2008

The Regulation of Mitochondrial Uncoupling Proteins in the Heart

Karl Robert Laskowski
Yale University

The Regulation of Mitochondrial Uncoupling Proteins in the Heart

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Joint Degree of Doctor of Medicine and
Master of Health Science

by

Karl Robert Laskowski

2008

ABSTRACT

THE REGULATION OF MITOCHONDRIAL UNCOUPLING PROTEINS IN THE HEART, Karl R. Laskowski, Maria Volkova, and Raymond R. Russell. Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT. The mitochondrial uncoupling proteins (UCPs) are a recently discovered group of proteins that are present in the inner mitochondrial membrane and mediate a variety of important functions. Involved in transmembrane proton transport, UCPs regulate cellular metabolism as well as prevent reactive oxygen species formation (ROS) and detoxify exogenous ROS. In the heart, these proteins may protect tissue during times of ischemic or metabolic stress. Also activated during metabolic stress is AMP-activated protein kinase (AMPK), which has been shown to provide cardioprotection during ischemia/reperfusion. We hypothesized that AMPK activation plays a role in upregulating the expression of uncoupling proteins UCP2 and UCP3 in the heart. Using both tissue and cellular models, we demonstrate that pharmacologic activation of AMPK with the AMP-analogue, AICAR, leads to increases in UCP2 and UCP3 mRNA and protein expression at both one-hour and twenty-four hour incubation time points. Furthermore, we identify a segment of the UCP3 promoter that can mediate AMPK-activated transcription. We conclude that AMPK activation appears to induce increased UCP expression, and that such an effect is mediated through an interaction with a specific portion of the UCP3 promoter. These findings support the idea that some degree of the cardioprotective effects observed with AMPK activation may be due to increased UCP expression in the heart.

ACKNOWLEDGMENTS

My PI, advisor, and mentor: Dr. Raymond R. Russell, whose frequent encouragement kept me energized when experiments were testing my patience, and whose kind manner and striking intelligence serve as model for the type of physician and scientist I hope to become.

Masha Volkova, April Kalinowski, and Monica Palmeri, without whom the time I spent in lab would have been far less productive, educational, and fun. I hope that all of my colleagues are as wonderful as each of you.

Drs. Kerry Russell, Lawrence Young, Ed Miller, and John N. K. Forrest who all provided helpful advice and demonstrated how to practice exemplary science.

Dr. John K. Forrest, Jr., Ms. Donna Carranzo, and the staff of the Office of Student Research, for all of the assistance you provide to each and every Yale Medical Student. Theses could not be written without you.

The NIH-NHLBI Medical Student Research Training Grant and the James G. Hirsch, M.D. Endowed Medical Student Research Fellowship for providing funding to support my research and education.

My family and friends for their ceaseless support and understanding, especially Zosia, who seldom let me wallow in self-pity and who makes every day something to look forward to.

I am grateful to you all for I could not have gotten here without you. I hope I make you proud.

TABLE OF CONTENTS

INTRODUCTION

Uncoupling Proteins

Structure and Function

The mitochondrial uncoupling proteins, or UCPs, are a group of recently discovered proteins found in the inner mitochondrial membrane in various mammalian tissues. Uncoupling proteins are members of the superfamily of anion carrier proteins that regulate the mitochondrial membrane potential. This membrane potential is created by the transfer of protons (generated by citric acid cycle flux) from the mitochondrial matrix to the space between the inner and outer mitochondrial membranes via the oxidation/reduction reactions of the electron transport chain.

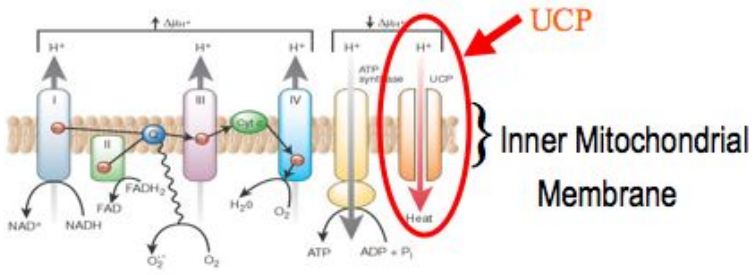

Figure 1. Uncoupling protein function. TCA cycle metabolism of NADH and $FADH_2$ generate a proton gradient that is utilized by ATP-synthase to create high-energy phosphates (ATP) or is "wasted" through uncoupling proteins that provide an alternate path for protons to cross the inner mitochondrial membrane. Adapted from Brownlee, M. Biochemistry and molecular cell biology of diabetic complications, Nature 2001; 414: 813-820.

The proton gradient drives mitochondrial ATP synthesis by F_0F_1-ATPase, which, in turn, dissipates the mitochondrial membrane potential. Under steady-state conditions, this membrane potential is determined by balance between the efflux of protons through

the electron transport chain and influx through the F_0F_1-ATPase. Like F_0F_1-ATPase, UCPs cause dissipation of the mitochondrial membrane potential through transport of protons from the space between the inner and outer mitochondrial membranes back into the mitochondrial matrix. However, in contrast to F_0F_1-ATPase, no ATP is synthesized by proton conductance through the UCPs and thus, they act as uncouplers of oxidative phosphorylation.

The first described uncoupling protein, UCP1 or thermogenin, was found to dissipate the proton gradient in an "energy wasting" manner, creating heat instead of the high-energy phosphate, ATP.[1] Largely restricted to brown adipose tissue, UCP1 is responsible for nonshivering thermogenesis. Subsequently, several proteins similar to UCP1 were identified based on sequence homology, establishing a family of UCPs. UCP2 was the first structural homolog of UCP1 to be described.[2, 3] UCP2 appears to be present in a variety of tissues throughout the body, although the presence of UCP2 protein in heart is the issue of some controversy and may be species-dependent.[4, 5]

Like UCP2, uncoupling protein 3 (UCP3) is an inner mitochondrial membrane protein with significant structural homology to UCP1. However, UCP3 appears to be present in the heart muscle of mice, rats, and humans.

While the expression of UCP3 in the heart is fairly well established, there remains conflicting data regarding the relative expression of UCP2 and UCP3 both between tissues and between species. There may also be substantial differences in the proteins' function depending on where they are located.[2, 6, 7] This variation has greatly complicated the study of the UCPs and much remains to be elucidated concerning the function of the UCPs. For example, while the primary function of UCP1 is widely agreed to be heat

generation in brown fat, the physiologic roles of UCPs 2 and 3 are less certain and remain the subject of ongoing experimentation and debate.[1, 8] Supporting the idea of different functions is data demonstrating that UCP1 is upregulated by exposure to cold temperatures and is critical for tolerance to cold, while UCP2 and UCP3 expression are not effected by cold, but are upregulated in response to fasting.[9, 10]

Multiple studies have suggested that these UCP2 and UCP3 may function in regulating metabolism: specifically, in the regulation of fatty acid oxidation or ATP production.[11] Some authors have hypothesized that the UCPs act as pores through which fatty acids can enter the mitochondria and undergo oxidation.[12, 13] While other previous studies have demonstrated that UCP3 knockout mice have lower rates of whole body fatty acid oxidation.[14] Furthermore, overexpression of UCP2 or UCP3 in cultured muscle or insulinoma cells increases the rate of fatty acid oxidation and decreases the rate of glucose oxidation when fatty acids are present.[15, 16] Yet metabolic effects may not be the primary function of the UCPs.

Given the similar structures of the mitochondrial uncoupling proteins and the known function of UCP1, it is not surprising that UCPs 2 and 3 also appear mediate some degree of proton leak within the mitochondria.[17] However, as mentioned above, UCP2 and UCP3 do not appear to be involved in significant thermogenesis. Although UCP2 and UCP3 are expressed in brown fat, previous research in UCP1-deficient adipocytes demonstrated that thermogenesis was almost entirely dependent on the presence of functional UCP1 proteins.[18] It has been proposed that the major function of UCP2 and UCP3 is related to the dissipation of the mitochondrial proton gradient, albeit not to produce heat. This function has important implications with respect to cellular protection.

Specifically, by reducing the electrical potential across the inner membrane, the UCPs reduce production of superoxide radicals at complex I of the mitochondrial respiratory chain.[19] Supporting this hypothesis are observations from isolated skeletal mitochondria harvested from mice: reactive oxygen species production is significantly increased in UCP3 null animals compared to wild-type animals.[20] In addition to preventing "endogenous" reactive oxygen species production by the mitochondria, UCPs may also detoxify "exogenous" reactive oxygen species. Specifically, overexpression of UCP1 in cultured cardiac myocytes protects against apoptosis induced by the addition of hydrogen peroxide.[23]

This role in mediating reactive oxygen species production has led to the hypothesis that the UCPs protect against oxidative damage. This has been best established in the neurologic system.[21, 22] However, as discussed below, this effect has also been demonstrated in pathologic states in the heart, such as ischemia/reperfusion injury, and may be relevant to other clinical scenarios such as heart failure and drug-induced cardiotoxicity (e.g. doxorubicin).

UCPs in Reperfusion, Ischemia, and Atherogenesis

As mentioned above, UCPs may perform essential functions in ischemic heart disease. In fact, increasing UCP expression in the heart may be a useful therapeutic strategy as multiple studies have demonstrated a significant protective effect of the UCPs in cardiac ischemia/reperfusion injury.[4] For example, H9c2 cells overexpressing UCP1 were more likely than their "wild-type" counterparts to survive hypoxia/reoxygenation exposure.[24] Similarly, overexpression of UCP1 in isolated perfused mouse hearts

improved functional recovery (as measured by contractility and oxygen consumption) after ischemia and reperfusion.[25]

Consistent with their potential role in limiting oxidative damage in "at-risk" myocardium, the UCPs have been observed to increase in cardiomyocytes adjacent to regions of recent infarction. In a study of post-MI ventricular dilation, myocardial infarction was induced in Sprague-Dawley rats resulting in a 60% increase in left ventricular end-diastolic dimension. The authors noted an almost two-fold increase in UCP2 mRNA expression in the tissue surrounding the infarct zone compared to sham-operated rats.[26] They hypothesized that this increased UCP expression may be contributing to the pathologic remodeling process by inducing a low-energy state in the surviving myocardium. However, given the cardioprotective data reviewed above, it seems more likely that UCP upregulation prevented further myocyte death through limiting oxidative damage in the vulnerable peri-infarct region. It is of interest to note that another "low-energy state", fasting, is associated with an increase in UCP2 and UCP3 expression, suggesting that the energy wasting effect of these UCPs is negligible.[27]

In addition to preventing cell damage and death due to the increased oxidative stress of reperfusion, there is evidence supporting the theory that the UCPs protect the heart during the preceding periods of ischemia. A recent study has demonstrated a direct correlation between the level of UCP2 and UCP3 expression and the degree of ischemic preconditioning attained by rat cardiomyocytes.[28] The authors further observed that UCP mRNA and protein expression were inversely associated with infarct size, and that UCP depletion (with RNAi) attenuated or abolished myocyte recovery in vitro after anoxic insult.

