MECHANISMS OF ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE-INDUCED PRECONDITIONING IN ISCHEMIA/REPERFUSION

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F. Spencer Gaskin
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

MECHANISMS OF ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE-INDUCED PRECONDITIONING IN ISCHEMIA/REPERFUSION

presented by Frederick Spencer Gaskin

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance.

_____ Ronald J. Korthuis, Ph.D.

_____ Douglas K. Bowles, Ph.D.

_____ Michael J. Davis, Ph.D.

_____ Virginia H. Huxley, Ph.D.

_____ Michael J. Rovetto, Ph.D.

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LIST OF ABBREVIATIONS USED

5-HD - 5-hydroxydecanoate

ACC - Acetyl-coenzyme A carboxylase

ADP – Adenosine diphosphate

AICAR - 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside

AICAR-PC – AICAR preconditioning

AMP – Adenosine monophosphate

AMPK – Adenosine monophosphate-activated protein kinase

Ara-A – Adenine 9-β-D-arabinofuranoside

ATP – Adenosine triphosphate

CaMKK – Calcium/calmodulin-dependent protein kinase kinase

cAMP – Cyclic adenosine monophosphate

CD – Cluster of differentiation

CFDASE - 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

CO – Carbon monoxide

Compound C - 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine

CuPP – Copper protoporphyrin

Cu/Zn SOD – Copper/zinc superoxide dismutase

EPC – Ethanol preconditioning

eNOS – Endothelial nitric oxide synthase

HMGR - HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase

HO – Heme oxygenase

HO-1 – Heme oxygenase-1

HO-2 – Heme oxygenase-2

HO-3 – Heme oxygenase-3

Hemin-PC – Hemin preconditioning

hr(s) – Hr(s)

HSP32 – Heat shock protein 32

ICAM-1 – Intercellular adhesion molecule-1

i.m. – intramuscular

iNOS – Inducible nitric oxide synthase

i.p. - Intraperitoneal

IPC – Ischemic preconditioning

I/R – Ischemia/reperfusion

i.v. – Intravenous

K_{ATP} – ATP-sensitive potassium channel

LA – Leukocyte adhesion

LEI – Leukocyte endothelial cell interactions

L-NAME - Nω-Nitro-L-arginine methyl ester hydrochloride

LR – Leukocyte rolling

min - Minutes

mK_{ATP} – Mitochondrial ATP-sensitive potassium channel

MMP - matrix metalloproteinases

MnSOD – Manganese superoxide dismutase

MODS – Multiple organ dysfunction syndrome

MPO - Myeloperoxidase

mRNA – Messenger RNA

NADPH oxidase - Nicotinamide adenine dinucleotide phosphate oxidase

NFκB – Nuclear factor kappa B

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOS – Nitric oxide synthase

PECAM-1 - Platelet endothelial cell adhesion molecule-1

PMN – Polymorphonuclear leukocyte

RGD - Arg-Gly-Asp sequence

ROS – Reactive oxygen species

sec - Seconds

Ser1177 – Serine 1177

siRNA – Small interfering RNA

SIRS – Systemic inflammatory response syndrome

sK_{ATP} – Surface (plasmalemmal) ATP-sensitive potassium channel

SnPP – Tin protoporphyrin

Thr172 – Threonine 172

TNFα – Tumor necrosis factor alpha

VCAM-1 - Vascular cell adhesion molecule-1

ZnPP – Zinc protoporphyrin

ABSTRACT

The phenomenon of ischemic preconditioning (IPC) to protect against the sequelae of ischemia/reperfusion (I/R) as seen clinically with heart attacks, strokes, and transplant surgeries, has received increasing attention since it was first discovered that brief bouts of ischemia and reperfusion prior to the prolonged ischemic insult paradoxically provides protection. Unfortunately, clinical trials aimed at harnessing this innate protective mechanism with various preconditioning stimuli have produced disappointing results. As the signaling mechanisms involved in the development of a protective phenotype are further elucidated, more specific and effective pharmacologic means can be developed to target key initiators, mediators, or effectors to elicit these salubrious events. One such potential preconditioning pathway is initiated by low dose ethanol consumption. We have shown previously that consuming the ethanol equivalent of one to two alcoholic beverages twenty-four hrs prior to a prolonged ischemic challenge (ethanol preconditioning, EPC), protects mice against I/R injury following forty-five min of ischemia and sixty min of reperfusion. We hypothesized that the serine/threonine kinase, adenosine monophosphate-activated protein kinase (AMPK), is activated by ethanol and in turn leads to endothelial nitric oxide synthase (eNOS) phosphorylation. The activation of eNOS to increase nitric oxide (NO) production has many beneficial effects both directly and indirectly. Here we show that AMPK activation by preconditioning with either ethanol or with direct pharmacologic activation via 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), twenty-four hrs prior to the

prolonged I/R (EPC and AICAR-PC, respectively), significantly reduced the number of rolling and adherent leukocytes in the post-capillary venules of the mouse small intestine as quantified using intra-vital microscopy. Ten single, unbranched venules with external diameters ranging from twenty-five to fifty microns were recorded for one minute each, following thirty and sixty min of reperfusion. Leukocytes were labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE), and determined to roll based on flow rates significantly below the mean centerline velocity of non-rolling leukocytes, and to be adherent if they remained stationary for a minimum of thirty sec. These leukocyte-endothelial cell adhesive interactions (LEI) were used as an index of the severity of the I/R injury, as they are some of the earliest and most sensitive indicators of this inflammatory response. Several other proteins in addition to AMPK and eNOS were also found to be involved in the protective signaling pathway, including both surface (or plasmalemmal) and mitochondrial adenosine triphosphate-sensitive potassium channels (sK_{ATP} and mK_{ATP}, respectively) and heme oxygenase (HO). Thus our overall hypothesis that EPC requires AMPK activation, and AMPK activation via AICAR-PC requires eNOS, K_{ATP} channels, and HO to induce the protective phenotype against postischemic leukocyte rolling and adhesion in postcapillary venules of the murine small intestine was proven to be true. By further identifying the participating proteins and signaling molecules involved in this complex event, the potential for therapeutically relevant targets can be examined and ultimately utilized to reduce

the morbidity and mortality associated with some of the leading causes on death in the modern world.

REVIEW OF THE LITERATURE

I. Introduction:

It has been shown that brief periods of coronary arterial occlusion followed by reperfusion prior to prolonged ischemia (a phenomenon known as ischemic preconditioning, or IPC), paradoxically protects the heart against deleterious inflammatory responses and limits infarct size seen in postischemic control animals without IPC. The ability to reduce ischemia/reperfusion (I/R) injury via the innate mechanism of IPC offers tantalizing potential to protect patients from the leading causes of death in the modern world. While the protective effects of IPC are impressive, the therapeutic utility of such an intervention is untenable, given the invasive nature of the approach. Ethanol preconditioning (EPC) offers a means of inducing the protective mechanism of IPC without the need for invasive and dangerous tissue or organ ischemia, and thus greatly increases its clinical relevance and potential. Additionally, IPC loses its protective effects in the presence of risk factors such as aging or diabetes, while EPC remains effective as a preconditioning stimulus under these conditions. By understanding the mechanisms whereby antecedent ethanol induces protection against I/R injury and the role of adenosine monophosphate-activated protein kinase (AMPK) in EPC, pharmacologic interventions may be developed that are more effective and practical to apply in clinical situations. The overall aim of the studies undertaken within are to determine the role of AMPK in EPC and further

elucidate the mechanisms involved in preconditioning to produce an anti-inflammatory phenotype in postcapillary venules via direct AMPK activation. The hypothesis tested was that the postischemic anti-inflammatory effects afforded by EPC are triggered by an AMPK-dependent mechanism and can be pharmacologically induced by direct activation of AMPK with AICAR (AICAR-PC). Moreover, antecedent AICAR triggers entrance into a preconditioned anti-inflammatory state by NO-derived from endothelial nitric oxide synthase (eNOS) and is mediated by an ATP-sensitive potassium (K_{ATP}) channel- and heme oxygenase-dependent mechanism as illustrated in Figure 1.

