ± 2.9	38.6 ± 1.4	4.4 ± 1.2
	AICAR	27.8 ± 3.6	32.1 ± 1.6	34.6 ± 3.1	10.9 ± 2.5
EDL	Control	10.1 ± 0.9	32.0 ± 4.9	28.4 ± 3.8	31.7 ± 2.8
	AICAR	10.9 ± 0.8	30.0 ± 1.7	24.9 ± 2.8	37.4 ± 3.2

B

		I	IIA	IID	IIB	I/IIA
Plant	Control	1739.9 ± 78.9	1622.5 ± 43.5	2294.0 ± 52.9	2284.0 ± 239.8	1296.3 ± 117.5
	AICAR	1676.0 ± 152.1	1770.7 ± 181.2	2211.0 ± 282.4	2483.8 ± 389.7	1539.1 ± 262.1
EDL	Control	944.5 ± 31.4	1137.0 ± 93.8	1803.8 ± 120.3	3012.2 ± 247.5	796.4± 86.2
	AICAR	1051.5 ± 90.5	1266.6 ± 174.8	1985.0 ± 233.9	3335.7 ± 419.7	993.2 ± 130.2

Data are expressed as mean ± SEM: (A) relative proportion (%) of fibers expressing each MHC isoform; (B) mean fiber area for each MHC-based fiber type. n=5 muscles for each group. (* = $p<0.05$)