In addition to protecting the heart from both ischemic and reperfusion-induced damage, UCPs may prevent ischemia entirely, through limiting atherosclerotic plaque formation. Reconstitution of the bone marrow of irradiated LDL-receptor deficient mice fed a high-cholesterol, atherogenic diet with UCP2 null bone marrow cells has been shown to result in larger atherosclerotic lesions in the aorta.[29] It is believed that the UCP2 null monocytes created by the bone marrow transplantation produce more hydrogen peroxide than wild-type monocytes. This increase in hydrogen peroxide production results in greater vascular inflammation and endothelial cell activation, important initial steps in atherogenesis. Also supporting this theory that UCPs protect against atherosclerotic plaque formation, a common single nucleotide polymorphism (-866 G→A) of the human UCP2 gene has been shown to be associated with the presence of carotid atherosclerosis.[30] Furthermore, individuals homozygous for this allele had double the risk for coronary heart disease as compared to people with the alternate allele.[31]

Heart Failure and ROS

The observations discussed above suggest a significant role for UCPs in protecting the heart from atherosclerosis, ischemia, and ischemia/reperfusion injury. Other research has demonstrated a clear association between ROS and the development of heart failure (implying a possible further connection between UCPs and CHF).[32, 33] In patients with heart failure, markers of increased oxidant stress have been demonstrated in both pericardial fluid and blood.[34, 35] Furthermore, alterations in the ability to regulate oxidant stress are often present. Specifically, in failing hearts, decreases in the expression and activity of enzymes involved in the detoxification of reactive oxygen species, including catalase and manganese superoxide dismutase, can be observed.[36, 37] While

these changes may be the result, rather than the cause of heart failure, the observed association between genomic changes in the expression of the detoxification enzymes and the risk of heart failure suggests a genetic predisposition, and a causal relationship. For example, polymorphisms in genes coding for superoxide dismustase are associated with an increased risk for the development of idiopathic dilated cardiomyopathy.[38-40]

Reactive oxygen species also contribute to the progression of heart failure. In addition to the serum markers of oxidant stress, increased levels of reactive oxygen species have been reported in heart tissue samples from patients with longstanding congestive heart failure.[37] In a study of the role of reactive oxygen species in established heart failure (in patients with hypertrophic cardiomyopathy), endomyocardial biopsy samples demonstrated that lipid peroxidation, a marker of oxidative stress, was significantly elevated as compared to individuals without heart failure.[41] Furthermore, this level of lipid peroxidation was directly correlated with both left ventricular end-diastolic diameter and end-systolic diameter, and inversely correlated with left ventricular ejection fraction. Taken together, these data suggest a possible pathophysiologic role for oxidative stress in the development and progression of heart failure.

With this background, the importance of decreasing oxidant stress in the failing heart is easily understood. The efficacy of many proven heart failure therapies involves, at least in part, protection of the myocardium from the detrimental effects of ROS. It has been suggested that the beneficial effects of angiotensin converting enzyme inhibitors (ACEI) in diabetic cardiomyopathy are related to the inhibition of ROS production.[42] This mechanism is supported by the existence of substantial evidence that ROS are involved in the development of diabetic heart failure.[43, 44]

Additionally, as a therapy for non-diabetic as well as diabetic heart failure, it appears that carvedilol derives some of its effectiveness through its potent antioxidant properties.[45] In one study utilizing an ischemic rabbit heart model, carvedilol, in comparison to bisoprolol (a beta-blocker without antioxidant properties), led to markedly decreased cardiac membrane lipid peroxidation.[46] In non-ischemic rabbit model of CHF, carvedilol treatment decreased measurements of oxidant stress (GSH to GSSG ratio) and attenuated ventricular remodeling, cardiac hypertrophy, and myocyte apoptosis whereas propranolol and doxazosin (which did not alter oxidative stress) had little effect.[45]

Other animal studies have confirmed the role of ROS in the development and progression of heart failure. A transgenic mouse model with heart and skeletal muscle-specific loss of manganese superoxide dismutase develops progressive congestive heart failure with specific molecular defects in mitochondrial respiration.[38] In this model, oxidative stress causes specific morphologic changes in the mitochondria, with excess formation of the reactive oxygen species, superoxide radical, reduction of ATP content, and transcriptional alterations of genes associated with heart failure. Conversely, overexpression of genes involved in the detoxification of reactive oxygen species, such as glutathione peroxidase, improved left ventricular function in mice following myocardial infarction.[47] As discussed below, animal studies of heart failure, including transgenic mouse models, have suggested that UCPs may be important in the response of the cardiac myocyte to oxidant stress. Given the robust evidence for ROS as a pathophysiologic mediator in the development and progression of heart failure, upregulation of UCPs may be a potential target for heart failure therapy.

UCPs in Heart Failure

In developed countries, the most common cause of heart failure is ischemic heart disease. Depending on the study population and the method of investigation, the etiology of heart failure is attributed to previous myocardial infarction in approximately 50% of cases.[48] Given the previous discussion of the role of UCPs in the prevention of ischemic injury it is easy to see how these proteins might also be relevant to heart failure (by preventing ischemic damage to the myocardium through slowing atherosclerosis as well as decreasing oxidant stress in ischemic/reperfused cardiomyocytes, UCPs prevent a major cause of heart failure). In addition, prior studies have demonstrated that the UCPs are downregulated, at least on the mRNA level, in the failing heart, suggesting that their downregulation may play a role in the development and/or progression of the disease (a hypothesis that would fit nicely with the evidence reviewed above implicating increased ROS as a mediator of CHF development and progression—i.e. decreased UCP→increased ROS→ CHF).[11, 49, 50]

As discussed above, mice with cardiac deletion of manganese-superoxide dismutase develop heart failure from oxidative damage.[38] In addition to the findings of enhanced oxidant stress, the authors also noted that there was decreased cardiac expression of UCP3, perhaps contributing to the pathologic process.

However, controversy remains as to whether UCPs play a beneficial or detrimental role, particularly in the setting of heart failure, because downregulation of UCPs in the failing heart could have theoretically opposing effects. A decrease in UCP expression would be expected to enhance the coupling between citric acid cycle flux and mitochondrial ATP synthesis, enhancing energetic efficiency. This effect would be

potentially important in cardiomyocytes that are energetically stressed through the many effects of heart failure. However, deceased UCP expression would also have the counterbalancing effect of allowing greater production of reactive oxygen species.

The question then becomes one of whether downregulation of UCP expression in the failing heart is beneficial or detrimental and should therapies be developed to increase or decrease UCP expression. Would the shunting of protons away from ATP production by increasing UCP expression contribute to a low-energy state?[51, 52] Given the preponderance of evidence implicating increased ROS in the pathogenesis of heart failure, it would seem that limiting oxidative stress would be the more pressing goal. Addtionally, it has been argued that, in addition to preventing oxidant stress and cardiomyocyte damage, the metabolic effects of the UCPs are actually beneficial to the failing heart, and represent an adaptive response to lipid accumulation within the mitochondria and subsequent toxicity, and not a maladaptive response.[53] Finally, one cannot ignore the evidence suggesting that UCP upregulation would prevent heart failure development through limiting ischemic damage.

Whether the decreased UCP expression in CHF is a protective response or a pathophysiologic one, it seems clear that the UCPs are intimately involved in the development and pathogenesis of heart failure.

Clinical Relevance

From a review of the literature, it seems clear that UCPs play an important role in two exceptionally important cardiovascular conditions: ischemic heart disease and heart failure. Ischemic heart disease causes more deaths and disability and incurs greater economic costs than any other illness in the developed world. In the United States, it is

estimated that more than 12 million people are afflicted with this disease. With the increasing rates of obesity, insulin resistance, and type 2 diabetes mellitus—all clearly linked to the development of heart disease—it is projected that the rates of ischemic heart disease will only continue to increase, likely becoming the most common cause of death worldwide by 2020.[54]

Like ischemic heart disease, congestive heart failure is a common and often devastating disease of great importance to the world health community. Heart failure is a common disease with a prevalence estimated at 1-2% in the general population.[55] Increasingly important as our population ages, this condition can afflict upwards of 7-10% of the elderly and is often underdiagnosed.[56] A diagnosis of heart failure is not a trivial matter with one year mortality rates estimated to be between 12 and 45% and worse long-term survival than most common cancers.[57, 58, 59] The substantial prevalence and significant complications of heart failure make it a source of major healthcare expenditure to the population at large. In the United States, more than 900,000 people are hospitalized each year for heart failure, creating annual expenses in excess of 6 billion dollars.[60] Given the important roles of the UCPs in common and devastating clinical diseases, a clear understanding of the regulation of these proteins would be extremely valuable in developing new potential therapies.

Regulation of UCPs

While the mechanisms responsible for regulating UCP expression in various tissues (including skeletal muscle, hepatocytes, pancreatic β-cells, and adipocytes) have been well studied, the regulation of UCP in the heart is not yet as well characterized. Data suggests that UCP expression in the heart is regulated, in part, through peroxisome

proliferator-activated receptor (PPAR) α-mediated mechanisms.[61] In a mouse heart model, PPAR α agonist WY-14,643 increased cardiac UCP2 and UCP3 protein in wild type mice but not in PPAR α -/- mice.[62]

In addition to the PPAR pathway, there is evidence that UCP expression can be increased in skeletal muscle through activation of AMP-activated protein kinase (AMPK), a signaling protein kinase that responds to metabolic stress. [63, 64] In one study, rats were injected with an AMPK activator (AICAR, described in detail below) for 28 days after which analysis of skeletal muscle revealed increased levels of UCP3 mRNA and protein. Another study utilizing an AMPKα2 knock out model demonstrated decreased levels of UCP3 mRNA content.[65] Prior to our experiments, it remained unclear whether these observations in skeletal muscle were applicable to the heart as well. Because of this uncertainty, and the potentially important clinical implications, it is the potential role of AMPK in the regulation of cardiac UCPs that is the focus of this research.

AMP-activated Kinase

Structure and Function

AMP-activated kinase (AMPK) is a heterotrimeric protein kinase that serves as a key energy regulator in the eukaryotic cell. First described in the late 1980s, AMPK has been implicated in a vast number of metabolic functions.[66] Chiefly, AMPK responds to changes in the energy stores of the cell by enhancing nutrient uptake, increasing the provision of energy, as well as decreasing energy-depleting cell activities with the combined result of restoring cellular energy balance.[67] This function has led some to

describe AMPK as the "fuel gauge" of the mammalian cell and the "guardian of energy status" in the heart.[68, 69]

The AMPK protein is a complex of three subunits: the catalytic α subunit, and the regulatory β and γ subunits. Each of these subunits has two or three isoforms, and twelve different isoform combinations appear to be stable as the functional heterotrimeric complex.[70, 71] However, the majority of cardiac myocyte AMPK appears as an α2β1γ1 combination, with lesser contributions from α1, β2, γ2, and γ3.[72, 73] The expression of different isoforms is tissue dependent, with γ3, for example, predominating in skeletal muscle but being absent in the heart.[68] Apart from the relative prevalence within specific tissues, some differences between the various isoforms have been described; specifically, variations in cellular localization and in response to cellular AMP levels.[70] For example, AMPK complexes containing the α2 isoform are preferentially localized within the cell nucleus whereas the α1-containing complexes are absent from this compartment.[74]

As briefly mentioned above, AMPK functions chiefly as an energy sensor and metabolic regulator. AMPK is activated when there is an increased AMP:ATP ratio—that is, when energy stores are low. AMP allosterically activates AMPK while also inducing phosphorylation of a threonine residue in the α subunit (Thr 172) by upstream kinases.[67] The AMPK kinases (AMPKK) include the tumor suppressor LKB1 as well as calcium calmodulin dependent kinase (CaMKK).[75, 76] (Interestingly, the CaMKK pathway appears to respond to increased intracellular calcium concentrations rather than AMP:ATP ratio and may be an alternative mechanism by which tissue specific regulation is achieved.)

In addition to inducing phosphorylation (and thus activation) of the α subunit, high levels of AMP prevent dephosphosphorylation and subsequent deactivation of the

AMPK complex.[77] Both the phosphorylation of AMPK by the upstream AMPKK and the inhibition of the AMPK phosphatases are prevented by increased concentrations of ATP. Thus it is the AMP:ATP ratio that ultimately controls the regulation of AMPK (as an aside: it has been shown that AMPK also responds to indirect signals of energy status, including glycogen levels—the β subunit of the AMPK protein has a glycogen binding domain that responds to high cellular glycogen levels [indicative of adequate energy stores] by inhibiting AMPK activation.[68, 78]). Perturbations in the AMP:ATP balance can be caused by physiologic mechanisms (such as exercise) or pathologic stimuli (e.g. heat shock and metabolic poisoning which limit ATP production, or ischemia and hypoxia which increase ATP consumption).[70] Previous research has clearly demonstrated that AMPK responds to a variety of these metabolic stressors, including hypoxia, ischemia, and oxidative stress.[67]

These physiologic stimuli perturb the "resting" balance of AMP relative to ATP. AMP is present in the heart in very low concentrations under normal physiologic conditions. During periods of ischemia or other types of metabolic stress, the amount of ADP rapidly increases.[68] In eukaryotic cells, a very active adenylate kinase enzyme maintains an equilibrium between ADP and AMP by catalysis of the reaction $2ADP \rightarrow ATP+AMP$. Thus an increase in ADP leads to a quick corresponding increase in AMP, and the AMP:ATP ratio varies as approximately the square of the ADP:ATP ratio.[70] This leads to an exquisitely sensitive mechanism for sensing the energy status of the cell.