Because the role of AMPK in EPC to attenuate I/R injury is unknown, the first specific aim to be examined focused on determining if EPC involves AMPK, and the ability of AICAR-PC to reduce the inflammatory response following I/R. Different AMPK catalytic subunits were examined to further elucidate the protective signaling pathway. The role of eNOS in AICAR-PC will be investigated in a second aim. NO and in particular NO, produced from eNOS will be examined because of the large body of evidence that implicates NO and eNOS as triggers in other forms of late preconditioning.

K_{ATP} channels have been shown to be essentially involved in many preconditioning pathways, thus the third aim will determine if AICAR-PC occurs via a K_{ATP}-dependent mechanism, and if so, which K_{ATP} channels are involved in triggering versus mediating the protective effects. The surface or plasmalemmal K_{ATP} channel has been shown to be a trigger of IPC, but is not involved in mediating the protection on day two (101). Conversely, mitochondrial K_{ATP}

channels have been shown to be involved in mediating the protective effect on day two during the ischemic insult but not in triggering this response on day one (9).

One of the key mediators of preconditioning is the stress induced, carbon monoxide (CO) producing protein heme oxygenase-1 (HO-1). For this reason, the fourth and final specific aim examines the role of HO-1 as an effector during I/R in AICAR-PC mice. It is likely that the protective effect of HO-1 is mediated by its degradation of heme to produce vasoactive CO, and the antioxidants biliverdin and secondarily derived bilirubin (39, 99). The enzymatic action of HO-1 yields protective breakdown products of heme degradation that are able to vasodilate vessels and prevent the burst of ROS following reperfusion of the previously ischemic tissue with normoxic blood. The studies proposed here have not been previously reported and are the first to tackle this complex mechanism.

Due to the lack of clinical applicability of organ and tissue protection induced by occlusion of conduit arteries, EPC and AICAR-PC are promising alternatives to IPC as they are notably less invasive and dangerous. If the mechanisms of EPC and AICAR-PC can be elucidated the potential for clinical PC without occlusion of an artery or arteries would be greatly increased. Eliciting a protective phenotype without inducing ischemia provides a significant benefit over IPC. This idea is strengthened by studies showing that, unlike classic IPC, EPC and late phase pharmacologic preconditioning can have greater magnitudes of protection than the early phase (68). Additional studies have shown that some pharmacologic preconditioning stimuli, such EPC, remain effective in models with

risk factors such as age and diabetes, where IPC has been shown to be less effective (103, 106, 123). If this mechanism can be deduced, possible pharmacologic tools could be utilized to induce protection therapeutically without the use of alcohol. This would provide a significant advantage as there are several drawbacks to ethanol consumption such as its addictive and negative health properties as well as social and religious customs that prohibit drinking alcohol. To this end several experiments have been devised to determine if EPC activates AMPK, whether AMPK activation with AICAR can substitute for ethanol and induce preconditioning, and the roles of eNOS, K_{ATP}, and HO-1 in AICAR-PC as proposed in Figure 1.

In summary, the ability to utilize the innate protective mechanisms that are activated by EPC and AICAR-PC would have great clinical relevance and the potential to greatly decrease the substantial mortality and morbidity involved with I/R injury. Cardiovascular disease, stroke, and the complications that follow these events result in extensive and costly damage to a large portion of our population. Inducing a protective phenotype in these patients would not only increase their chances of surviving an ischemic event (e.g. stroke, heart attack, or gastrointestinal infarct) initially but also greatly reduce the occurrence of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction (MODS) which often lead to death following organ ischemia. Preconditioning could also greatly decrease inflammation and complications resulting from transplant surgery, angioplasty, and bypass surgery. The enormous physical and economic burdens of these pathologies demand

aggressive research in order to explore means to reduce the otherwise irreversible damage by both prevention and treatment. The proposed studies would contribute significantly to the basic understanding of a poorly understood but vastly promising mechanism that may ultimately lead to treatments for a leading cause of mortality.

II. Mechanisms of Ischemia/Reperfusion (I/R) Injury:

Ischemia/reperfusion (I/R) injury refers to the subsequent pathology from a reduction or stoppage of blood flow followed by the re-establishment of oxygenated arterial blood. This is commonly seen clinically following a heart attack, stroke, transplant surgery, or gastrointestinal and limb ischemia. I/R injury includes several deleterious results in the microcirculation: reduced bioavailability of NO, impaired endothelium-dependent dilation in arterioles and endothelial cell growth, enhanced fluid filtration and leukocyte plugging of capillaries, leukocyte trafficking and protein extravasation in postcapillary venules, causing apoptosis, increased endothelial cell migration, and activation of adhesion molecules and inflammatory reactions (16, 156). An interesting observation is the increased injury following I/R versus ischemia alone, where there is increased injury following 1 hr of ischemia and 3 hrs of reperfusion compared to 4 hrs of ischemia alone (100, 139). As the previously ischemic tissue is reperfused, there is a large oxidative burst as reactive oxygen species (ROS) are rapidly produced by leukocytes, endothelial cells, and vascular

smooth muscle cells. These highly reactive molecules set off many inflammatory processes which will be discussed in further detail below. Leukocytes are able to produce ROS via nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), which is also present in vessel walls, and myeloperoxidase (MPO) which produces hypochlorous acid (HOCl). Other sources of ROS include xanthine oxidase in the vessel wall. During I/R, the host antioxidant defense mechanisms are overwhelmed by the oxidative burst which occurs upon reperfusion of the previously ischemic tissue. These defenses include the superoxide dismutases which degrade the highly reactive superoxide to the less cytotoxic hydrogen peroxide, catalase which catalyzes the breakdown of hydrogen peroxide, and glutathione peroxidase and other peroxidases which also degrade hydrogen peroxide. An additional consequence of increased ROS is that the highly reactive NO will form the reactive nitrogen specie peroxynitrite (ONOO-) when ROS levels increase as seen with I/R. Thus, one of the main sources for the ROS at reperfusion are leukocytes that produce many different reactive oxygen metabolites and increase oxidative stress via several enzymatic reactions.

a. Role of Leukocytes:

Leukocytes have been shown to play an essential role in the pathophysiology of I/R, though it is unknown whether the leukocytes are also essential to recovery or wound repair. The inflammatory response that is normally activated to locate and eradicate foreign material at sites of infection is

altered in response to I/R and these same leukocytes identify and attack the area of injury in an autoimmune manner. For leukocytes to become activated and damaging they require the oxidative burst at reperfusion and in effect attack and induce apoptosis and necrosis in tissue that was still viable at the end of ischemia prior to reperfusion. The concept that the leukocytes roll, adhere, and ultimately transmigrate the endothelium and basement membrane in an attempt to begin wound repair has credence and is further supported by the requirement for leukocytes in the induction of the protective effects of IPC with brief periods of ischemia and reperfusion. The normally beneficial leukocyte actions may simply overwhelm the host defenses against such a large burst of ROS at reperfusion following prolonged ischemia where they have a detrimental effect. Another cytotoxic protein that functions in the immune response is the heme enzyme, myloperoxidase, and its activity can be used to quantify leukocyte infiltration by measuring its activity in inflamed tissue. This enzyme catalyzes the formation of strong oxidizing acids such as HOCl.

i. Mechanism of Injury:

The mechanism of leukocyte-derived cellular damage and death is likely tightly coupled with their ability to emigrate into the parenchyma where they can release ROS, proteases, and other cytotoxic products that results in tissue damage and impaired barrier function as the cells become apposed to and adherent to parenchymal cells. In addition, leukocyte-mediated exposure of matricryptic sites and Arg-Gly-Asp sequences (RGDs) and activation of matrix

metalloproteinases (MMPs) can further degrade the integrity of the surrounding cells and tissue.