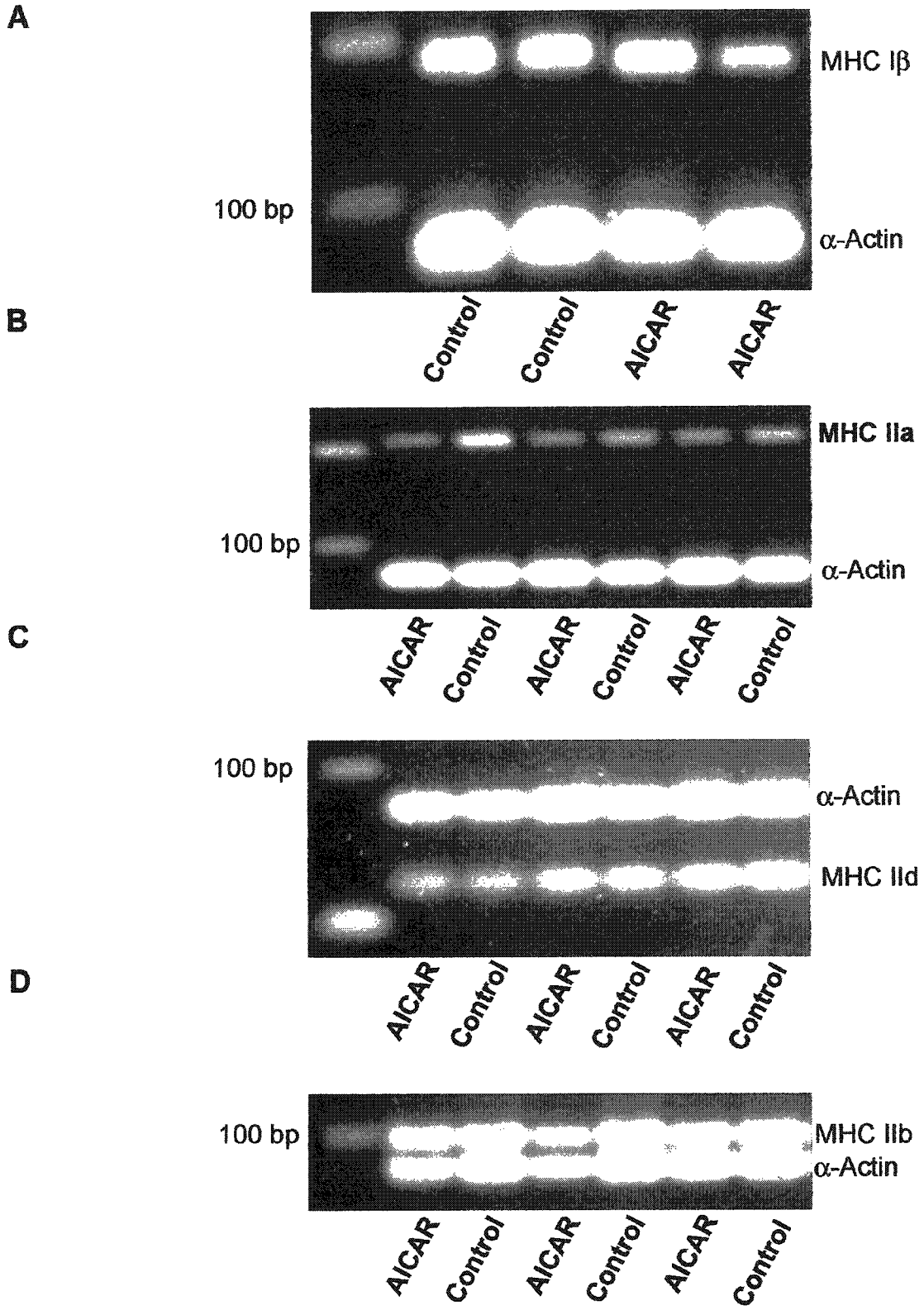

Figure 4-6 Representative RT-PCR gels. (A) MHC Iβ; (B) MHC IIa; (C) MHC IId; (D) MHC IIb

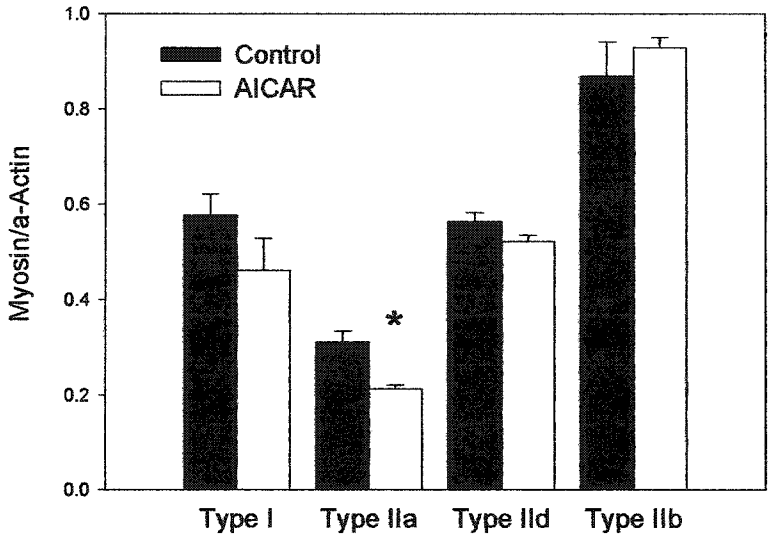

Figure 4-7 MHC mRNA from Gastroc muscle. Data are mean ± SEM. (* = $p<0.05$)

Chapter V

Discussion

Due to its possible role as a cellular fuel gauge and its putative ability to modify transcription in skeletal muscle previous authors have speculated that AMPK may be responsible for regulating metabolic (52) and contractile (90) phenotype in response to stressful circumstances such as exercise. According to this reasoning, AMPK is hypothesized to be a homeostatic mechanism designed to stabilize cellular energetics both acutely and chronically. The acute stabilization may be achieved by increasing the output of ATP producing catabolic pathways while inhibiting ATP consumption in biosynthesis. AMPK could also act chronically by regulating protein expression in these same catabolic and biosynthetic pathways while at the same time altering contractile protein expression in skeletal muscle in order to produce a more efficient fibre. In addition to being better able to maintain a stable energy charge the adapted fibre would be capable of greater workloads and less susceptible to fatigue.

AMPK Activation and Nucleotides

To test the hypothesis that AMPK would regulate phenotype in skeletal muscle, AICAR was administered via subcutaneous injections in a stepwise fashion for 28 days. The average dose over 28 days was 0.3 mg/g and the dose for the final 14 days was 0.5 mg/g, substantially less than the dose of 1.0 mg/g

used in previous studies by others (51,131). Recently, another group administered AICAR in a similar manner at 0.5 mg/g for 7 weeks (16). Despite the different treatment protocols ZMP was significantly elevated to similar levels in four different studies suggesting that lower doses are equally effective at inducing the accumulation of intramuscular ZMP, and presumably activating AMPK (Table 5-1).