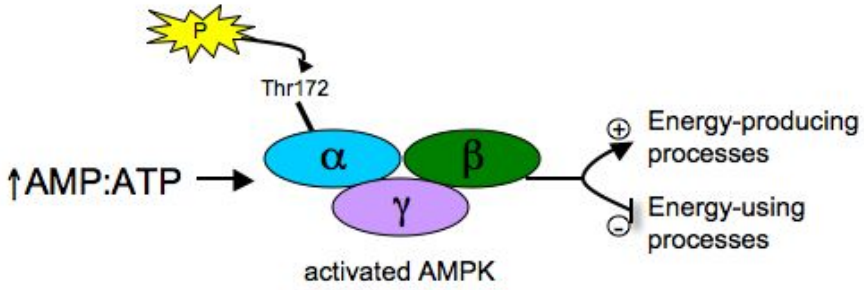

Figure 2. AMP-activated Kinase (AMPK) regulation and function. An increased AMP:ATP ratio indicates a negative energy balance in the cell and induces phosphorylation of a threonine residue on the catalytic α unit of AMPK by upstream AMPK kinases (e.g. LKB1). The activated AMPK phosphorylates various downstream targets with the net result of increasing cellular energy production while limiting cellular energy-expenditure

In response to depleted energy stores, AMPK has a range of effects on tissues throughout the body with the goal of limiting ATP consumption while increasing its production to restore cellular energy balance. While many of the molecular pathways remain to be elucidated, it is clear that AMPK phosphorylates a range of target proteins, "switching on catabolic pathways that generate ATP, while switching off anabolic pathways and other nonessential ATP-consuming processes."[70] The energy-expending processes that AMPK activation limits include fatty acid, cholesterol and protein synthesis. This occurs through a combination of direct protein modification as well as modulation of cellular genetic expression.[70] In the heart, AMPK activation increases fatty acid oxidation and glycolysis as well as glucose uptake by cardiomyocytes.[79] Similar effects are observed in skeletal muscle. In the liver, fatty acid synthesis, cholesterol synthesis and gluconeogenesis are inhibited by AMPK activation. Fatty acid synthesis and lipolysis are also decreased in adipocytes. And in the hypothalamus, appetite is stimulated.[69] The end result of this regulation is to conserve energy within the cell during times when stores are low.

As stated previously, the exact mechanism of action of many of these pathways has yet to be fully defined. It seems likely that many of these effects are mediated through signaling cascades involving protein phosphorylation by the AMPK enzyme, but some degree of transcription factor modification and differential gene expression is likely as well. The uncertainty surrounding the downstream effectors of the AMPK pathways make this research project an important endeavor, particularly because AMPK has already been proven to exert important protective effects in the heart, and a more clear understanding of signaling pathways would allow for potential pharmacologic manipulation.

AMPK and the Heart

Previous research has demonstrated that AMPK is important in maintaining cardiovascular energy balance. Under normal physiologic conditions, AMPK plays a role in controlling both skeletal and heart muscle metabolism during exercise.[68, 80] Under pathologic conditions, AMPK activation leads to translocation of cardiac GLUT4 glucose transporters to the sarcolemma and a subsequent increase in cellular glucose uptake.[68, 81] In the ischemic heart, this transition from fatty-acid based metabolism to a greater reliance on glucose as an energy source is key to cell survival as nutrients become more scarce. [81] In addition, AMPK has been shown to be critical to maintaining myocardial energy homeostasis during ischemia by stimulating glycolysis.[82, 83]

Past research in our lab has indicated an important role for AMPK in the myocardial response to ischemia. To better characterize the function of AMPK during ischemic and post-ischemic reperfusion conditions, transgenic mice with a kinase dead (KD) form of the AMPK protein were utilized in a series of experiments. During low-

flow ischemia and post-ischemic reperfusion in vitro, KD hearts failed to augment glucose uptake and glycolysis, although glucose transporter content and insulin-stimulated glucose uptake were normal. KD hearts also failed to increase fatty acid oxidation during reperfusion. Furthermore, KD hearts demonstrated impaired recovery of LV contractile function during postischemic reperfusion that was associated with a lower ATP content and increased injury compared with wild type hearts. These data suggest that AMPK is responsible for activation of glucose uptake and glycolysis during low-flow ischemia and that it plays an important protective role in limiting damage and apoptotic activity associated with ischemia and reperfusion in the heart.[79]

AMPK and the UCPs

AMPK activation has additional effects that are related to regulation of key proteins on a transcriptional level. One important effect of AMPK activation in skeletal muscle is an increased expression of the UCPs as well as other key metabolic proteins, including hexokinase II and GLUT4.[81, 84-86] In addition, AMPK activation has been shown to be a critical step in the regulation of several nuclear transcription factors, including NRF-1, HIF-1α and p300.[84, 87, 88] These observations suggest that AMPK may exert some of its metabolic effects through direct modification of genetic expression in the nucleus.[86]

While AMPK activation has been shown to upregulate both UCP3 mRNA and protein expression in rat skeletal muscle, a similar association has not been demonstrated in the heart. There is some circumstantial evidence that implies such a role. For example, PPARγ agonists have been shown to activate AMPK, probably through a mechanism that involves increasing the cellular AMP:ATP ratio.[89] PPAR pathways are also involved in

the upregulation of UCPs, though most evidence for the cardiac UCPs supports a role for PPARα rather than PPARγ (which has been shown to activate UCPs in tissues outside the heart).[90-92]

Given the potential role for UCPs in protecting the cell from oxidative stress, an association between increased ROS load and AMPK activation would seem to suggest a possible regulatory link. In a series of experiments with NIH-3T3 cells, H_2O_2 induced phosphorylation at the Thr172 activation site of AMPK. This activation was blocked by pretreatment with 0.5% dimethyl sulfoxide, a potent free-radical scavenger.[93] Others have suggested that AMPK may have an as yet unidentified role in the prevention of reactive oxygen species generation.[68] These observations favor a potential role for AMPK in the regulation of UCPs: decreased energy or increased reactive oxygen species leading to AMPK activation would increase expression of the UCPs and prevent excess ROS production as well as detoxify existing ROS. We set out to experimentally examine the hypothesis that AMPK activation upregulates UCP transcription and translation, and to more clearly define the mechanism by which this occurs.

STATEMENT OF PURPOSE AND SPECIFIC AIMS

Mitochondrial uncoupling proteins appear to play critical roles in cardiovascular metabolism, in protection of the heart during periods of ischemia and post-ischemic reperfusion, and in the pathogenesis and potential prevention of heart failure. A more complete description of their regulation may lead to important advances in understanding the pathophysiology of various heart diseases, and may contribute to the development of promising, novel therapies. AMPK controls the metabolic state of virtually all mammalian cells, including cardiomyocytes, and is activated under clinically relevant stimuli that appear to also induce the upregulation of the UCPs in the heart (ischemia, for example). Given these observations, it has been suggested that AMPK controls the cardiac expression of UCPs. The set of experiments described in this thesis examined the following hypothesis:

Activation of AMPK leads to an increased expression of the mitochondrial uncoupling proteins, UCP2 and UCP3, in the cardiomyocyte.

Specific Aims:

1. To determine whether pharmacologic activation of AMPK induces an increase in UCP2 and UCP3 mRNA utilizing both isolated tissue and cellular cardiomyocyte models.
2. To determine whether pharmacologic activation of AMPK induces an increase in UCP2 and UCP3 protein in a cellular cardiomyocyte model.

3. To identify any sites of interest in the UCP3 promoter where AMPK, or its downstream mediators, may interact to affect an upregulation of UPC3 transcription and translation.

METHODS

Pharmacologic AMPK activation

To determine whether mitochondrial uncoupling proteins in the heart are upregulated via a mechanism involving the activation of AMPK, a series of experiments were planned utilizing a pharmacologic activator of AMPK. While AMPK is usually activated by an increased AMP:ATP ratio as described above, alterations in this ratio are often impractical for experimental investigations. AMPK can be pharmacologically activated through the addition of 5-aminoimidazole-4-carboxamide riboside (AICAR). AICAR is a cell-permeable adenosine analog that is taken up by the cells and phosphorylated to form 5-aminoimidazole-4-carboxamide-1-d-ribofuranosyl-5′-monophosphate (ZMP), an AMP mimetic.[67] ZMP mimics all of the activating effects of AMP, although it is much less potent on a mol:mol basis.[70, 94] AICAR also blocks adenosine re-uptake (as described in more detail below) and this was the motivation behind its previous use in clinical trials.

Throughout all of the experiments described below, AMPK activation was achieved using AICAR. In all cases, the activation of AMPK was confirmed by evaluation of Thr172 phosphorylation by immunoblot analysis. As described above, phosphorylation of the threonine-172 residue on the α-subunit by upstream AMPKK is a critical step in the activation of the AMPK complex, and phosphorylation is an accepted marker of enzyme activation.

Tissue Model

To examine the role of AMPK in the regulation of cardiac UCP expression, a mouse tissue model was used first. C57/BL6 wild-type mice were sacrificed using pentobarbital (150 mg/kg, i.p.) and the hearts were harvested and immediately placed in ice-cold 0.9% saline. The ventricles were isolated by trimming away any remnants of the great vessels as well as the atria. The remaining tissue was placed in an acrylic mouse brain slicer with 1.0-mm coronal slicing intervals. The heart was then cut into 1-mm thick rings that were then placed in 100-mL Erlenmeyer flasks with either a control buffer solution or an "experimental" AICAR solution as described below.

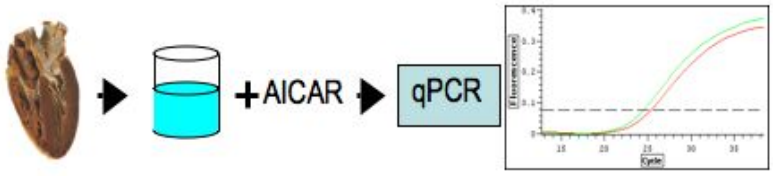

Figure 3. Schematic Representation of Tissue Model Experiments. C57/BL6 wild type mouse ventricles were harvested, sectioned, and incubated with or without AICAR. After homogenization, RNA was isolated, and quantitative real time PCR performed to determine relative expression of UCPs.

The basic incubation solution consisted of 1x Dulbecco's phosphate buffered saline (DPBS) with 1 mM calcium chloride, 1 mM magnesium chloride, 1% bovine serum albumin (BSA), 5 mM glucose and 0.4 mM oleate. To make the control solution, 5 µl of DMSO was added (as a vehicle control) to 5 ml of the incubation solution described above. The "experimental" solution was identical except that 25 µl of 200 mM AICAR was added to the 5-mL of incubation solution to yield a 1 mM concentration of AICAR.

The heart slices were incubated in either control solution or AICAR solution on a shaking water bath at 37°C with 100% oxygen for 1 hour or 24 hours. At the end of the

incubation, the ventricular slices were snap frozen in liquid nitrogen and stored at -80°C until analysis.

Frozen sections were homogenized using a rotor-stator homogenizer followed by 5 passes through a 20-gauge needle. RNA was then isolated using the RNeasy mini kit (Qiagen) using the protocol suggested by the manufacturer. RNA concentration was determined spectrophotometrically as A 260/280.