1. Direct:

Once leukocytes have been extravasated into the parenchyma they are able to release their cytotoxic contents such as: ROS, proteases, and HOCl (29). Due to the large shift in the ratio of ROS:NO, the vasculature loses its ability to vasodilate. NO production by eNOS is not only decreased but the available NO rapidly reacts with ROS to produce peroxynitrite. It has been shown that impaired endothelium-dependent vasodilatation is not a problem with vascular smooth muscle, as administration of NO donors can induce vasodilation, and NOS inhibitors or NO scavengers can simulate this effect. Additionally NO has anti-adhesive properties, thus the reduction in NO bioavailability increases LEI directly. Increased arginase expression that is induced by tumor necrosis factor α (TNFα) is another means for I/R injury to reduce the bioavailability of NO, as arginase competes with NOS for arginine (8).

2. Indirect (capillary no-reflow):

Activated leukocytes are known to plug capillaries as they become increasingly rigid and adhesive, and frequently stick to platelets and other leukocytes forming thrombi. The main cause of capillary no-reflow has been shown to be neutrophil plugging via CD18-dependent adhesion (64). In addition, leukocyte-dependent microvascular barrier disruption leads to edema formation,

which in turn elevates interstitial fluid pressure, which physically compresses capillaries, leading to no-reflow. This pathology has been shown to be dependent on P-selectin-dependent rolling and ICAM-1-adhesion (63).

ii. Margination and Capture:

As leukocytes exit capillaries and venules, the smaller and more flexible erythrocytes exert hydrodynamic forces that cause leukocytes to marginate, which is to say that they are forced to the periphery of blood flow away from the center and most rapid flow and into close proximity with the endothelium. In inflamed states, the endothelium is activated and expresses adhesive structures that allow for capture of the circulating leukocytes. Marginating leukocytes are exposed to activating stimuli released from inflamed tissues including activated endothelial cells. As this process continues the likelihood of capture is increased and soon the first molecular bond between a leukocyte and the endothelium is formed. These initial events in the inflammatory response lead to increased leukocyte-endothelial cell interactions that are more complex and significant.

iii. Leukocyte-Endothelial Cell Interactions (LEI):

There are two main types of LEI, a weaker adhesive interaction characterized by leukocyte rolling and stronger adhesive interactions that allow the rolling leukocytes to become firmly adherent or stationary. These interactions are mediated by the expression of different adhesion molecules on the surface of both leukocytes and endothelial cells. The initial interactions that lead to rolling

are primarily mediated by mobilization and surface expression of preformed pools of adhesion molecules and clustering of already expressed adhesive structures. Upregulated expression of new gene products regulates adhesive events that occur after 3 or more hrs of prolonged exposure to proinflammatory stimuli.

1. Rolling:

Leukocyte rolling is an early event in the inflammatory cascade and precedes the more detrimental LEI as leukocytes become increasingly activated, activating, and adhesive. The interactions involved in this LEI are primarily mediated by preformed endothelial platelet selectin (P-selectin) which is rapidly mobilized from Weibel-Palade bodies to the cell surface and leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) initially, and endothelial and leukocyte selectin (E- and L-selectin, respectively) after several hrs.

2. Adhesion:

Leukocyte rolling allows these cells to monitor their local environment for the presence of activating factors, which in turn induces clustering of CD11/CD18. CD11/CD18 interacts with intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, allowing rolling leukocytes to transition to stationary adhesion. Stationary leukocytes are able to penetrate the endothelium and extravasate into parenchymal tissue, events that appear to be mediated by ICAM-1 and platelet endothelial cell adhesion molecule-1 (PECAM-1).

3. Effects of Shear Rate:

Shear rate has many important effects on LEI. The greater the shear rate, the more difficult it is for the leukocytes to interact with the endothelium and form adhesive bonds as the adhesion molecules on the leukocytes and endothelium must resist increased force and form stronger or more numerous bonds to support rolling or adhesion. The consequences of ischemia and reperfusion which are reduced flow and capillary plugging reduce shear rate and increase LEI.

4. Extravasation:

Extravasation refers to the leakage or migration of leukocytes out of the blood vessels, particularly capillaries and venules. This transmigration allows the leukocytes and other cell types and molecules that follow access to the extracellular space and parenchyma. Although somewhat controversial, many believe that microvessel barrier function is compromised by this process and promotes edema formation and other inflammatory responses.

iv. Methods to Measure:

There are many ways to measure inflammation clinically and experimentally. For the purpose of this review only the experimentally used methods will be discussed. These vary from cytokine and adhesion molecule

quantification, leukocyte infiltration, histological examination, and methods that observe the tissue and vasculature directly in-vivo for real time analysis.

1. Leukocyte infiltration:

One of the most damaging consequences of I/R is leukocyte infiltration. Once leukocytes extravasate into the parenchyma they degrade releasing their cytotoxic contents leading to apoptosis and necrosis. Leukocyte infiltration can be measured by myeloperoxidase (MPO) enzyme activity as discussed below, in addition to histological examination by staining for leukocyte-specific cellular markers such as adhesion molecules or their ligands.

2. Histology:

Histological examination is commonly used to measure morphologic changes in cells and tissues following I/R. Perhaps the most common use of this technique is staining of the myocardium to measure the area of infarct relative to the area at risk using tetrazolium staining. This method allows comparison of infarct size or tissue damage in response to I/R and different preconditioning stimuli.

3. Cell Specific Markers:

One of the leukocyte-specific proteins that can be used to quantify leukocyte infiltration is MPO. Assays which measure MPO activity can be used

to determine the amount of MPO present and thus extrapolate the number of leukocytes that are present in the tissue as a function of the enzyme activity.

4. Intravital Microscopy and other imaging modalities:

One of the major means of quantifying LEI and other in vivo markers of I/R injury is intravital microscopy. This method allows observation and measurement of various cell types such as red blood cells, white blood cells, platelets, etc. This method is a useful tool as it is conducted in intact animals under different experimental conditions, and can be used to investigate various tissues. This type of microscopy has been utilized to examine effects of I/R on the vasculature of skeletal and smooth muscles, the intestinal wall, and in the brain. Unfortunately, this technique has not been successfully applied to the lungs or heart, as their anatomical positioning and structure do not lend themselves to this method.