Table 5-1 Subcutaneous AICAR injections: ZMP and AMPK response with varying protocols

Study	Duration	Dose	Muscle	ZMP (μmol/g)	Fold Increase in AMPK Activity
Buhl et. al. 2002 (16)	1 day	0.5 mg/g	Red Gastroc	0.70 ± 14*	2.9 fold *
			White Gastroc	0.49 ± 17*	5.5 fold *
Holmes et. al. 1999 (51)	1 day	1.0 mg/g	Gastroc/Plant	0.91 ± 0.02*	2.4 fold *
Winder et. al. 2000 (131)	28 days	1.0 mg/g	Red Quadriceps	0.94 ± 0.08*	0.9 fold
			White Quadriceps	0.42 ± 0.04*	0.9 fold
			Soleus	1.15 ± 0.07*	1.4 fold
Present Study	28 days	0.1 mg/g, 0.5 mg/g	Gastroc	1.08 ± 0.15*	1.0 fold
			Sol	0.82 ± 0.34*	1.2 fold

Data taken from 3 other studies and the present work. All studies employed subcutaneous AICAR injections in sterile saline solution. Duration of injections and dose administered are listed along with ZMP and AMPK response. All data are mean ± SEM. AMPK activity is represented as the fold increase (i.e. AICAR/Control), (* = $p<0.05$)

ZMP Accumulation

ZMP accumulation in intact muscle can be seen as the net result of three processes: 1) AICAR import into the cell, possibly via an adenosine transport system (25); 2) adenosine kinase mediated AICAR phosphorylation to ZMP; 3) phosphorylation of ZMP to ZTP catalyzed by 5-phosphoribosyl-1-pyrophosphate

synthetase (PRPP synthetase) (101). When reviewing the data from other studies and the present one (Table 5-1) it becomes apparent that ZMP accumulates to a higher concentration in red and mixed muscle after a single injection of AICAR. This may be explained by the observation that PRPP synthetase activity is upregulated by the administration of glycogenolytic hormones (9). Therefore it may be more active in white glycolytic muscle resulting in faster depletion of ZMP and production of ZTP in this tissue.

Following chronic injections, muscles accumulate ZMP to concentrations similar to those observed after acute injections, suggesting that the rate of whole body AICAR metabolism has not increased with time despite the possibility of liver hypertrophy (16,131) following chronic AICAR injections. In addition, ZMP metabolism in either red or white muscle does not appear to be altered. As during acute injections, ZMP accumulates to a higher degree in red or mixed muscle after a series of repeated treatments. Interestingly, ZMP appears to reach similar concentrations at both 0.5 and 1.0 mg/g after acute and chronic administration.

AMPK Activation

Surprisingly, the largest accumulation of ZMP does not lead to the greatest increase in AMPK activity. Following acute AICAR injections, the largest AMPK response is found in white muscle which also has the lowest accumulation of ZMP (Table 5-1). In another study, AMPK activity was increased 4-fold in the white epitrochlearis but only 2.4 fold in mixed gastrocnemius/plantaris muscle

following a 1.0 mg/g injection (51). The same group showed 2.9, 1.4 and 2.5 fold increases in white quadriceps, red quadriceps and soleus, respectively, following an acute dose of 1.0 mg/g (131). Thus, acute doses of AICAR cause the largest increases in ZMP within mixed and red muscle, but the AMPK response is greatest in white muscle which contains predominantly IID and IIB fibres. Data from a recent report suggests that white muscle contains considerably more of both the α_1 and α_2 isoform than red muscle (3) which may explain the greater responsiveness of AMPK in white muscle. In addition, Corton et. al. have suggested that at higher concentrations of ZMP, the nucleotide produces an inhibitory effect, possibly by competing with ATP at the catalytic site (25). ZMP mediated inhibition may be a factor in red and mixed muscles since they accumulate the most ZMP and have the lowest AMPK levels. It is possible that red muscle AMPK would be stimulated by much lower concentrations of ZMP.

Interestingly, this study and a previous one (131) both observed a blunted AMPK activity response following chronic AICAR treatment, although ZMP accumulation did not appear to diminish. Despite this, AMPK is capable of upregulating mitochondrial enzymes and hexokinase activity (131) (and this study) following chronic activation. Taken together, these data suggest a disconnect between ZMP accumulation and AMPK activation following both acute and chronic subcutaneous AICAR injection. The lack of comprehensive dose-response data for AICAR administration creates the possibility that previous and current administration schemes are using excess amounts of the drug.