Formatted: Bullets and Numbering

Using the RNA samples, reverse transcription PCR was performed to determine the relative expression of UCP2 and UCP3 mRNA. RNA was diluted to 1 µg/11 µl concentration. For each reaction, 11 µl of the RNA mixture was added to 1 µl of oligo$(dT)_{15}$ primer (Promega) and mixed. Reverse transcription mastermix was prepared (per reaction: 4 µl of 5x first strand synthesis buffer [Roche] + 2 µl of 0.1 M DTT [Roche] +1 µl of 10mM dNTPs per reaction [Roche]). Seven microliters of this reverse transcription mastermix was added to each reaction and incubated at 42°C for 2 minutes. One microliter of Superscript II (Roche) was added to each reaction and the mixture returned to 42°C incubation for another 50 minutes. The mixture was then incubated at 70°C for 15 minutes. RNase H (0.6 µl, Roche) was added to each sample and then incubated for 20 minutes at 37°C. The total volume was adjusted to 100 µl by adding 79 µl of sterile water, and the sample was boiled for 10 minutes. The sample was immediately placed on ice and used immediately in PCR reactions, or stored at -80°C.

The cDNA from the RT-PCR described above was used for qualitative real time PCR (qRTPCR) reactions as follows: primers and Taqman probes using a "fam" dye were created (Biosearch Technologies) using sequences previously published in the scientific literature.[61]

GENE	PRIMER/ PROBE	SEQUENCE
rat/mouse UCP2	Forward	5'-TCATCAAAGATACTCTCCTGAAAGC-3'
	Reverse	5'-TGACGGTGGTGCAGAAGC-3'
	Probe	5'-FAM-TGACAGACGACCTCCCTTGCCACT-TAMRA-3'
rat UCP3	Forward	5'-GTGACCTATGACATCATCAAGGA-3'
	Reverse	5'-GCTCCAAAGGCAGAGACAAAG-3'
	Probe	5'-FAM-CTGGACTCTCACCTGTTCACTGACAACTTCC-TAMRA-3'
mouse UCP3	Forward	5'-TGCTGAGATGGTGACCTACGA-3'
	Reverse	5'-CCAAAGGCAGAGACAAAGTGA-3'
	Probe	5'-FAM-AAGTTGTCAGTAAACAGGTGAGACTCCAGCAA-TAMRA-3'
rat/mouse cyclophilin	Forward	5'-CTGATGGCGAGCCCTTG-3'
	Reverse	5'-TCTGCTGTCTTTGGAACTTTGTC-3'
	Probe	5'-FAM-CGCGTCTGCTTCGAGCTGTTTGCA-TAMRA-3'

Table 1. Taqman Primers and Probes for quantitative Real Time PCR. Mouse primers and probes were used for tissue model experiments (mouse ventricle) while rat primers and probes were used for cellular model experiments (H9c2 cells). Table adapted from Young, ME, et. al.[61]

For the qRTPCR experiments, cyclophilin was selected as a reference "housekeeping" gene on the basis that it is present in the cells of interest and thought to be unaffected by treatment with AICAR. In addition, previous work by others in the lab had demonstrated that the amplification efficiencies of the cyclophilin reference gene and the target UCP2 and UCP3 genes are similar. This observation is critical for meaningful analysis with the delta-delta method (described in detail below).

A "primer-probe" mix was prepared for each gene of interest (for example, UCP2, UCP3 and cyclophilin) with 5 μl of 100 μM probe, 10 μl of 100 μM forward

primer, 10 µl of 100 µM reverse primer, and 375 µl of DNAse/RNAse free water. Eight µl of cDNA was mixed with this primer-probe mix and 12.5 µl of taqman mastermix (Applied Biosystems).

The qRTPCR was conducted with a DNA Engine Opticon 2 two-color PCR Detection System (Bio-Rad) connected to a standard IBM desktop computer. A typical qRTPCR protocol was employed (e.g. incubation at 95°C for 5 minutes followed by 50 cycles of 95°C for 30 seconds, 60°C for 1 minute, and a plate reading). Quality control for the specificity of the amplification product was achieved through gel analysis revealing a single band of appropriate size.

Results were analyzed using the "delta-delta" or "comparative C_t" method. A "threshold value" is arbitrarily set along the logarithmic portion of the amplification curve. The cycle at which the sample crosses the threshold (or C_t value) is recorded for each sample, including the reference gene samples. The target samples, both experimental and control (in this case AICAR treated and control incubation buffer treated, respectively) are each normalized to the reference gene by subtracting the C_t of the reference sample from the C_t values of the experimental samples yielding ΔC_t's for each of the target samples. As mentioned above, it is critical that the sample gene has an amplification frequency similar to the chosen reference gene—that is, that the slope of the logarithmic portion of the curve is similar. If this is not the case, than normalization to the reference gene will introduce error into the analysis and make meaningful comparison impossible.

After normalizing to the reference gene, the "delta delta" value is then calculated by subtracting the ΔC_t of the mean value for the control sample from the ΔC_t of each experimentally treated sample. Expressed mathematically, the calculation is as follows:

delta delta C_t= ($C_t$experimental - $C_t$reference) - ($C_t$control - $C_t$reference)

If the amplification efficiencies are similar, this value is an expression of the quantitative amount of cDNA that was present at the start of the qRTPCR procedure—that is, the relative expression of the gene of interest. To complete the determination of relative gene expression, the following calculation is performed:

$$\text{relative mRNA} = 2^{-\text{delta delta Ct}}$$

In this set of experiments, cDNA from the AICAR treated and "control" (or non-AICAR treated) ventricular samples was analyzed as above. Mean expression values were calculated for both experimental and control samples and paired student t-tests were used to determine statistical significance (GB-Stat PPC SchoolPak).

Cellular Model

Next, experiments were performed utilizing a cellular model. The cellular model was used to further support a role for AMPK in the regulation of UCP expression (including important quantification of UCP protein levels as well as mRNA) and to perform the genetic studies outlined below.

H9C2 cells were selected because of their rapid doubling time, ease of use, and similarity to adult cardiomyocytes. The cells are derived from a neonatal rat myoblastic line and retain some of the characteristics of true cardiomyocytes. Unlike cardiomyocytes, however, H9C2 cells need not be isolated from harvested tissue, are far

easier to maintain, and replicate indefinitely.[95] For these experiments, the cells were used from passage 1 through passage 15 and then discarded because later generations can develop morphology significantly different from typical cardiomyocytes.

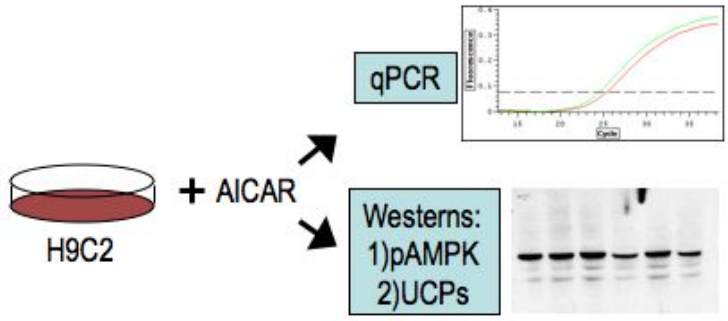

Figure 4. Schematic Representation of Cellular Model Experiments. H9C2 Cells were incubated with or without AICAR. In one set of experiments mRNA was isolated and then used for quantitative real time PCR to determine UCP expression in AICAR treated vs. control cells. In a second set of experiments, cells were harvested and protein isolated to determine phospho-AMPK and UCP expression in AICAR treated vs. control populations.

Cells were plated under sterile conditions onto 100-mm tissue culture plates with high glucose, L-glutamate containing Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and a 1% penicillin-streptomycin antibiotic mixture. Subconfluent (70-80%) plates were passaged in a typical manner (cells were detached from plates by addition of trypsin, which was subsequently inactivated by addition of 5 ml of DMEM/10% FBS media; the cells were then collected and sedimented by centrifugation, resuspended in fresh media, and aliquoted onto new plates). All cells were stored in a sterile incubator at 37°C and 5% CO_2.

Originally, there was some concern that the 10% FBS media might contain growth factors or other substances that would affect AMPK activation and introduce error to the planned experiments. Therefore, H9C2 cells were incubated with either high glucose (4.5 g/l) or low glucose (1 g/l) DMEM with 0.1% or 1% FBS for 24 hours prior

to harvest and immunoblots for phospho-AMPK were performed to determine if these culture conditions activated AMPK (described below). However, all combinations of low-FBS media led to super-activation of AMPK such that no difference between AICAR treated and control samples could be determined (all samples were maximally phosphorylated). A decision was therefore made to incubate all cells with 10% FBS for the entirety of the experiments.

The H9C2 model was used to examine the effect of AMPK activation on UCP2 and UCP3 mRNA levels in a manner similar to the tissue model experiments described above. When 70-80% confluent, H9C2 cells were washed with DPBS, then treated with 1 mM AICAR in 5-ml of 10% H9C2 media (as described above) or with 5-ml of media alone for 1 hour or 24 hours. The cells were again washed with DPBS and harvested as per the "cells" protocol in the RNeasy minikit (Qiagen). RNA concentration was again determined spectrophotometrically, and reverse transcription PCR was performed in the same manner as described above. Using this cDNA, qRTPCR was then performed as previously described. Analysis was again performed using the Opticon 2 (Bio-Rad) system and the "delta delta" method.

To verify that AICAR was in fact activating AMPK, immunoblotting for phospho-AMPK was performed. H9C2 cells that were 70-80% confluent were washed with cold DPBS. Vacuum suction was then employed to remove all DPBS. Lysis buffer mix was prepared by combining 10-ml of CellLytic M Cell Lysis reagent (Sigma), 1 Complete Proteinase Inhibitor complex tablet (Roche), 100 µl of 1 M sodium fluoride, and 10 µl of 1M sodium orthovanadate (Na_3VO_4). Two hundred microliters of this mix was added to each plate. The plates were scraped and the cell lysate was transferred to a

microcentrifuge tube. The mixture was centrifuged at 10,000g for 10 minutes. The supernatant was removed and stored (precipitate was discarded) and the protein concentration subsequently measured spectrophotometrically using the Bradford-Standard method with a BSA standard.

Twenty micrograms of protein was combined with 5 µl of 6x protein loading buffer and water (if necessary) to a total volume of 25 µl. This mixture was heated to 100°C for 10 minutes. The protein was then loaded into a 10% NuPage gel (Invitrogen) with a kaleidoscope marker (Biorad) and electrophoresis carried out in MES containing buffer for 35 minutes at 200V. The separated proteins were then transferred to a nitrocellulose membrane (Millipore) by electroblotting at 250 mA for 1.25 hours. The membrane was blocked in 5% BSA solution for 1 hour and then incubated overnight at 2°C with 10 µl of rabbit anti-phosphoAMPK antibody (Cell Signaling) in 10-ml of 5% BSA. The next morning, the membrane was washed with tris-buffered saline with tween (TBST) buffer three times for 15 minutes each. Secondary antibody (4-µl of donkey anti-rabbit IgG-HRP [Santa Cruz] in 5 ml of 2.5% BSA solution) was then added for 1 hour at room temperature. The membrane was washed again 3 times for 15 minutes each. After washing was complete, the membrane was incubated with Chemiluminescent Developer Solution (Pierce) for 1 minute, and x-ray film (Kodak) placed in the developer cassette with the membrane for approximately 15 minutes. The blot was then stripped to remove the antibodies, washed two more times with TBST buffer and blocked in 5% milk for 1 hour. Then the membrane was incubated overnight at 2°C with 25 µl of goat anti-AMPK α2 (R&D Systems) in 10 ml of 5% milk to confirm equal loading of samples. The next morning the membrane was washed three times and then incubated with 4 µl of donkey

anti-goat IgG-HRP (Santa Cruz). The membrane was again developed with detection buffer and an x-ray film record obtained after approximately 10 minutes of exposure. Immunoblots were later scanned into a computer. Band intensities from the images were quantified using Image J (National Institutes of Health).