III. Ischemic Preconditioning (IPC):

Since the discovery of ischemic preconditioning (IPC) which was first described in the landmark paper by Murry et al. (68), a vast body of research has emerged attempting to elucidate the mechanisms and mediators of this innate protective mechanism against ischemia/reperfusion (I/R) injury. Murry has shown that brief periods of ischemia induced via arterial occlusion followed by reperfusion prior to prolonged ischemia, paradoxically protects the heart against

the deleterious effects seen in control animals without IPC. Subsequent work has shown that minimum thresholds of time of ischemia and reperfusion must be reached to induce preconditioning (34), thus if either is too short in duration, no protection will result. Another significant discovery was that of the biphasic time course of IPC with two windows of protection: an early or acute phase, and a late or delayed phase. The initial window occurs immediately following IPC and dissipates within several hrs (early phase), and a second and more prolonged window of protection appears twenty-four hrs after IPC and lasts up to seventy-two hrs (late phase) (2, 5). The early phase of preconditioning is relevant to studying ways of attenuating I/R injury following predictable events such as cardiopulmonary bypass or transplant surgeries. The late phase of preconditioning is more clinically relevant to developing and maintaining a chronic protective phenotype prior to unpredictable events such as myocardial infarct or stroke. Late phase preconditioning also increases the amount of time that can pass before therapeutic reperfusion occurs prior to irreversible damage (32). As such, late phase preconditioning is viewed to be the most promising clinical therapy to ablate I/R injury, especially as it can also prevent myocardial stunning (4). For these reasons, the mechanism of late phase preconditioning will be studied in the proposed experiments. There is, however, a major drawback of late local IPC: it requires the occlusion of an artery that feeds the heart (or other target organ or tissue) which is to be protected, thus it requires an invasive surgical intervention to initiate. Thus the search for another means of inducing the protection seen with late preconditioning without the need of

inducing occlusion or ischemia of a major organ would be much more advantageous and clinically relevant.

a. Initiators of IPC:

At present the main triggers, or initiating events of the preconditioning cascade, of late phase IPC are believed to be: adenosine, bradykinin (BK), K_{ATP} channels, NO, and reactive oxygen species (ROS) (3, 22). Adenosine, BK, NO, and ROS are produced during the bouts of preconditioning ischemia and reperfusion. The early phase of local IPC preconditioning utilizes preformed pools of mediators and effectors, whereas the late phase involves altered gene expression and protein synthesis (16, 110). In particular, studies have reported increased expression of prosurvival proteins such as: inducible NO synthase, cyclooxygenase-2, heme-oxygenase-1, aldose reductase, and Mn superoxide dismutase following late phase local IPC (107, 127).

i. Adenosine:

As with many of the triggers that have been shown to be essential to IPC via the abrogation of protection with pharmacologic inhibition and induction with activators or exogenous compounds, administration of exogenous adenosine can induce a comparable degree of protection to that seen with IPC (2). Likewise, blockade of adenosine receptors can block the protective effects of IPC .

ii. Nitric Oxide:

NO has been shown to be an essential mediator of many forms of preconditioning. There is evidence for a role of NO as a trigger, mediator, and effector of various preconditioning paradigms, with specific isoforms being involved in the different functions.

iii. Surface ATP-sensitive Potassium Channels:

The surface or plasmalemmal ATP-sensitive potassium (sK_{ATP}) channel has been shown to be a trigger of IPC, but is not involved in mediating the protection on day two (62, 101).

b. Downstream Signaling Elements:

The initiating events of IPC have many downstream targets. These elements have been less strenuously examined than the triggering mechanism, though they may provide the most therapeutic potential, as they are activated by the triggers and may be more specific with fewer inimical consequences on other signaling pathways. Several potential targets exist which may provide more direct and specific induction of protection without activating some of the unnecessary targets activated by the common upstream elements involved in additional signaling pathways.

i. Nitric Oxide Synthase:

In addition to the role of NO in IPC as an initiator, there is also evidence that NO functions as a mediator of the protective mechanism, acting as an intermediary between triggering events and the end effectors. These various roles may be mediated by different isoforms of NOS such as inducible NOS (iNOS) or neuronal NOS (nNOS).

c. Effectors:

The most significant shortcoming in the preconditioning literature is the lack of information concerning the end effectors and their mechanisms to induce protection. The effectors are the proteins and molecules involved in conferring protection during the I/R injury on Day 2, unlike the upstream mediators that have their actions on Day 1 24 hrs prior to the injurious event.

i. Mitochondrial ATP-sensitive Potassium Channels:

Mitochondrial K_{ATP} channels have been shown to be involved in mediating the protective effect on day two during the ischemic insult, but not in triggering this response on day one (9).

ii. Heme-oxygenase-1:

Heme oxygenase (HO) is a ubiquitously expressed protein that catalyzes the oxidative degradation of protoheme IX into equimolar quantities of biliverdin, divalent iron, and carbon monoxide (CO) (71). Biliverdin is further metabolized to

bilirubin, a powerful endogenous antioxidant, by the action of biliverdin reductase (134). Three isoforms of the HO enzyme, HO-1, HO-2, and HO-3, have been described (87). HO-3 appears to exhibit lower activity and is less well characterized than HO-1 and HO-2. HO-2 is a constitutively expressed and non-inducible gene product. On the other hand, HO-1 is an inducible enzyme which is also called heat shock protein 32 (HSP32). Heme-oxygenase is a likely candidate for being an end effector in preconditioning due to the direct protective effects of its products.

IV. Ethanol Preconditioning (EPC):

Preconditioning with ethanol (EPC) provides several advantages over IPC, and the elucidation of its mechanisms may yield targets for intervention with greater therapeutic potential. These benefits include: a means of inducing a protective mechanism like IPC without the need for the prohibitively hazardous tissue or organ ischemia, IPC loses its protective effects in the presence of risk factors such as aging or diabetes, while EPC remains effective as a preconditioning stimulus under these conditions, and unlike IPC, EPC and late phase pharmacologic preconditioning can have a greater magnitude of protection than the early phase (68, 103, 106, 123, 124). These results suggest that EPC is a superior means of inducing protection against I/R than IPC.

a. Initiators of EPC:

Several pharmacologic agents are now known to induce a protective phenotype similar to that seen with IPC, such as preconditioning with: ethanol, adenosine receptor agonists, bradykinin, nitric oxide (NO) donors, and exogenous calcitonin gene-related peptide (CGRP) (14, 22, 81, 131, 140). Indeed, our own work has established that these agents induced the development of a protective anti-inflammatory phenotype in postcapillary venules, such that these vessels fail to support adhesion molecule expression, leukocyte rolling and adhesion, and increased vascular permeability when the small bowel is subsequently exposed to prolonged I/R (32, 33, 68, 69, 121, 122, 152).

i. Adenosine Monophosphate:

A rapid consequence of ethanol consumption or treatment is the increase in AMP levels (82). AMP has been shown to activate AMPK and increase its affinity for upstream kinases to further activate the enzyme, which in turn phosphorylates and activates eNOS, it is likely that this is one of the mechanisms utilized by EPC to induce a protective phenotype. Similarly, EPC has shown to be mediated by adenosine itself (19, 98, 152).

ii. Nitric Oxide:

NO has been shown to be an essential mediator in EPC by several groups (12, 70, 79). Work in the Korthuis laboratory has also shown that this is an essential molecule (68).

iii. Surface ATP-sensitive Potassium Channels:

A role for surface or plasmalemmal ATP-sensitive potassium (sK_{ATP}) channels has been shown to be one of the early events in EPC as well as triggering other forms of preconditioning (9).

b. Downstream Signaling Elements:

Several of the downstream targets of the initiators of EPC are shared with IPC. Though preconditioning pathways have many redundancies, it is likely that there are divergences between the various forms that exist. Only the mediators proposed to be involved in EPC will be discussed here.

i. Adenosine Monophosphate-activated Protein Kinase:

As discussed previously, EPC increases AMP levels which in turn activate AMPK. AMPK also phosphorylates and activates eNOS, actions that have been implicated as essential triggering elements in the development of ischemic preconditioning (24, 49). Furthermore, antecedent ethanol ingestion, which induces late phase preconditioning and prevents postischemic leukocyte rolling and adhesion by an eNOS-dependent mechanism, also activates AMPK (65). These observations led us to postulate that ethanol preconditioning may be triggered by an AMPK-dependent mechanism.

ii. Nitric Oxide Synthase:

NO is thought to play a role in mediating the protective effects of EPC in addition to its action as an initiator. Specific NOS isoforms may play different roles as initiators, mediators, and effectors in preconditioning. Evidence suggests that eNOS is the primary isoform involved in triggering EPC, though it is unknown if other isoforms may be involved in mediating this protective signal.

c. Effectors:

As with mediators of EPC, some of the target effectors involved on Day 2 during I/R are shared with IPC. Only the most relevant will be discussed below, though this area remains the most undefined.

i. Mitochondrial ATP-sensitive Potassium Channels:

mK_{ATP} channels have been shown to be essential effectors on Day 2 of a vast array of preconditioning stimuli (9). They are one of the most commonly studied end effectors, though their role in AICAR-PC and EPC has not been well studied. It has been suggested that their activation is able to maintain mitochondrial membrane integrity and prevent the opening of the mitochondrial permeability pore. It is thought that this prevents the release of cytochrome c and other pro-apoptotic signaling molecules.

ii. Heme-oxygenase-1:

HO has several lines of evidence supporting its role in EPC. First, heme oxygenase activity is exquisitely sensitive to upregulation by NO donors (80) and NO appears to play an important role in initiating the effects of EPC (154) and NO induces the expression HO-1 mRNA (84).