Blunted AMPK Response

In the present study, no significant changes in AMPK activity in either the total or active fractions were detected, although the total AMPK activity was increased 1.3 fold in Sol muscle ($p = 0.05$). Despite this, there were significant changes in the activity of HXK and CS, responses which have been previously shown to be AMPK mediated (51,81,98,110), indicating that AMPK did have an effect in the current study. It is important to note that the assay for AMPK can only measure the portion of AMPK activated due to phosphorylation by its upstream kinase, AMPKK. Thus the effects of allosteric activation of AMPK by ZMP would have been lost in preparation of the extract for assay. Since there was no changes in AMPK activity following chronic AICAR treatment, the *in vivo* activation of AMPK in this study may have been due to allosteric activation by ZMP. In addition, there were no decreases in total AMPK activity (Table 4-1) suggesting that, as compared to acute exposure to AICAR, chronic AICAR administration has some down-regulatory effect on AMPK phosphorylation. This creates two possibilities following chronic AICAR treatment: 1) that AMPKK becomes less responsive to the ZMP stimulus; or 2) that AMPKK expression is diminished. In order to test this hypothesis, future experiments should be undertaken to determine the level of AMPK phosphorylation at Thr 172, and thus the AMPKK activity level, following chronic AICAR treatment. Previous authors have used this method (42) and there is currently a commercially available antibody suitable for this purpose (Phospho-AMPK-alpha (Thr 172), Cell

Signaling Technology, Beverly, MA). Interestingly, other authors have also noted a blunted response of AMPK to chronic AICAR injections (131).

Along with increases in ZMP levels there was a significant increase in ATP in Gastroc muscle (Table 4-1). Similar results were reported by Winder et. al. in chronically treated white and red quadriceps and after acute and chronic AICAR treatments in red quadriceps (131). This may be due to the role of ZMP in *in vivo* purine biosynthesis. AICAR administration has been shown to increase ATP levels in chinese hamster fibroblasts (102) but not in hepatocytes or adipocytes (25). The increase in ATP may have played a role in the blunted AMPK response seen in this study since ATP can inhibit AMPK activity (46). However, the most important determinants of AMPK activity *in vivo*, the ATP/AMP and ATP/ADP ratios, were not significantly altered suggesting that any changes in AMPK activity were due to AICAR treatment alone.

Under normal circumstances AMPK is activated during stressful situations such as exercise which would likely represent a more intermittent and random stimulus for AMPK than chronic AICAR injections. A more intermittent and short term AICAR treatment may be more effective in chronically activating AMPK since it likely represents a stimulatory pattern that better approximates *in vivo* conditions (131).

Hexokinase and Glycolysis

Evidence that AMPK was indeed activated following AICAR treatment is provided, in part, by the increase in HXK activity and expression. Although

others have shown increases in HXK activity following AMPK activation (51,81) this is the first study to show concomitant increases in expression *in vivo*. Interestingly, the significant increases in HXK expression and HXK phosphorylation closely mirrored the increases in HXK activity. The increases in HXK activity in Gastroc and Plant muscles were 2.08 and 1.86 fold respectively while the corresponding increases in HXK expression for these muscles were 2.11 and 2.06 fold. In Gastroc muscle the increase in HXK II phosphorylation at threonine residues was increased 2.15 fold. The similarity between values for activity, expression and phosphorylation in Gastroc muscle suggests that the increased HXK activity is due to changes in expression and not to some direct mechanism of HXK phosphorylation by AMPK. The increased phosphorylation of Gastroc HXK II (Figure 4-1) is likely reflective of the change in expression. Neither HXK activity nor expression was significantly altered in Sol muscle.

Hexokinase activity can be regulated by binding to porin at the outer mitochondrial matrix and formation of a hexokinase tetramer (14). In skeletal muscle this binding is augmented following low-frequency stimulation resulting in a marked increase in HXK activity (84). The increased activity of HXK in brain appears to be due directly to the formation of a tetramer (14). However, skeletal muscle mitochondria are located close to the plasmalemma suggesting that binding of HXK also allows it preferred access to exogenous glucose and to mitochondrial ATP (for review see:(2)) produced by oxidative phosphorylation. In fact, HXK may provide the motive force for glucose import by removing free glucose from the cytosol and "pulling" glucose across the plasmalemma (93).