Expression of UCP mRNA and protein

There is some controversy in the literature as to whether UCP mRNA levels truly correlate with protein expression.[96, 97] For example, while UCP2 mRNA is found in adult myocardium, skeletal muscle, and brown adipose tissue, UCP2 protein is generally not detected in these tissues.[97, 98] In recognition of this potential discrepancy, H9C2 cells were treated with AICAR or control solution and then harvested for immunoblot analysis of protein expression. Because the uncoupling proteins are found in the mitochondria, a mitochondrial isolation protocol was employed to improve the detection of UCP2 and UCP3 protein. Plates containing H9C2 cells that were 90-100% confluent were trypsinized and the cells collected into centrifuge tubes (4 plates per tube) and spun at 600g for 5 minutes. The cells were washed with ice cold DPBS and then spun down again after which the supernatant was discarded. This step was repeated a second time. Approximately 1.5 ml of lysis buffer (5 ml of lysis reagent [Sigma], 1/2 of a Complete Protease Inhibitor mini tablet [Roche], 500 µl of 1 M sodium fluoride, and 50 µl of 1M sodium orthovanadate) was added to each tube. The samples were then incubated on ice for five to ten minutes. At 1-minute intervals, the samples were mixed by drawing them up and down 3 times through a 25-gauge needle into a 3 ml syringe. Three milliliters of mannitol mitochondrial extraction buffer were added to each sample (extraction buffer

contained 5 mM HEPES, 210 mM mannitol, 70 mM sucrose and 1mM EGTA). The homogenate was centrifuged at 600g and 4°C for 10 min. The pellet was set aside for nuclear protein isolation and subsequently stored for later analysis. The supernatant was transferred to a fresh tube and centrifuged at 11,000g and 4°C for 10 min. The resulting supernatant was then discarded and the pellet resuspended in a storage buffer and stored at -80°C until analysis.

Immunoblot analysis was performed in a manner similar to that described above. Electrophoresis was performed using 12% NuPage gels (Invitrogen) in MES buffer for 35 minutes at 200V. Transfer to the nitrocellulose membrane was carried out via the same procedure as above. Blocking was achieved with 5% milk solution and the membranes were incubated overnight in primary antibody solution (25 µl of rabbit anti-UCP2 [BioLegend] or 25 µl of anti-UCP3 [Affinity Bioreagents] in 5 ml of 5% milk). Secondary antibody (4 µl of donkey anti-rabbit IgG-HRP [Santa Cruz]) was applied the next day after washing, and development achieved as above with 20-30 minute exposures. After stripping, washing and blocking in 5% milk for one hour, 10 µl of goat anti-cytochrome C antibody in 10 ml of 5% milk was applied to the membrane (as a loading control) and incubated overnight at 2°C. Secondary antibody (2 µl of anti-goat IgG-HRP [Santa Cruz] in 10 ml of 2.5% milk) was then applied and the membrane incubated at room temperature for one hour. Development (with approximately 2 minute exposure) and analysis were carried out as above.

Analysis of the Promoter Region of UCP3

In order to identify the promoter region of the UCP3 gene that interacts with activated AMPK or one of its downstream messengers, luciferase reporter vectors were created incorporating different portions of the UCP3 promoter.

The sequence of the 2100 bp segment preceding the start of transcription was downloaded from GeneBank (NIH). Using computer software (Vector NTI [Invitrogen]) this promoter was analyzed and divided into three overlapping segments. The full 2000 bp sequence was termed "long," the segment from -1977 to -1077 "left," from -1600 to -697 "middle," and -1000 to +20 "right."

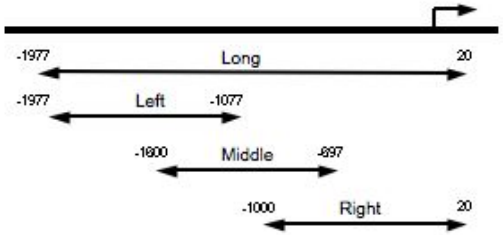

Figure 5. UCP3 Promoter Constructs. In order to better characterize the site of interaction between activated AMPK and the promoter segment of the UCP3 gene, the 2000 bp segment preceding the start of transcription was cloned, as were three "fragments" named "Left," "Middle," and "Right."

Heart tissue from a Sprague-Dawley rat was used to extract DNA using a DNeasy tissue kit (Qiagen). After obtaining all appropriate primers from the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University), PCR of the aforementioned DNA was employed to create the promoter constructs. The primers used in this experiment are presented below in Table 2.

	Promoter Name	Sequence	Tm	Length
Full Promoter	PRU3F1	ACCCTCAGCCCTACCTGTCCCACAT	63	25
	PRU3R1	CCGGAGATCTTACCTGTGAGTCTAGCCAAGGT	66	32
Left	PRU3F2	GCTTGAGAAATGTCAGCCCACACTT	60	25
	PRU3R2	CCGGAGATCTTGACATAGAAGGGAGACTAAAA	63	32
Middle	PRU3F4	GCTGCAGGCAAAGTGGAGGTCTGAA	64	25
	PRU3R4	CCGGAGATCTAGCAGGATCGTACAGAATGTAT	64	32
Right	PRU3F3	AAGGAATTCTGCTCACCTGCCCCTT	63	25
	PRU3R3	CCGGAGATCTCCCCTCCTCACCATTCACTGTT	71	32

Table 2. UCP3 Promoter Primers for Fragment Construction. In order to create UCP3 promoter "fragments," the above PCR primers were designed using Vector NTI software (Invitrogen). All primers also included a BglII restriction enzyme site to allow for easy transfer from the T-easy vector to the pGL4.18 vector.

After PCR, all of the fragments were subsequently cloned into the pGEM T-easy vector (Promega) and subcloned into the luciferase reporter vector pGL 4.18 (Promega). Fragment presence, orientation, and quality (absence of nucleotide mismatches) were confirmed by direct sequence analysis (Yale Keck Facility).

Together with pGL4.18/fragment transfection, H9C2 cells were transfected with the pGL4.75 vector containing the CMV promoter to be used as a control. This control plasmid was later used to normalize signal to the number of transfected cells. Briefly, H9C2 cells were plated on 60-mm plates and transfected at 65-80% confluency. FuGene 6.0 (Roche) was used as a transfection reagent. Maximum transfection efficiency was achieved by using 6 µl of FuGene 6.0 and 2 µg of target and 2 µg of control plasmid.

After transfection, half of the cells were treated with 1 mM AICAR while the other half of the cells remained in control culture media. One hour later, all cells were harvested with 300 µl of 0.25% trypsin/EDTA, centrifuged to sediment the cells, resuspended in 100 µl of fresh media, and mixed with 100 µl of DualGlo (Promega) luciferase reagent/buffer mixture as described in the kit's instructions. After twenty

minutes of incubation at room temperature, chemiluminescence was measured using a luminometer (Turner Designs). Results were recorded and 100 µl of 1:100 Stop and Glo (Promega) reagent/buffer mixture was added to each sample. The samples were incubated at room temperature for an additional twenty minutes after which luminescence was measured again. The firefly luciferase chemiluminescence (measured after DualGlo reagent addition) revealed UCP3 transcription in response to AMPK activation or in the absence of this stimulus (control). The CMV Renilla promoter-driven luciferase, measured after addition of Stop and Glo reagent, served as a control to standardize data between samples. Luciferase chemiluminescence for each sample was normalized to CMV Renilla chemiluminescence for the same sample to control for transfection efficiency and cell number. The normalized data from experimental samples were then compared to control samples for the same promoter fragments and a relative expression calculated.

Statistical analysis

Data are reported as mean values +/- standard deviation. Means were compared to control values and to each other with student's t-tests (GB-Stat PPC SchoolPak) and two-tailed p-value less than 0.05 was considered statistically significant.

RESULTS

Activation of AMPK by AICAR

As discussed above, phosphorylation of the threonine-172 residue of the α-subunit of AMPK is necessary for covalent enzyme activation. As was expected, AICAR induced phosphorylation (and activation) of AMPK at both one and twenty-four hours of treatment in H9C2 cells. These data confirm that AMPK is activated under the experimental conditions in which UCP mRNA and protein expression were to be evaluated.

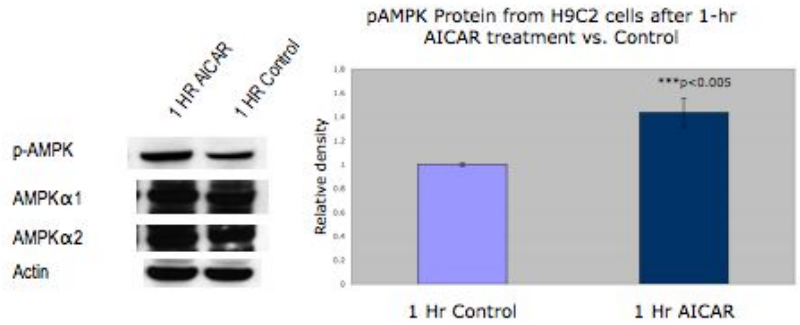

Figure 6. AMPK activation as a result of 1 hour of AICAR treatment. Phosphorylation of the Thr172 residue of the AMPKα subunit is indicative of activation. Treatment of H9C2 cells and subsequent protein isolation and immunoblotting demonstrated that pAMPK levels were significantly increased in AICAR treated samples as compared to controls after 1 hour of treatment. pAMPK density was normalized to Actin. N=5.

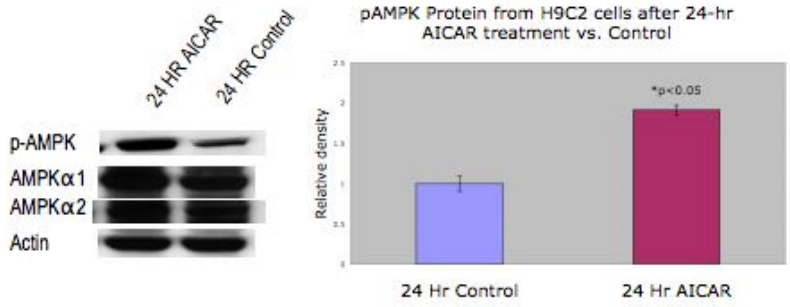

Figure 7. AMPK activation as a result of 24 hours of AICAR treatment. After 24 hours of treatment of H9C2 cells with AICAR a significant difference in Thr172 phosphorylation (AMPK activation) is observed. pAMPK density was normalized to Actin. N=5.

AICAR effect on UCP mRNA in mouse ventricle

In the isolated tissue model utilizing mouse ventricular slices, activation of AMPK by AICAR induced significant increases in UCP2 and UCP3 mRNA at both the 1-hour and 24-hour time points. Interestingly, there was a much greater increase in the expression of UCP3 mRNA after 24 hours of AMPK activation when compared to UCP2 mRNA. These data suggest that AMPK activation may play a role in upregulating the expression of the mitochondrial uncoupling proteins in the heart.

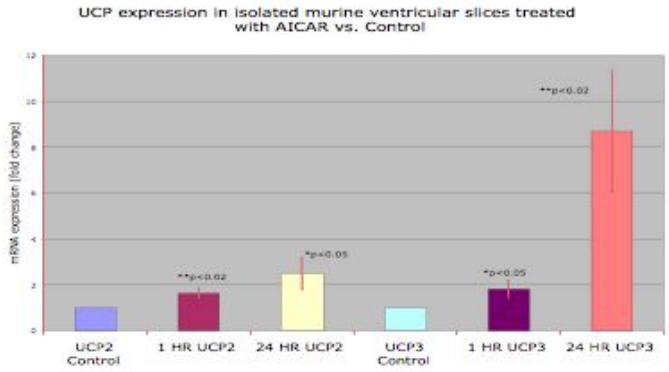

Figure 8. Mouse ventricle incubation with AICAR leads to increased UCP2 and UCP3 mRNA levels. Isolated 1 mm slices of mouse ventricle were incubated in a buffer solution with or without AICAR. After 1 hour or 24 hours, samples were snap frozen in liquid nitrogen and subsequently mRNA isolated from them. qRTPCR was performed yielding data as summarized above. Both UCP2 and UCP3 mRNA were significantly increased at both time points as compared to 1 hour and 24 hour controls.