V. Adenosine Monophosphate-activated Protein Kinase (AMPK):

The heterotrimeric serine/threonine kinase, AMPK, is composed of a catalytic alpha subunit and two regulatory subunits, beta and gamma. This protein is ideally located in a myriad of signaling pathways to regulate metabolism at the cellular, tissue, organ, and organism levels. AMPK is activated during times of metabolic stress such as exercise and ischemia/hypoxia. AMPK has been shown to be phosphorylated on threonine 172 on its catalytic alpha subunit and thus activated by at least two AMPK kinases (AMPKK). The two known AMPKKs are LKB1, a tumor suppressor, and calcium/calmodulin-dependent protein kinase kinase (CaMKK), though others likely exist. AMPK has been termed a metabolic master switch, which refers to its activities that result in a transition from ATP consuming anabolic pathways during times of high cellular energy status, to ATP producing catabolic pathways that maintain falling energy levels. As its name implies, AMPK is activated by AMP, which makes it extremely sensitive to fluctuations in energy status. AMPK not only effects acute alterations in metabolism but it also affects gene transcription and protein synthesis which have much more prolonged effects.

a. Role in Cardioprotection:

The role for AMPK in cardioprotection has been well documented. AMPK activation, as determined by phosphorylation at Thr172, has been shown to increase in rats fed a caloric restricted diet. These mice also had improved left ventricular recovery following I/R in young and aged animals, suggesting that perhaps AMPK activation is cardioprotective (124). IPC has been shown to activate myocardial AMPK, and hearts from transgenic mice lacking the catalytic alpha 2 subunit of AMPK could not be preconditioned (128). In another study, preconditioning the myocardium with short bouts of ischemia has been shown to activate AMPK, an effect that led to activation of surface (sarcolemmal or plasmalemmal) K_{ATP} (sK_{ATP}) channels and was blocked with the sK_{ATP} channel blocker HMR-1098 (128).

b. Role in Non-cardiac Tissues:

The brain is another organ that has been considered regarding I/R and AMPK, as stroke is a common clinical occurrence and AMPK activity is centrally tied to the hypothalamus were satiety and metabolism are regulated. Other tissues or organs that have been studied are the lung, liver, skeletal muscle, and the gastrointestinal tract. These organs are all susceptible to I/R injury and subsequent dysfunction.

c. Upstream Signaling Elements:

As a central mediator of energy homeostasis, AMPK has many upstream proteins and molecules that regulate its function. It is known to be activated by at least two upstream kinases, LKB1 and CaMKK, as well as increases in the AMP:ATP ratio. AMPK is dephosphorylated and thus inactivated by protein phosphatase 2C and 2A. It is also allosterically inhibited by ATP and phosphocreatine. There is also evidence that it is under hormonal control by orexigenic and anorexigenic peptides such as ghrelin and leptin, respectively.

i. Adenosine Monophosphate:

AMPK can be activated by many stressful stimuli such as exercise, ischemia, and other events that shift the cellular energy balance. One of the main consequences of these incidents is ATP-depletion, which is exquisitely monitored by AMPK. AMPK is activated in response to a shift in the ATP:AMP ratio, which is orders of magnitude more sensitive to reductions of ATP levels than the ATP:ADP ratio. By this highly regulated mechanism, AMPK is centrally involved in energy homeostasis, and is activated by AMP. This occurs via binding of AMP to the gamma regulatory subunit, which activates AMPK, as well by increasing the affinity of AMPK for the activating phosphorylation on the activation loop at Thr172 by its upstream kinase LKB1 (114). AMP also inhibits the activity of protein phosphatase-2Cα, the phosphatase that dephosphorylates and inactivates AMPK (30).

ii. LKB1:

The tumor-suppressor LKB1 was the first known kinase to phosphorylate AMPK. This AMPK kinase (AMPKK) phosphorylates AMPK in response to cellular stress and increases in AMP (114).

iii. Calcium/calmodulin-dependent protein kinase kinases:

The second AMPKK discovered was calcium/calmodulin-dependent protein kinase kinase (CaMKK). This enzyme imparts calcium-sensitivity to AMPK by phosphorylating AMPK when intracellular Ca^{++} increases.

iv. Other Modulators (Anti-diabetes Drugs and Protein Phosphatases)

In addition to PP2C, palmitate has recently been shown to inhibit AMPK activity by its activation of the protein phosphatase 2A (145). The glucose lowering agent metformin activates AMPK and prevents the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α (53). This study also demonstrated that adhesion molecule expression was reduced by metformin or AICAR administration, a protective effect that was abrogated by the addition of a siRNA directed towards AMPK α1. These activities have previously been attributed to eNOS by others (10, 148). There is also evidence that AMPK can be activated by statins such as atorvastatin, which may explain the pleiotropic effects of these drugs (130). This last report is not surprising as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) was one of the first enzymes shown to be phosphorylated and inhibited by AMPK

activity (18). These results are intriguing in light of the recent observation by Fisslthaler et al. (37) that fluid shear stress activates AMPK and NO production which modulate HMGR expression and activity.

d. Downstream Signaling Elements:

AMPK was first discovered based on its sensitivity to AMP, and its ability to inhibit acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Since then, it has been found to be centrally involved in many complex signaling pathways, particularly those involving metabolic regulation.

i. Nitric Oxide Synthase:

AMPK activation, like EPC, has been shown to increase endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 and its activity (24, 55, 91, 150, 151). The phosphorylation and activation of eNOS has been shown to have a salubrious effect, whereas a decrease in eNOS activity leads to the pathology of I/R by increasing leukocyte infiltration and decreasing vasodilatation (70, 79, 95). IPC has been shown to involve an eNOS-dependent mechanism in numerous studies such as seen in the recent study by Xuan et al. (150) who showed that late phase IPC was ineffective at reducing infarct size in eNOS deficient mice. Similarly, it has been demonstrated that AICAR-PC is dependent on NOS, and is loses its protective reduction of postischemic leukocyte rolling in eNOS deficient mice (42).

ii. Surface ATP-sensitive Potassium Channels:

In a recent study by Sukhodub et al. (128), it was shown that IPC occurred by an AMPK-dependent mechanism, which involved recruitment and activation of sK_{ATP} channels. This study also showed that hearts from mice that had a dominant negative mutation in the gene for the alpha 2-subunit of AMPK could not be protected against I/R by IPC.

e. Effectors:

Though AMPK is involved in a myriad of signaling pathways with diverse effects, little is known about the end effectors that carry out its protective effects in preconditioning.

i. Mitochondrial ATP-sensitive Potassium Channels:

A common end effector in the preconditioning literature is the mK_{ATP} channel. It has been believed that one of the primary effects of preconditioning is to prevent apoptosis by maintaining mitochondrial membrane integrity with these channels through the prevention of the mitochondrial permeability transition pore opening (76). This literature has been under attack recently due to the nonspecific effects of the pharmacologic agents used to modulate its activity and varied reports concerning mitochondrial membrane potential recordings during preconditioning and I/R. The fact that mK_{ATP} channels have been implicated as an important mediator for most forms of preconditioning while treatment with K_{ATP} channel agonists prevent leukocyte infiltration when administered during

reperfusion after prolonged ischemia (13, 89, 90, 137, 142) led us to first evaluate their potential role as effectors of AICAR-PC.

ii. Heme-oxygenase-1:

The induction of the heat shock protein HO-1 is a likely effector in many forms of preconditioning. This enzyme not only produces a vasodilator (CO) that can compensate for the decreased bioavailability of NO, but it also produces two antioxidants: biliverdin and bilirubin. Additionally heme oxygenase's activity is exquisitely sensitive to upregulation by NO donors (80) and NO appears to play an important role in initiating the effects of AICAR-PC (42). Additionally, NO induces expression of HO-1 mRNA by a mechanism that involves transcription factor NFκB (84), which is also inhibited by AMPK (53). cAMP, a downstream signaling molecule that is produced in response to adenosine A_2-receptor activation, increases HO-1 mRNA, protein, and activity (52), and produces a preconditioned phenotype in the small intestine (74). Adenosine A_2 receptor activation is another important trigger for AICAR-PC in the heart (27). Furthermore, induction of HO-1 suppresses P-selectin expression and leukocyte adhesion induced by hydrogen peroxide or ischemia/reperfusion in the small intestine (54, 138), inflammatory processes which are also prevented by AICAR-PC. It has also been shown that HO-1-derived CO inhibits the expression of proinflammatory cytokines, as does AMPK activation (44, 126). This, in addition to the observation that hemin-induced HO-1 expression exerts infarct-sparing effects in the setting of myocardial I/R (50) while the protective effects of IPC

against I/R could be inhibited by pharmacologic inhibition of HO-1 with zinc protoporphyrin (ZnPP) or siRNA (61). Finally, and perhaps most importantly, the reaction products of HO-1-catalyzed heme degradation exert powerful anti-adhesive and antioxidant effects (87, 126, 134). Moreover, HO-1 activity appears to be particularly rich in postcapillary venules of the small intestine (54).

VI. Summary of the Literature:

According to the most recent report from the Centers for Disease Control, the leading cause of death for men and women of all races is heart disease (146, 147). Stroke was the fourth and third leading cause of morbidity and death, respectively. A significant portion of the morbidity and mortality resulting from these events is attributed to the pathology resulting from both ischemia and reperfusion (I/R). The inflammatory response to I/R results in edema, arteriolar vasoregulatory dysfunction, capillary no-reflow, contractile dysfunction in the myocardium, absorptive and mucosal barrier dysfunction in the gut, apoptosis, and necrosis (45, 47, 75, 157). Depending on the severity of the I/R injury, this inflammatory response can lead to organ dysfunction, failure, and ultimately death. Clinically the severity is determined by the degree and duration of ischemia prior to reperfusion, and usually results from longer periods of ischemia. In the initial stages of this response which occur at the onset of reperfusion, leukocytes become activated by proinflammatory mediators released by the reperfused vasculature and tissue. The leukocytes begin to roll along the

endothelium of vessels, leading to further activation of leukocytes, platelets, and the endothelium itself. As the inflammatory cascade continues, cytokines, chemoattractants, and adhesion molecules are released, expressed, or mobilized, resulting in increasing adhesive interactions between the leukocytes and endothelium leading to an escalation in leukocyte rolling (LR), firm adhesion (LA), and emigration along and through the endothelium. Once leukocytes have extravasated into the interstitium they release cytotoxic oxidants and hydrolytic enzymes, causing tissue damage and cell death. These are essential events in I/R injury, as studies have shown that treatments that reduce neutrophils, inhibit neutrophil activation, or inhibiting leukocyte/endothelial cell adhesive interactions with adhesion molecule binding antibodies significantly reduce injury in a variety of models (35, 36, 83, 97, 113). The adhesive interactions between leukocytes and endothelial cells are initiating events and mediators of this, making their quantification an early and sensitive indicator of injury.

Prior to 1986 little was known about ways to prevent or reduce I/R injury. In that year the concept of ischemic preconditioning (IPC) was introduced, which set the benchmark for preventing the inimical consequences of I/R. Since the discovery of IPC which was first described in the landmark paper by Murry et al. (94) a vast body of research has emerged that attempted to elucidate the mechanisms and mediators of this innate protective mechanism against I/R. Murry et al. showed that brief periods of ischemia induced via arterial occlusion followed by reperfusion prior to prolonged ischemia paradoxically protects the heart against the deleterious effects seen in control animals without IPC.

Subsequent work has shown that minimum thresholds of time of ischemia and reperfusion must be reached to induce preconditioning (149), thus if either is too short in duration, no protection will result. Another significant discovery was the biphasic time course of IPC with two windows of protection an early or acute phase, and a late or delayed phase. The initial window occurs immediately following IPC and dissipates within several hrs (early phase), and a second and more prolonged window of protection appears twenty-four hrs after IPC and lasts up to seventy-two hrs (late phase) (4, 12). The early phase of preconditioning is relevant to studying ways to attenuate I/R injury following predictable events such as cardiopulmonary bypass or transplant surgeries. The late phase of preconditioning is more clinically relevant to developing and maintaining a chronic protective phenotype prior to unpredictable events such as myocardial infarction or stroke and may be especially important for patient populations at risk for ischemic events, such as the obese, diabetic, smoker, and hypertensive and hypercholesterolemic individual. Late phase preconditioning also increases the amount of time that can pass before therapeutic reperfusion occurs prior to irreversible damage, which is an important determinant of survival (133). As such, late phase preconditioning is viewed to be the most promising clinical therapy to ablate I/R injury, especially as it can also prevent myocardial stunning (11). For these reasons, the mechanism of late phase preconditioning will be studied in the proposed experiments. There is, however, a major drawback of late IPC: it requires the occlusion of the arterial supply that feeds the target organ or tissue which is to be protected. Thus, it requires an invasive surgical

intervention to initiate in most tissues and itself may pose a risk for life threatening arrhythmias and other manifestations of tissue injury. These concerns have led to the search for other means of inducing the protection seen with late IPC without the need of inducing occlusion or ischemia of a major organ as such approaches may be much more advantageous, clinically relevant, and practical.

A promising alternative that has recently emerged is the phenomenon known as ethanol preconditioning (EPC). Epidemiological data have supported a role for alcohol consumption in cardioprotection, a phenomenon known as the French Paradox (6, 26, 41, 108). Consumption of wine in France has been suggested to underlie the paradoxically low ischemic heart disease in a population that has a high dietary fat intake (26, 105). Aside from ethanol, wine and particularly red wine, contains many other reported protective compounds including, resveratrol, quercetin, and other polyphenolic antioxidants (25, 116), leading to the belief that there could be molecules other than ethanol that account for the protective effects seen with wine consumption. Later studies show that both ethanol and the antioxidant components of wine exert protection against I/R, but that the protection is induced by different signaling pathways (28, 115, 117). The first experimental evidence that ethanol was protective against I/R injury was gathered by Kobayashi et al. (73). In these experiments, the addition of physiologically relevant ethanol concentrations to the perfusion media during the I/R protocol in isolated rat hearts reduced cell injury compared to controls. In a later study by Chen et al. (22) brief exposure to low ethanol levels

immediately prior to I/R was shown to protect both isolated cardiac myocytes and Langendorff perfused hearts from adult rats in a PKCε-dependent manner. Miyamae et al. (88), was the first to demonstrate prolonged EPC in an animal model by chronic ethanol administration to guinea pigs for three to twelve weeks, which mimicked the protective effects of IPC. This study also demonstrated that this protective effect was dependent on adenosine A_1 receptors.