This could account for the increased glucose transport following acute bouts of exercise where HXK mRNA transcripts and activity are upregulated with no change in glucose transporter 4 (GLUT4) mRNA (2). It appears that the rate-limiting step for glucose import during exercise is in fact phosphorylation by HXK (55,56). The other advantage of giving HXK preferable access to mitochondrial ATP is to replenish ADP and increase the efficiency of oxidative phosphorylation. Thus the binding of HXK to mitochondria and subsequent increase in activity serves to increase glucose uptake and phosphorylation in the short term and perhaps to increase the efficiency of oxidative phosphorylation.

The findings of increased expression and activity of HXK is especially important in light of the recent interest in AMPK as a potential pharmacological target in treating diabetes (130). Previous work has shown that AMPK activation causes an increase in GLUT4 content (137), translocation to the plasmalemma (60), glucose import (72) and an increased responsiveness to insulin administration (17). More recently, the anti-diabetic drug metformin has been used to activate AMPK in skeletal muscle and has been associated with increased glucose disposal and ACC phosphorylation (34,76).

Another potential activator of AMPK in skeletal muscle is the peroxisome proliferator-activated receptor-α agonist, fenofibrate. Fenofibrate has been previously shown to lower serum triglycerides and suppress the expression of ACC, fatty acid synthase and the activity of HMG-CoA reductase in hepatic hamster cells (39). In rats, chronic fenofibrate treatment resulted in significant decreases in serum and muscle triglyceride content and increases in HADH

activity and insulin sensitivity in soleus muscle (35). The convergence of downstream effects of AMPK and fenofibrate treatment suggest that fenofibrate may act through AMPK. Further study should be performed to determine the specificity of fenofibrate and metformin in activating AMPK and to determine their role in the treatment of diabetes mellitus.

AMPK is capable of increasing HXK activity in the short term (81) and long term, along with a concomitant increase in expression (this study). It is likely that this increase in activity is due to an increased fraction of HXK bound to the mitochondria. Interestingly, porin also binds creatine kinase (CK) to the inside of the outer membrane (i.e. within the inter-membrane space) causing the formation of tetramers and octamers (14). In light of the fact that AMPK is known to regulate CK activity through phosphorylation and inhibition (92), future studies should be devoted to the quantification of subcellular fractions and activity of HXK and CK following acute and chronic AMPK activation.

AMPK is implicated in the regulation of glucose transport (72) and HXK activity (this study) as well as glycogen breakdown (136). However, no studies have as yet determined a role for AMPK in the activation of glycolysis in skeletal muscle. Generally, glycolysis is thought to be stimulated by metabolic by-products such as AMP and inhibited by ATP and intermediates such as glucose 6-P, fructose 1,6-P_2 and pyruvate. This is in keeping with the need for rapid upregulation of a system necessary for immediate, high-intensity exercise. AMPK activation is unlikely to be a rapid enough response mechanism for the direct stimulation of glycolysis. Further, the increase in HXK expression and

activity is most probably not an adaptive response to increase glycolytic flux or alter metabolic phenotype but in order to increase the import of glucose GLUT4 (72). This may also explain the negative finding in this study that GAPDH activity was not increased. In fact, increased fatty acid oxidation rates and AMPK activation have been associated with decreases in transcription of the glycolytic enzymes L-type pyruvate kinase (28,63,133) and aldolase B (63). However, a recent study showed that AMPK does play a role in the activation of the heart isozyme phosphofructokinase-2 (PFK2) by direct phosphorylation (69). The authors propose that AMPK stimulates PFK2 to produce fructose 2,6-bisphosphate, a potent stimulator of PFK1. The PFK2 isozyme is not found in skeletal muscle or liver and it seems unlikely that this mechanism can respond quickly enough in cardiac muscle to the demands of immediate, high-intensity exercise.

AMPK and Metabolic Phenotype

Recent studies have shown that AMPK has the ability to increase mitochondrial enzyme activities (131), especially those of the citric acid cycle, and induce mitochondrial biogenesis (80). This is in keeping with our results that CS and HADH activity are upregulated following chronic AICAR administration (Figure 4-2). In addition, the GAPDH/HADH ratio was significantly decreased in Gastroc muscle, suggesting a fast-to-slow transformation in metabolic phenotype of this fast twitch muscle. This represents the significant finding that AMPK is capable of altering fibre phenotype in rat skeletal muscle. To the author's

knowledge this is the first study to establish this connection. Interestingly, previous authors showed no increase in HADH following chronic AICAR injections (131). This discrepancy may be related to the differences in HADH assay procedures between this work and the work by Winder and colleagues. It is also possible that the differences in HADH activity may be specific to the different muscles they were assayed in.