AICAR effect on UCP3 mRNA in H9C2 cells

To further confirm this association, H9C2 cells were treated with AICAR for 1- or 24-hour periods, and mRNA expression was quantified by qRTPCR. After 1 hour incubation with AICAR, a trend towards an increase in the expression of UCP2 on the mRNA level was observed. In contrast, the expression of UCP3 mRNA was statistically unchanged from control levels.

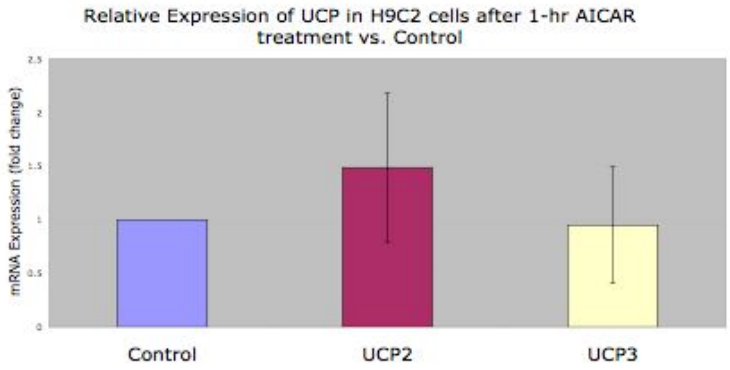

Figure 9. UCP mRNA expression in a cellular model after 1 hour AICAR treatment. H9C2 cells were treated with 1 mM AICAR for 1 hour after which cells were harvested and mRNA isolated. qRTPCR revealed a trend towards increased UCP2 mRNA with inconclusive UCP3 results. N=4.

After H9C2 cells were incubated with AICAR for 24-hours, a small but statistically significant increase in UCP2 mRNA was observed, while UCP3 mRNA levels remained unchanged compared to control levels.

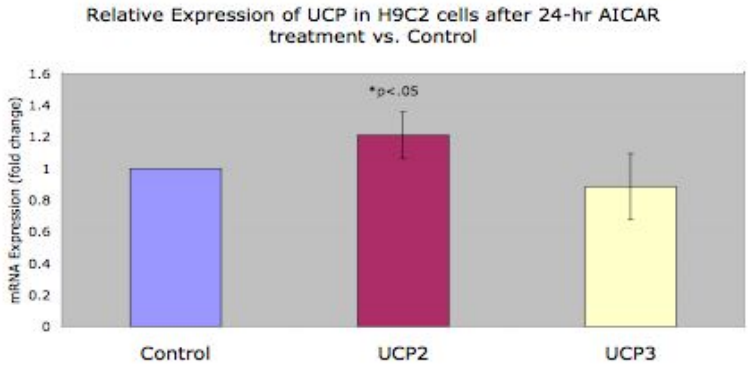

Figure 10. UCP mRNA expression in a cellular model after 24 hours of AICAR treatment. H9C2 cells were treated with 1mM AICAR for 24 hours after which cells were harvested and mRNA isolated. qRTPCR revealed a small but significant increase in UCP2 mRNA but no significant change in UCP3 mRNA. N=4.

AICAR effects on UCP protein expression in H9C2 Cells

Consistent with the mRNA results in the cellular model, immunoblots of UCP2 protein from H9C2 cells treated with AICAR demonstrated significantly increased UCP2 protein expression as compared to the controls samples after both one and twenty-four hours of incubation with AICAR.

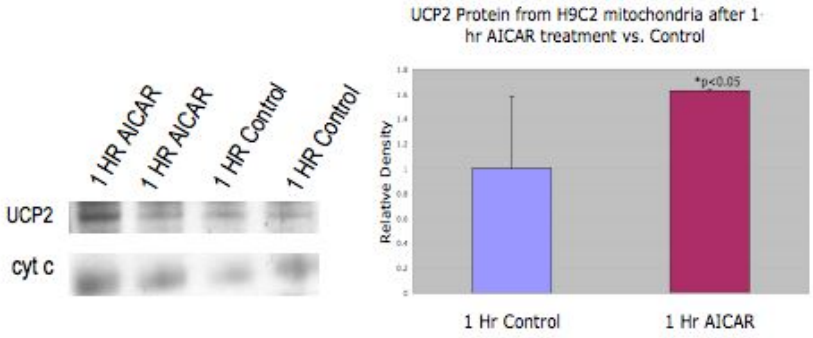

Figure 11. UCP2 protein expression in H9C2 cells after 1 hour of AICAR treatment. Western Blot densitometric analysis demonstrated a significant increase of UCP2 protein in AICAR treated cells as compared to controls. Data normalized to cytochrome c loading controls. N=5.

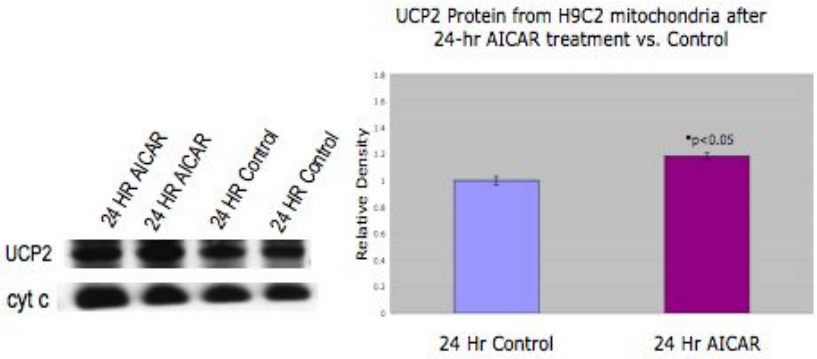

Figure 12. UCP2 protein expression in H9C2 cells after 24 hours of AICAR treatment. Western blot densitometric analysis demonstrated a small but statistically significant increase in UCP2 protein after AMPK activation through AICAR treatment for 24 hours. Data normalized to cytochrome c loading controls. N=6.

Treatment of H9C2 cells with AICAR for 1 hour did not result in an increase in the level of UCP3 protein expression (data not pictured). In contrast, H9C2 incubation with AICAR for 24 hours demonstrated a dramatic increase in UCP3 protein expression, with the AICAR treatment resulting in UCP3 protein levels that were more than 15-fold higher than control samples.

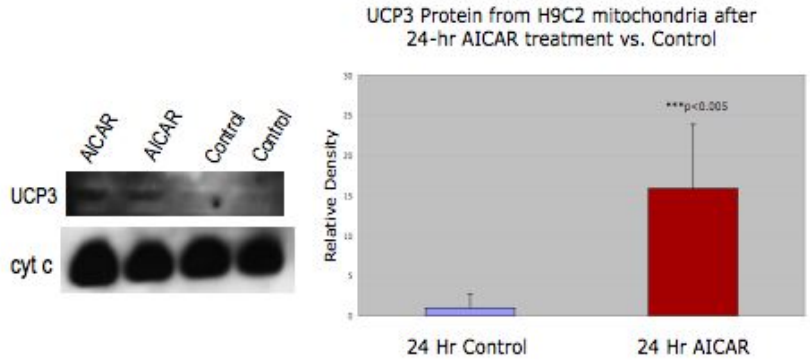

Figure 13. UCP3 protein expression in H9C2 cells after 24 hours of AICAR treatment. Western blot reveals a dramatic increase in UCP3 protein in AICAR treated cells as compared to controls. Data normalized to cytochrome c loading controls. N=6.

AMPK activation and the UCP3 promoter

In the UCP3 promoter studies, a clear increase in firefly luciferase activity was noted when H9C2 cells with the "middle" promoter construct were treated with AICAR for one hour. In contrast, there was no increase in transcriptional activity in response to AICAR for the "left" or "right" sections of the promoter. A large increase in luciferase activity for the "long" construct with AICAR treatment was noted as well, though there was a large degree of variation between measurements for this construct, and the increased response of the "long" fragment, while impressive, remained statistically insignificant.

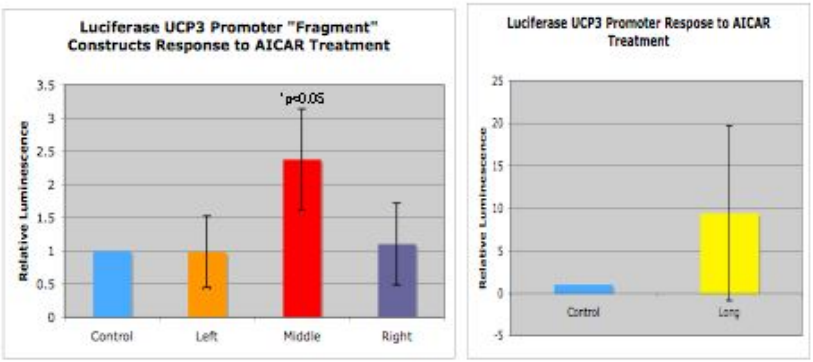

Figure 14. Luciferase UCP3 promoter constructs response to AMPK activation with AICAR. Left panel summarizes data from "fragment" UCP3 promoter constructs while right panel summarizes entire promoter response, both compared to control. After 1 hour of incubation with 1mM AICAR, the entire promoter, "Long," demonstrates trend towards increased luminescence. The "middle" fragment construct has significantly increased luminescence after AICAR treatment relative to control.

DISCUSSION

These studies were performed to determine if AMPK activation results in an increase in the expression of UCPs in the heart. We first demonstrated that the pharmacologic activator, AICAR, increased AMPK phosphorylation in heart muscle both after one hour and 24 hours of incubation. We then demonstrated that AMPK activation was associated with increased expression of UCP2 and UCP3 mRNA at either 1 or 24 hours of stimulation with AICAR in adult mouse heart slices. In contrast, in H9C2 myocyte cultures, the changes in UCP mRNA were much less pronounced with only UCP2 reaching statistical significance. However, we found little correlation between the expression of the UCP mRNAs and expression of the UCPs on the protein level. Specifically, there was only a small increase in UCP2 protein with AICAR incubation while there was a dramatic 15-fold increase in the expression of UCP3 by 24 hours of AMPK stimulation by AICAR. With respect to the regulation of UCP expression by AMPK activation, we found that there is a region within the promoter region between -1600 and -697 bp upstream of the start codon that contains element(s) potentially regulated by AMPK activation.

	UCP2		UCP3	
	1hr AICAR	24hr AICAR	1hr AICAR	24hr AICAR
Mouse tissue mRNA	+	+	+	+ +
H9C2 mRNA	(+)	+	No change	No change
H9C2 protein	+	+	No change	+ +
Rat UCP promoter	N/A	N/A	+	N/A

Table 3. Summary of UCP2 and UCP3 response to AICAR.

Using isolated mouse ventricle slices as a tissue model, we have demonstrated that AMPK activation by AICAR leads to statistically significant increases in UCP2 and UCP3 mRNA at both one-hour and twenty-four-hour time points. Interestingly, when using a similar protocol in the H9C2 cellular model, UCP mRNA response was less striking. UCP2 mRNA appeared to trend towards an increase at one hour and a small but statistically significant increase was observed at twenty-four hours. No significant difference in UCP3 mRNA was detected after either one hour or twenty-four hour incubation with AICAR. This was particularly surprising given the robust response of UCP3 at 24 hours in the mouse model.

These discrepant results might be explained by the fact that there is considerable variation in UCP expression (and by extension, regulation) between different species and even between different tissues within the same species. However, one would not expect the observed UCP3 immunoblot results: while there was no difference between UCP3 protein levels in H9C2 mitochondrial isolations after one hour of AICAR incubation, there was a striking increase in UCP3 protein in the cells incubated for 24 hours with AICAR as compared to control samples. It is possible that there was a transient increase in UCP3 mRNA at some time between one and twenty-four hours which had decayed by the time points that were analyzed while the increase in protein expression remained detectable. Alternatively, another potential explanation for the discrepancy between UCP protein and mRNA results is suggested by the following previous studies. UCP2 protein has been shown to increase up to 12-fold in mouse lung and stomach following lipopolysaccharide treatment without any corresponding increase in UCP2 mRNA, a result that these authors attributed to translational regulation.[98] Such translational

regulation may also contribute to determining UCP3 protein expression levels. In either case, it was clear that 24-hour incubation of H9C2 cells with 1mM AICAR led to a significant increase in UCP3 protein levels.