Like IPC, EPC has been shown to have a temporally similar biphasic response in humans and other animals, as well as have many major signaling pathways in common with IPC, such as: reactive oxygen species (ROS), ATP-sensitive potassium channels (K_{ATP} channels), nitric oxide (NO), and calcitonin-gene related peptide (CGRP) (7, 9, 13, 57, 69, 101, 154). At present, the main triggers of late phase IPC are believed to be: adenosine, bradykinin (BK), K_{ATP} channels, NO, and ROS (5, 101), many of which are also shared with EPC (33, 152-154). EPC has been shown to reduce postischemic leukocyte rolling and adhesion (152), though the role for AMPK in this protective effect has not been examined.

The reason for the hypothesis that AMPK activation is involved in EPC stems from work showing that ethanol exposure leads to increased AMP:ATP ratio and an increase in AMPK phosphorylation and activity as measured by the phosphorylation state of AMPK and its downstream targets such as acetyl coenzyme A carboxylase (ACC) (65). AMPK is ubiquitously expressed and a highly conserved heterotrimeric serine/threonine kinase, with two isoforms of the catalytic alpha subunit, and two and three isoforms of the regulatory beta and

gamma subunits, respectively (85, 135). AMPK is a central regulator of metabolism that is activated in times of stress such as exercise, hypoxia, and ischemia, when ATP concentrations decrease resulting in increased AMP:ATP ratios, and acts to inhibit ATP consuming processes while activating ATP producing pathways (85). AMPK activation as determined by phosphorylation at Thr172, has been shown to increase in rats fed a caloric restricted diet. An effect that coincided with improved left ventricular recovery following I/R in young and aged animals (124). Likewise, ischemic preconditioning has been shown to activate myocardial AMPK, and hearts from transgenic mice lacking the catalytic alpha 2 subunit of AMPK could not be preconditioned (128). Also, like EPC, AMPK activation has been shown to increase endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 and its activity (24, 55, 91).

eNOS has been shown to be an essential mediator of preconditioning and I/R injury. Furthermore, phosphorylation and activation of eNOS has been shown to have a salubrious effect, whereas a decrease in eNOS activity leads to the pathology of I/R by increasing LEI and decreasing vasodilatation (70, 79, 95).

Preconditioning the myocardium with short bouts of ischemia has been shown to activate AMPK, an effect that led to activation of surface (sarcolemmal or plasmalemmal) K_{ATP} channels and was blocked with the surface K_{ATP} channel blocker HMR-1098 (128). In contrast, studies looking at the effects of AICAR activation of AMPK on K_{ATP} channels in mouse pancreatic cells showed that AMPK activation lead to closing of the channels (141). There is clearly a tissue-specific effect of AICAR-PC which may be explained by differential catalytic

alpha subunit isoform expression. Little is known about the downstream mediators of AMPK activation on Day 2, and the effectors involved in the induction of the protective phenotype. One such possible effector is HO-1.

HO-1 is an inducible enzyme that produces CO and biliverdin which is quickly reduced to bilirubin. HO-1 has been shown to be involved in several preconditioning paradigms including IPC, EPC, and NO mediated pathways (34, 61, 78). Because exogenous CO prevents LR and LA, and bilirubin and biliverdin are powerful antioxidants we hypothesized that AICAR increases HO-1 activity and expression during I/R and thus serve as an effector of the anti-inflammatory protection induced by antecedent AMPK activation.

The ability to reduce I/R injury via the innate mechanism of IPC offers tantalizing potential to protect patients from the leading causes of death in the modern world. Though the protective effects of IPC are impressive, the therapeutic utility of such an intervention is untenable, given the invasive nature of the approach. EPC offers a means of inducing the protective mechanism of IPC without the need for invasive and dangerous tissue or organ ischemia, and thus greatly increases its clinical relevance and potential. Additionally, IPC loses its protective effects in the presence of risk factors such as aging or diabetes, while EPC remains effective as a preconditioning stimulus under these conditions. By understanding the mechanisms whereby antecedent ethanol induces protection against I/R injury and the role of AMPK in EPC, pharmacologic interventions may be developed that are more effective and practical to apply in clinical situations. The purpose of this dissertation was to determine the role of

AMPK in EPC and further elucidate the mechanisms involved in preconditioning to produce an anti-inflammatory phenotype in postcapillary venules via direct AMPK activation. The hypothesis that postischemic anti-inflammatory effects afforded by EPC are triggered by an AMPK-dependent mechanism and can be pharmacologically induced by direct activation of AMPK with AICAR-PC, and that AICAR-PC is mediated by eNOS, K_{ATP} channels, and HO-1 as illustrated in Figure 1.

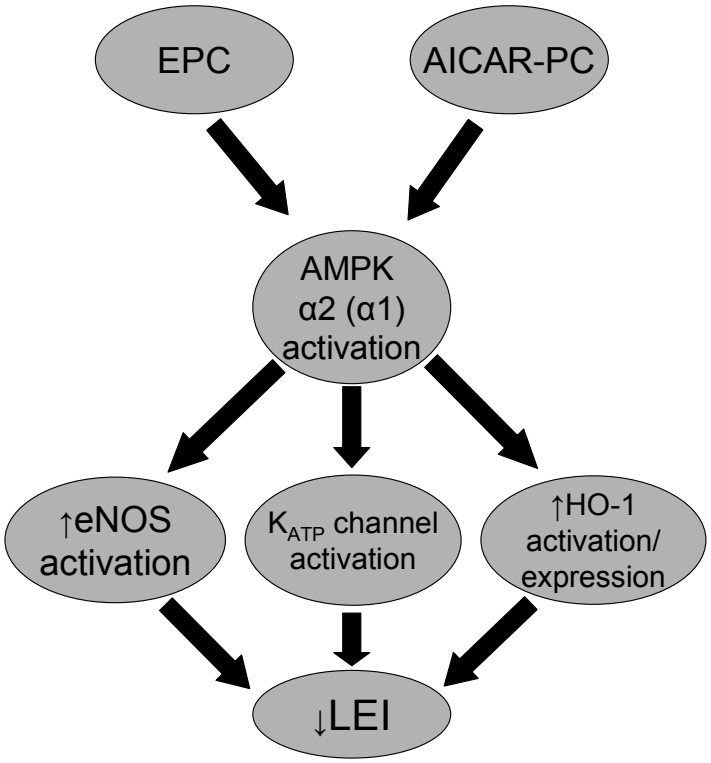

Figure 1. Proposed mechanism of ethanol and AICAR preconditioning.