Mitochondrial biogenesis is an important adaptation during fast-to-slow transformation of metabolic phenotype. Recently, the nuclear respiratory factor 1 (NRF1) transcription factor was identified as an important stimulator of mitochondrial biogenesis (for review see (105)). NRF1 is a transcriptional activator whose genetic targets include those involved in respiration, heme biosynthesis and mitochondrial transcription and replication (see Table 1.0 of (105) for a comprehensive list of target genes). In muscle, increases in cytochrome *c* content and δ-aminolevulinate synthase (ALAS) mRNA transcripts have been linked to AMPK mediated increases in NRF1 activation (8). This is in keeping with the observation that NRF1 is activated in 5-day endurance trained rats following an acute bout of exercise (74). In addition to NRF1, mitochondrial biogenesis has been linked to the transcriptional coactivator peroxisome proliferator activated receptor γ coactivator-1 (PGC1) (135). Currently, PGC1 is thought to act through binding to and activating NRF1. Evidence for this theory is provided by the findings that PGC1 and NRF1 coimmunoprecipitate together and interact in an *in vitro* binding assay (106). Moreover, PGC1 expression induces mitochondrial biogenesis which can be inhibited by expression of a dominant

negative mutant of NRF1 (106). In skeletal muscle, PGC1 expression caused an increase in the mRNA for NRF1 and NRF2α (135) and a 77% increase in O_2 consumption *in vitro*. In addition, PGC1 was able to bind and coactivate NRF1 leading to an increase in transcriptional activity of the gene for the mitochondrial transcription factor, an important promoter of mitochondrial DNA replication (135). The effects of PGC1 activity *in vivo* have been recently investigated with the use of transgenic mouse lines. Results of these experiments have shown that PGC1 overexpression leads to increases in myoglobin, mitochondrial enzymes and a fast-to-slow shift in fibre phenotype, although the authors point out that the phenotypic transformation is not complete (64). Also quite recently, PGC1 mRNA was shown to be increased in rat muscle following either swimming exercise, electrical stimulation or AICAR administration (121).

It appears that AMPK is capable of increasing the expression of NRF1 and PGC1 following exercise or AICAR administration (121). This suggests that AMPK may be a regulator of mitochondrial biogenesis in skeletal muscle, an idea that fits well with the present conclusions that AMPK is capable of altering metabolic phenotype *in vivo*.

AMPK and Contractile Phenotype

MHC isoform content was unaltered by the current treatment protocol as measured by SDS-PAGE and immunohistochemistry. There was, however, a significant decrease in MHC IIa mRNA transcripts following chronic AICAR administration and nonsignificant decreases for both MHC I and MHC IId.

However, these decreases were not coordinated as might be seen during exercise induced fast-to-slow transformation of the MHC content. Indeed, the putative existence of a default fibre phenotype implies tight coordination of MHC isoform expression during transformation (108). Instead, the greatest decreases were found in the slower MHC isoforms (i.e. MHC I and MHC IIa), with smaller decreases in MHC IId and no change in MHC IIb (Figure 4-7). This may relate to the fact that MHC IIb has the lowest turnover rate of all the MHC isoforms and therefore the longest half-life (122). Based on the results of this study, AMPK is not likely to be responsible for altering contractile phenotype. In retrospect this result does not seem surprising in light of the established role of AMPK in inhibiting cell growth (57,102) and differentiation (40). In addition, AMPK inhibits global protein synthesis during stressful situations (10). In keeping with the "cellular fuel gauge" hypothesis of AMPK function it is unlikely that AMPK would undertake the energetically demanding process of remodelling muscle tissue during or immediately following an energetically stressful event. Our findings suggest that chronic AICAR administration is insufficient to alter contractile phenotype *in vivo*.