Given the evidence that AMPK activation regulates increased UCP expression in skeletal muscle, and that this effect is likely mediated through modification of genetic expression through AMPK translocation to the nucleus and/or AMPK-driven activation of various transcription factors, we hypothesized that AMPK would employ a similar mechanism in the heart. Specifically, AMPK activation was hypothesized to regulate UCP2 and 3 expression by influencing cellular transcription. In constructing UCP3 promoter "fragments" we hoped to identify a region of the gene where activated AMPK acts to induce increased expression of UCP3. Our results support the conclusion that such a region exists somewhere between -1600 and -697 base pairs upstream of the transcription start site. Further characterization of this site and the possible mechanism of interaction remain to be investigated.

Limitations

A limit to our study includes the incomplete identification of the UCP3 promoter site referenced above, as well as a lack of *in vivo* data to support our experimental models. Given the cross-species variation of UCP expression, it is also not unreasonable to question whether data from mouse and rat models can be generalized to humans. Although most data concerning UCP expression and function in the heart thus far support a system relatively conserved across these species, further investigation in humans is warranted.

Similarly, although recent research has indicated that UCP3 protein is present in

the adult heart, while there remains a considerable degree of controversy as to whether UCP2 protein is expressed in cardiomyocyte mitochondria.[97] Our decision to investigate the UCP3 promoter, and not UCP2, was influenced by this uncertainty. Nevertheless, our findings of UCP2 protein in H9c2 preparations support the conclusion that UCP2 may, in fact, be expressed in the myocardium.

Yet our choice of cellular model may also be interpreted as a limitation. The H9C2 cell line was derived from fetal rat cardiomyocytes to allow for multiplication in culture.[95] Adult cardiomyocytes are terminally differentiated and must be repeatedly obtained from sacrificed animals. This is labor-intensive and difficult. While advantageous in this respect, H9C2 cells are not ideal in that they do not completely retain the characteristics of isolated (or *in vivo*) adult cardiomyocytes. For example, H9C2 cells lack the gap junctions, caveolae, T tubules, and microfibriller sarcomeres of freshly prepared cardiomyocytes. Nevertheless, the composition of the surface coat as well as many of the ion channels and signaling proteins remain similar or identical between the immortalized H9C2 cell line and the isolated cardiomyocte.[99] Further studies employing an isolated cardiomyocyte model, while laborious, would strengthen the observations made in this study.

A further minor limitation of our study is our method of verifying AMPK activation. In addition to pAMPK immunoblotting, we could have checked downstream ACC activation or performed AMPK activity assays to prove AMPK activation. However, given the general agreement that phosphorylation is indicative of activation, we felt that such measures would be superfluous.

It should be noted, however, that AICAR is a relatively nonspecific activator of AMPK and, as such, upregulation of the UCPs upon AICAR treatment does not specifically prove a cause and effect relationship between AMPK activation and increased UCP expression, although it does strongly support such a conclusion.[94] To remove any doubt that activated AMPK causes UCP upregulation, further experiments could be conducted treating an AMPK deficient model (AMPK KD cardiomyocytes or H9C2 cells transfected with an AMPK KD vector) with AICAR in an attempt to observe a lack of UCP response.

Clinical Support

Despite the limitations of our study, previous data support our conclusions. The chemical activator of AMPK, AICAR, has previously been used in clinical trials as a cardioprotective agent (although this was not done specifically to upregulate UCPs, or even to phosphorylate AMPK). A meta-analysis of studies employing acadasine (the formulary name for AICAR) to treat patients before and after coronary artery bypass graft surgery demonstrated that treatment with the drug yielded moderate benefits. Specifically, acadesine decreased the need for left ventricular assist devices, the number of peri-operative myocardial infarctions, and the number of post-operative cardiac deaths when compared to placebo treatment.[100] Acadasine was developed as an adenosine reuptake inhibitor to promote activation of adenosine receptors in the ischemic heart and to potentially help replenish depleted myocardial stores of GTP and ATP.[69] The drug was ultimately abandoned because it only weakly augmented the adenosine pathway. The relatively modest effects could be explained by the fact that AICAR is only a weak activator of AMPK.[70, 94] Furthermore, AICAR is not an ideal activator because it must be

converted to ZMP through phosphorylation to exert its effects, and has significant side effects including bradycardia and hypoglycemia.[68]

At the time the clinical studies using AICAR were conducted, the actions of this chemical were incompletely understood. Neither activation of AMPK nor upregulation of the UCPs was considered as a potentially cardioprotective mechanism. Nevertheless, the moderately successful results of this drug may be explained by this pathway, and provide important human, clinical evidence that AICAR activation of AMPK (and potential upregulation of cardiac UCPs) improves outcomes after exposure to hypoxia/ischemia.

Future Directions

UCPs have many important potential clinical benefits including protection of the heart during periods of ischemia and reperfusion, as well as a role in the prevention of heart failure. Our results support the idea that AMPK activation induces UCP upregulation through a mechanism involving interaction with the UCP promoter. However, some pieces of the puzzle remain missing. For example, we do not yet have data as to the promoter response after 24 hours of AICAR incubation, though we expect that the "Middle" fragment will yield increased luciferase activity as was observed in the 1 hour incubations. Further experiments will cull the size of the promoter fragments until we have isolated the nucleotide sequence that appears to be responsible for the AMPK-induced upregulation of UCP3. We also hope to identify potential transcription factors that might interact with this region. Finally, we will confirm the involvement of AMPK in the upregulation of the UCPs through the use of AMPK KO mouse models or AMPK deficient cell lines (as mentioned above). We believe that future studies of the role of

AICAR in UCP regulation utilizing *in vivo* animal models and human subjects will clarify this important pathway and may lead to novel cardioprotective therapies.

CONCLUSIONS

We hypothesized that one of the pathways responsible for increased expression of cardiac uncoupling proteins involves activation of AMP-activated protein kinase. Our data show that pharmacologic AMPK activation with the AMP-mimetic, AICAR, leads to increased levels of UCP2 and UCP3 mRNA in an isolated tissue model. We have further shown that UCP2 and UCP3 protein expression in a cellular cardiomyocyte model is increased as a result of AICAR treatment. Finally, we have identified a 1000 base pair region of the UCP3 promoter which appears to mediate AMPK-activated UCP transcriptional upregulation. Taken together, these data support the idea that activation of AMPK is a critical step in increasing myocardial expression of the mitochondrial uncoupling proteins.

REFERENCES

1. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. Physiol Rev 1984;64:1-64.

2. Fleury C, Neverova M, Collins S, et al. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet 1997;15:269-72.

3. Gimeno RE, Dembski M, Weng X, et al. Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. Diabetes 1997;46:900-6.

4. Sack MN. Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance. Cardiovasc Res 2006;72:210-9.

5. Murray AJ, Anderson RE, Watson GC, Radda GK, Clarke K. Uncoupling proteins in human heart. Lancet 2004;364:1786-8.

6. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB. UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. Biochem Biophys Res Commun 1997;235:79-82.

7. Matsuda J, Hosoda K, Itoh H, et al. Cloning of rat uncoupling protein-3 and uncoupling protein-2 cDNAs: their gene expression in rats fed high-fat diet. FEBS Lett 1997;418:200-4.

8. Jaburek M, Varecha M, Gimeno RE, et al. Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. J Biol Chem 1999;274:26003-7.

9. Kontani Y, Wang Z, Furuyama T, Sato Y, Mori N, Yamashita H. Effects of aging and denervation on the expression of uncoupling proteins in slow- and fast-twitch muscles of rats. Journal of Biochemistry 2002;132:309-15.

10. Bing C, Brown M, King P, Collins P, Tisdale M, Williams G. Increased gene expression of brown fat uncoupling protein (UCP)1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. Cancer Res 2000;60:2405-10.

11. Taegtmeyer H, Razeghi P, Young ME. Mitochondrial proteins in hypertrophy and atrophy: a transcript analysis in rat heart. Clin Exp Pharmacol Physiol 2002;29:346-50.

12. Jezek P. Fatty acid interaction with mitochondrial uncoupling proteins . Review 66 refs. Journal of Bioenergetics & Biomembranes;31:457-66.

13. Jezek P. Possible physiological roles of mitochondrial uncoupling proteins -- UCPn. Review 100 refs. International Journal of Biochemistry & Cell Biology;34:1190-206.

14. Bezaire V, Hofmann W, Kramer JK, Kozak LP, Harper ME. Effects of fasting on muscle mitochondrial energetics and fatty acid metabolism in Ucp3(-/-) and wild-type mice. American Journal of Physiology - Endocrinology & Metabolism 2001;281:E975-82.

15. Garcia-Martinez C, Sibille B, Solanes G, et al. Overexpression of UCP3 in cultured human muscle lowers mitochondrial membrane potential , raises ATP/ADP ratio, and favors fatty acid vs. glucose oxidation. FASEB Journal 2001;15:2033-5.

16. Hong Y, Fink BD, Dillon JS, Sivitz WI. Effects of adenoviral overexpression of uncoupling protein-2 and -3 on mitochondrial respiration in insulinoma cells. Endocrinology 2001;142:249-56.

17. Douette P, Sluse FE. Mitochondrial uncoupling proteins : new insights from functional and proteomic studies. Review 108 refs. Free Radical Biology & Medicine 2006;40:1097-107.

18. Matthias A, Ohlson KB, Fredriksson JM, Jacobsson A, Nedergaard J, Cannon B. Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty scid-induced thermogenesis. J Biol Chem 2000;275:25073-81.

19. Liu SS. Generating, partitioning, targeting and functioning of superoxide in mitochondria. Biosci Rep 1997;17:259-72.

20. Vidal-Puig AJ, Grujic D, Zhang CY, et al. Energy metabolism in uncoupling protein 3 gene knockout mice. J Biol Chem 2000;275:16258-66.

21. Mattiasson G, Shamloo M, Gido G, et al. Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. Nat Med 2003;9:1062-8.

22. Bechmann I, Diano S, Warden CH, Bartfai T, Nitsch R, Horvath TL. Brain mitochondrial uncoupling protein 2 (UCP2): a protective stress signal in neuronal injury. Biochem Pharmacol 2002;64:363-7.

23. Teshima Y, Akao M, Jones SP, Marban E. Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes. Circ Res 2003;93:192-200.

24. Bienengraeber M, Ozcan C, Terzic A. Stable transfection of UCP1 confers resistance to hypoxia/reoxygenation in a heart-derived cell line. J Mol Cell Cardiol 2003;35:861-5.

25. Hoerter J, Gonzalez-Barroso MD, Couplan E, et al. Mitochondrial uncoupling protein 1 expressed in the heart of transgenic mice protects against ischemic-reperfusion damage. Circulation 2004;110:528-33.

26. Guo P, Mizushige K, Noma T, et al. Association of uncoupling protein-2 expression with increased reactive oxygen species in residual myocardium of the enlarged left ventricle after myocardial infarction. Heart Vessels 2005;20:61-5.

27. Boss O, Samec S, Dulloo A, Seydoux J, Muzzin P, Giacobino JP. Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting or cold. FEBS Lett 1997;412:111-4.

28. McLeod CJ, Aziz A, Hoyt RF, Jr., McCoy JP, Jr., Sack MN. Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. J Biol Chem 2005;280:33470-6.

29. Blanc J, Alves-Guerra MC, Esposito B, et al. Protective role of uncoupling protein 2 in atherosclerosis. Circulation 2003;107:388-90.

30. Oberkofler H, Iglseder B, Klein K, et al. Associations of the UCP2 gene locus with asymptomatic carotid atherosclerosis in middle-aged women. Arterioscler Thromb Vasc Biol 2005;25:604-10.

31. Dhamrait SS, Stephens JW, Cooper JA, et al. Cardiovascular risk in healthy men and markers of oxidative stress in diabetic men are associated with common variation in the gene for uncoupling protein 2. Eur Heart J 2004;25:468-75.

32. Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest 2005;115:500-8.

33. Givertz MM, Colucci WS. New targets for heart-failure therapy: endothelin, inflammatory cytokines, and oxidative stress. Lancet 1998;352 Suppl 1:SI34-8.

34. Diaz-Velez CR, Garcia-Castineiras S, Mendoza-Ramos E, Hernandez-Lopez E. Increased malondialdehyde in peripheral blood of patients with congestive heart failure. Am Heart J 1996;131:146-52.

35. Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, Tedgui A. Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for in vivo oxidant stress in ventricular dilatation and progression to heart failure. Circulation 1998;97:1536-9.

36. Baumer AT, Flesch M, Wang X, Shen Q, Feuerstein GZ, Bohm M. Antioxidative enzymes in human hearts with idiopathic dilated cardiomyopathy. J Mol Cell Cardiol 2000;32:121-30.

37. Sam F, Kerstetter DL, Pimental DR, et al. Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium. J Card Fail 2005;11:473-80.

38. Nojiri H, Shimizu T, Funakoshi M, et al. Oxidative stress causes heart failure with impaired mitochondrial respiration. J Biol Chem 2006;281:33789-801.

39. Hiroi S, Harada H, Nishi H, Satoh M, Nagai R, Kimura A. Polymorphisms in the SOD2 and HLA-DRB1 genes are associated with nonfamilial idiopathic dilated cardiomyopathy in Japanese. Biochem Biophys Res Commun 1999;261:332-9.

40. Valenti L, Conte D, Piperno A, et al. The mitochondrial superoxide dismutase A16V polymorphism in the cardiomyopathy associated with hereditary haemochromatosis. J Med Genet 2004;41:946-50.

41. Nakamura K, Kusano KF, Matsubara H, et al. Relationship between oxidative stress and systolic dysfunction in patients with hypertrophic cardiomyopathy. J Card Fail 2005;11:117-23.

42. Ye G, Metreveli NS, Ren J, Epstein PN. Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. Diabetes 2003;52:777-83.

43. Boudina S, Abel ED. Diabetic cardiomyopathy revisited. Circulation 2007;115:3213-23.

44. Boudina S, Abel ED. Mitochondrial uncoupling: a key contributor to reduced cardiac efficiency in diabetes. Physiology (Bethesda) 2006;21:250-8.

45. Kawai K, Qin F, Shite J, Mao W, Fukuoka S, Liang CS. Importance of antioxidant and antiapoptotic effects of beta-receptor blockers in heart failure therapy. Am J Physiol Heart Circ Physiol 2004;287:H1003-12.

46. Gao F, Chen J, Lopez BL, et al. Comparison of bisoprolol and carvedilol cardioprotection in a rabbit ischemia and reperfusion model. Eur J Pharmacol 2000;406:109-16.

47. Shiomi T, Tsutsui H, Matsusaka H, et al. Overexpression of glutathione peroxidase prevents left ventricular remodeling and failure after myocardial infarction in mice. Circulation 2004;109:544-9.

48. Fox KF, Cowie MR, Wood DA, et al. Coronary artery disease as the cause of incident heart failure in the population. Eur Heart J 2001;22:228-36.

49. Young ME, Patil S, Ying J, et al. Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. FASEB Journal 2001;15:833-45.

50. Razeghi P, Young ME, Ying J, et al. Downregulation of metabolic gene expression in failing human heart before and after mechanical unloading. Cardiology 2002;97:203-9.

51. Neubauer S. The failing heart--an engine out of fuel. New England Journal of Medicine 2007;356:1140-51.

52. Ashrafian H, Frenneaux MP, Opie LH. Metabolic mechanisms in heart failure. Circulation 2007;116:434-48.

53. Hesselink MK, Schrauwen P. Uncoupling proteins in the failing human heart: friend or foe? Lancet 2005;365:385-6.

54. Selwyn AP, Braunwald E. Ischemic Heart Disease. In: Kasper DL, et. al., eds., ed. Harrisons's Internal Medicine. 16th edition ed. Online Resource: McGraw-Hill Companies; 2007.

55. Mosterd A, Hoes AW. Clinical epidemiology of heart failure. Heart 2007;93:1137-46.

56. Redfield MM, Jacobsen SJ, Burnett JC, Jr., Mahoney DW, Bailey KR, Rodeheffer RJ. Burden of systolic and diastolic ventricular dysfunction in the community: appreciating the scope of the heart failure epidemic. JAMA 2003;289:194-202.

57. Stewart S, MacIntyre K, Hole DJ, Capewell S, McMurray JJ. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. Eur J Heart Fail 2001;3:315-22.

58. Blackledge HM, Tomlinson J, Squire IB. Prognosis for patients newly admitted to hospital with heart failure: survival trends in 12 220 index admissions in Leicestershire 1993-2001. Heart 2003;89:615-20.

59. Brophy JM, Joseph L, Rouleau JL. Beta-blockers in congestive heart failure. A Bayesian meta-analysis. Ann Intern Med 2001;134:550-60.

60. Mark DB. Economics of treating heart failure . American Journal of Cardiology 1997;80:33H-8H.

61. Young ME, Patil S, Ying J, et al. Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. FASEB J 2001;15:833-45.

62. Murray AJ, Panagia M, Hauton D, Gibbons GF, Clarke K. Plasma free fatty acids and peroxisome proliferator-activated receptor alpha in the control of myocardial uncoupling protein levels. Diabetes 2005;54:3496-502.

63. Zhou M, Lin BZ, Coughlin S, Vallega G, Pilch PF. UCP -3 expression in skeletal muscle : effects of exercise, hypoxia, and AMP-activated protein kinase. American Journal of Physiology - Endocrinology & Metabolism 2000;279:E622-9.

64. Stoppani J, Hildebrandt AL, Sakamoto K, Cameron-Smith D, Goodyear LJ, Neufer PD. AMP-activated protein kinase activates transcription of the UCP3 and HKII genes in rat skeletal muscle. Am J Physiol Endocrinol Metab 2002;283:E1239-48.

65. Jorgensen SB, Wojtaszewski JF, Viollet B, et al. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. FASEB J 2005;19:1146-8.

66. Carling D, Zammit VA, Hardie DG. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Letters 1987;223:217-22.

67. Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation . Review 112 refs. Journal of Clinical Investigation 2006;116:1776-83.

68. Young LH, Li J, Baron SJ, Russell RR. AMP - activated protein kinase : a key stress signaling pathway in the heart. Review 93 refs. Trends in Cardiovascular Medicine 2005;15:110-8.

69. Hardie DG. AMP-activated protein kinase: the guardian of cardiac energy status . comment . Review 20 refs Comments Comment on: J Clin Invest. 2004 Aug;114(4):495-503; PMID: 15314686. Journal of Clinical Investigation 2004;114:465-8.

70. Hardie DG. Minireview : the AMP-activated protein kinase cascade : the key sensor of cellular energy status . Review 66 refs. Endocrinology 2003;144:5179-83.

71. Woods A, Cheung PC, Smith FC, et al. Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. Journal of Biological Chemistry 1996;271:10282-90.

72. Stapleton D, Mitchelhill KI, Gao G, et al. Mammalian AMP-activated protein kinase subfamily. Journal of Biological Chemistry 1996;271:611-4.

73. Li J, Coven DL, Miller EJ, et al. Activation of AMPK alpha- and gamma-isoform complexes in the intact ischemic rat heart. Am J Physiol Heart Circ Physiol 2006;291:H1927-34.

74. Salt I, Celler J, Hawley S, et al. AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem J 1998;334:177-87.

75. Kemp BE, Stapleton D, amp, et al. AMP - activated protein kinase , super metabolic regulator . Review 78 refs. Biochemical Society Transactions 2003;31:162-8.

76. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA. The Ca2+/calmodulin-dependent protein kinase kinase s are AMP - activated protein kinase kinase s. Journal of Biological Chemistry 2005;280:29060-6.

77. Davies SP, Helps NR, Cohen PT, Hardie DG. 5'-AMP inhibits dephosphorylation , as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Letters 1995;377:421-5.

78. Polekhina G, Gupta A, Michell BJ, et al. AMPK beta subunit targets metabolic stress sensing to glycogen. Current Biology 2003;13:867-71.

79. Russell RR, 3rd, Li J, Coven DL, et al. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury . Journal of Clinical Investigation 2004;114:495-503.

80. Coven DL, Hu X, Cong L, et al. Physiological role of AMP-activated protein kinase in the heart : graded activation during exercise. American Journal of Physiology - Endocrinology & Metabolism 2003;285:E629-36.

81. Russell RR, 3rd, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. American Journal of Physiology 1999;277:H643-9.

82. Xing Y, Musi N, Fujii N, et al. Glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative alpha2 subunit of AMP-activated protein kinase. Journal of Biological Chemistry 2003;278:28372-7.

83. Marsin AS, Bertrand L, Rider MH, et al. Phosphorylation and activation of heart PFK -2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Current Biology 2000;10:1247-55.

84. Jager S, Handschin C, St-Pierre J, Spiegelman B. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. proc Natl Acad Sci U S A 2007;104:12017-22.

85. Putman C, Kiricsi M, Pearcey J, et al. AMPK Activation Increases UCP-3 Expression and Mitochondrial Enzyme Activities in Rat Muscle without Fibre Type Transitions -- Putman et al., 10.1113/jphysiol.2003.040691 -- The Journal of Physiology Online. J Physiol 2003;551:169-78.

86. McGee S, Howlett K, Starkie R, Cameron-Smith, D, Kemp B, Hargreaves M. Exercise increases nuclear AMPK alpha2 in human skeletal muscle. Diabetes 2003;52:926-8.

87. Lee M, Hwang J, Lee H, et al. AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. J Biol Chem 2003;278:39653-61.

88. Bergeron R, Ren J, Cadman K, et al. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis -- Bergeron et al. 281 (6): E1340 -- AJP - Endocrinology and Metabolism. In; 2001:E1340-E6.

89. Fryer LG, Parbu-Patel A, Carling D. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP - activated protein kinase through distinct signaling pathways. Journal of Biological Chemistry 2002;277:25226-32.

90. Van Der Lee KA, Willemsen PH, Van Der Vusse GJ, Van Bilsen M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. FASEB Journal 2000;14:495-502.

91. Kageyama H, Osaka T, Kageyama A, et al. Fasting increases gene expressions of uncoupling proteins and peroxisome proliferator-activated receptor-gamma in brown adipose tissue of ventromedial hypothalamus-lesioned rats. Life Sciences 2003;72:3035-46.

92. Teruel T, Smith SA, Peterson J, Clapham JC. Synergistic activation of UCP-3 expression in cultured fetal rat brown adipocytes by PPAR alpha and PPAR gamma ligands. Biochemical & Biophysical Research Communications 2000;273:560-4.

93. Choi SL, Kim SJ, Lee KT, et al. The regulation of AMP-activated protein kinase by H(2)O(2). Biochemical & Biophysical Research Communications 2001;287:92-7.

94. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole- 4-carboxamide ribonucleoside . A specific method for activating AMP-activated protein kinase in intact cells? European Journal of Biochemistry 1995;229:558-65.

95. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. Experimental Cell Research;98:367-81.

96. Sivitz WI, Fink BD, Donohoue PA. Fasting and leptin modulate adipose and muscle uncoupling protein : divergent effects between messenger ribonucleic acid and protein expression. Endocrinology 1999;140:1511-9.

97. Krauss S, Zhang CY, Lowell BB. The mitochondrial uncoupling - protein homologues . Review 114 refs. Nature Reviews Molecular Cell Biology;6:248-61.

98. Pecqueur C, Alves-Guerra MC, Gelly C, et al. Uncoupling protein 2, in vivo distribution, induction upon oxidative stress , and evidence for translational regulation. Journal of Biological Chemistry 2001;276:8705-12.

99. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. Circulation Research 1991;69:1476-86.

100. Mangano DT. Effects of acadesine on myocardial infarction, stroke, and death following surgery. A meta-analysis of the 5 international randomized trials. The Multicenter Study of Perioperative Ischemia (McSPI) Research Group. Jama 1997;277:325-32.