Future Research Directions

Convergence of Pathways

Recent evidence has shown that calcineurin, a Ca^{2+}/calmodulin responsive serine/threonine phosphatase is capable of altering MHC content,

upregulating glycolytic enzymes and regulating metabolic and contractile phenotype in response to increased intracellular Ca^{2+} (6,30). As reviewed above, both calcineurin and CaMK IV are capable of activating the transcription factor MEF2 independently or in a synergistic manner. MEF2 factors appear to be necessary to upregulate the myoglobin and troponin I enhancers found in slow (type I and IIA) fibres (134) and are capable of binding to MHC promoter sequences (134) which suggests that they may play a role in the expression of a slow fibre genetic program. AMPK has also been implicated in the upregulation of MEF2 transcription factors (80).

PGC1, a transcriptional coactivator which plays a significant role in mitochondrial biogenesis (105,106) has been shown to be regulated by both AMPK (121) and calcineurin (64). Althought there is currently a great deal of research into energy and Ca^{2+} responsive pathways it seems apparent that neither of these stimuli is sufficient on its own to regulate muscle plasticity. However, owing to the increase in reports of their convergence it remains a distinct possibility that Ca^{2+} and energetically responsive pathways such as AMPK converge in a complex system to coordinately alter contractile and metabolic phenotypes.

AMPK, Hexokinase and Cancer

An interesting observation in a number of tumour cell lines is the increased dependence on glucose metabolism for proliferation and cell growth (for review see (111)). As hepatocarcinogenesis proceeds, an isozyme switch

takes place such that the muscle form of HXK (HXK II) is overexpressed at the expense of the liver specific glucokinase (58). Findings such as these have lead cancer researchers to search for the control mechanisms regulating both the increase in HXK II and its preferential binding to mitochondria (70). The current study points to AMPK as a likely regulator of HXK increase and speculates on the role of AMPK in inducing mitochondrial binding in skeletal muscle. It is possible that AMPK could also represent a control point in the induction of aerobic glycolysis in neoplastic carcinomas. Many of the phenomena observed in tumour cell lines have been shown to be under the control of AMPK in other tissues including excessive glycogen storage (73), reduced gluconeogenesis (125,126), mitochondrial biogenesis (80), increases in citric acid cycle and electron transport enzymes (131) and the increase in HXK II (this study).

Recently, AICAR treatment and AMPK activation was shown to phosphorylate hepatic wild type p53 *in vitro* leading to an inhibition of cell growth (53) in a cancerous liver cell line. This result is in keeping with the function of wild type p53 in cell cycle arrest and fits a previously established role for AICAR and AMPK in inhibiting cell growth (57,102) and differentiation (40). If, under energetically stressful conditions AMPK were activated it could act via phosphorylation of wild type p53 to upregulate HXK and inhibit cell growth and differentiation, in keeping with its role as a cellular fuel gauge. In support of this, cellular AMP is a known inhibitor of proliferation in cancer (71). In addition, AMP and creatine analogues have been previously used to inhibit cell proliferation by interfering with energy metabolism (for review see (71)). The use of glucose

analogues and inhibitors of glycolysis are also under investigation in the hopes of producing treatments that result in the starvation of rapidly growing and proliferating tumour cells.

Conclusions

The most common method for *in vivo* or *in vitro* AMPK activation is administration of AICAR. However, it appears that current AICAR injection protocols are administering the drug in excess amounts. This may sexplain why many studies have shown the large accumulation of ZMP in red muscle but no corresponding activation of AMPK. Chronic AICAR administration, both in this study and a previous one, failed to show increases in AMPK activity following the treatment period. However, known downstream targets of AMPK were significantly altered suggesting that the AMPKK response to ZMP has been downregulated and that the affects of AMPK after chronic activation are attributable to allosteric modification by ZMP. If AICAR remains a popular method for activating AMPK there is a need for conclusive dose-response data concerning its use *in vivo* and *in vitro*.

To this author's knowledge this is the first study to establish a role for AMPK in the regulation of metabolic phenotype in skeletal muscle. In contrast, this study did not find a link between AMPK and contractile phenotype. A number of previous studies have shown partial or incomplete transformations after activating various signal transduction pathways. It seems likely that no one pathway is an exclusive regulator of muscular plasticity. Rather, numerous

intracellular signals and hormonal messengers are likely interwoven into a complex scheme in order to regulate phenotype. The advantage of such a scheme is that it would allow the muscle fibre to be exquisitely regulated and afford a great deal of heterogeneity in order to meet the specific environmental demands imposed upon the fibre.

Chapter VI

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