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5'-AMP-ACTIVATED PROTEIN KINASE ACTIVATION PREVENTS POSTISCHEMIC LEUKOCYTE-ENDOTHELIAL CELL ADHESIVE INTERACTIONS

F. Spencer Gaskin[1], Kazuhiro Kamada[1], Mozow Yusof[1], and Ronald J. Korthuis[1,2]

[1] Department of Medical Pharmacology and Physiology and the [2] Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65212

Running Title: AMPK PRECONDITIONING AND POSTISCHEMIC LEUKOCYTE ADHESION

Address for mailing proofs:

Ronald J. Korthuis, PhD
Department of Medical Pharmacology and Physiology
University of Missouri-Columbia
One Hospital Drive
Columbia, MO 65212

Telephone number: (573) 882-8059
Fax number: (573) 884-4276
E-mail address: korthuisr@health.missouri.edu

ABSTRACT

Preconditioning (PC) with nitric oxide (NO) donors or agents that increase endothelial nitric oxide synthase (eNOS) activity 24 hrs prior to ischemia/reperfusion (I/R) prevents postischemic leukocyte rolling (LR) and stationary leukocyte adhesion (LA). As 5'-AMP-activated protein kinase (AMPK) phosphorylates eNOS at Ser1177, resulting in its activation, we postulated that AMPK activation may trigger the development of a preconditioned anti-inflammatory phenotype similar to that induced by NO donors. Wild-type (WT) C57BL/6J and eNOS-/- mice were treated with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) 30 min (early AICAR PC) or 24 hrs (late AICAR PC) prior to I/R; LR and LA were quantified in single postcapillary venules in the jejunum using intravital microscopy. I/R induced comparable marked increases in LR and LA in WT and eNOS-/- mice relative to sham (no ischemia) animals. Late AICAR PC prevented postischemic LR and LA while early AICAR PC prevented LA in WT mice. Late AICAR PC was ineffective in preventing I/R-induced LR but not LA in the eNOS-/- mice and the same pattern was seen in wild-type animals treated with the NOS inhibitor L-NAME. Early AICAR PC remained effective in preventing LA in eNOS-/- mice. Our results indicate that both early and late PC with an AMPK agonist produces an anti-inflammatory phenotype in postcapillary venules. Because the protection afforded by late AICAR PC on postischemic LR was prevented by NOS inhibition in WT mice and absent in eNOS-deficient mice, it appears that eNOS triggers

this protective effect. In stark contrast, antecedent AMPK activation prevented I/R-induced LA by an eNOS-independent mechanism.

Key Words: ischemia, reperfusion, leukocyte rolling and adhesion, preconditioning, endothelial nitric oxide synthase deficient mice

INTRODUCTION

Since ischemic preconditioning (IPC) was first described by Murry et al. (94) in 1986, this intrinsic protective mechanism has been studied extensively. This seminal work demonstrated that subjecting the myocardium to brief periods of vascular occlusion followed by reperfusion just prior to induction of a prolonged ischemic insult (index ischemia) significantly reduced myocardial infarct size. Subsequent work demonstrated that increasing the reperfusion time interval between the preconditioning stimuli and the onset of index ischemia resulted in a progressive decline and eventual loss of the infarct-sparing effects of IPC. However, a second less powerful phase of protection emerged 24 hrs after preconditioning, an observation that gave rise to the concept that IPC induced biphasic (early vs. late phase) preconditioned responses (4, 129, 132). Due to the impracticality of inducing IPC in the clinical arena an intensive research effort has been directed at identifying other interventions that trigger the development of innate preconditioning mechanisms. As a result of this work several pharmacologic agents are now known to induce a protective phenotype similar to that seen with IPC, such as preconditioning with: ethanol, adenosine receptor agonists, bradykinin, nitric oxide (NO) donors, and exogenous calcitonin gene-related peptide (14, 22, 81, 131, 140). Indeed, our own work has established that these agents induce the development of a protective anti-inflammatory phenotype in postcapillary venules, such that these vessels fail to support adhesion molecule expression, leukocyte rolling and adhesion, and

increased vascular permeability when the small bowel is subsequently exposed to prolonged I/R (32, 33, 68, 69, 121, 122, 152).

Most of the agents which induce the development of preconditioned states do so by a triggering mechanism that involves formation of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS). AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric serine/threonine kinase that regulates a diverse array of enzymes and substrates. Among its many enzymatic targets for phosphorylation, AMPK-mediated eNOS activation plays a prominent role in regulating downstream activities of a variety of therapeutic agents (31, 93, 118). Interestingly the glucose lowering agent metformin has been shown to activate AMPK and prevent the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α (53). This study also demonstrated that adhesion molecule expression was reduced by metformin or AICAR administration, a protective effect that was abrogated by the addition of an siRNA directed towards AMPKα1. These activities have previously been attributed to eNOS by others (10, 148).

Taken together with the fact that AMPK has been shown to stimulate eNOS, the aforementioned observations led us to hypothesize that direct activation of AMPK should result in the development of an anti-inflammatory phenotype similar to that seen in tissues preconditioned with ethanol or brief periods of ischemia. Moreover, we postulated that such beneficial effects would occur by an eNOS-dependent mechanism. To test these hypotheses, we examined the effects of an AMPK activator, 5-aminoimidazole-4-carboxamide 1-

β-D-ribofuranoside (AICAR), as an early and late phase preconditioning stimulus, using postischemic leukocyte-endothelial cell adhesive interactions in single postcapillary venules of the small intestine of wild-type C57BL/6J mice by intravital microscopy as indices of injury. In addition, we evaluated the role of NO in AMPK preconditioning via NOS inhibition with L-NAME prior to AICAR administration in wild-type animals and by evaluating the effectiveness of AICAR preconditioning in mice that were genetically deficient in eNOS (eNOS-/-).

MATERIALS AND METHODS

Animals: Wild-type male C57BL/6J and eNOS-/- mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and water ad libitum with 12 hr light and dark cycles, and used at 8-10 weeks of age. The experimental procedures described have been described previously (69), and were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee.

AICAR Preconditioning, Surgical Procedures, and Induction of I/R: C57BL/6J and eNOS-/- mice were preconditioned with the AMPK agonist 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml) by intraperitoneal injection either 30 min or 24 hrs prior to induction of I/R. Early and late phase preconditioned mice were subsequently anesthetized initially with a mixture of ketamine (150 mg/kg body wt, i.m.) and xylazine (7.5 mg/kg body wt, i.m.). After attaining a surgical plane of anesthesia a midline abdominal incision was performed and the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After these procedures the right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood

pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). The left jugular vein was cannulated for administration of carboxyfluorescein diacetate, succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR, USA) a fluorescent dye that labels leukocytes. CFDASE was dissolved in DMSO at a concentration of 5 mg/ml divided into 25 µl aliquots and stored in light-tight containers at -20 °C until use. During the preparation and storage of CFDASE care was taken to minimize light exposure. After the 45 min ischemic period the clip was gently removed and leukocytes were labeled with CFDASE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at a rate of 20 µl/min over 5 min. The sham group had an equivalent 45 min period without occlusion of the SMA prior to CFDASE administration. Leukocyte/endothelial cell adhesive interactions were observed over min 30-40 and 60-70 of reperfusion via intravital fluorescence microscopy.

Intravital Fluorescence Microscopy: The mice were positioned on a 20 x 30 cm Plexiglas^{TM} board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 x 3 cm hole centered in the Plexiglas^{TM}. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize tissue dehydration, temperature changes, and the influence of

respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant temperature circulator (Model 1130; VWR). Body temperature of the mice was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lump. The Plexiglas™ board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20x objective lens. Fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on DVD using a DVD video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over min 30-40 and 60-70 of reperfusion or at equivalent time points in the sham control groups, as described below. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm in diameter, 100 μm in length) were observed for at least 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules followed by calculation of

the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for at least 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per mm^2 vessel area.

Experimental Protocol: Figure 2 illustrates the general design of the experimental protocols for the study. The number of animals used in each group is described below with the protocols for Groups 1 through 4 being conducted in both wild-type C57BL/6J and eNOS-/- mice. Drug doses were selected based on previous experiments in our laboratory and reports in the literature (20, 104, 122).

Group 1: Sham. As a time control for the effects of experimental duration, wild-type C57BL/6J (n=7) or eNOS-/- (n=6) mice in this group received an intraperitoneal (i.p.) injection of 0.5 ml saline which was used as a vehicle for AICAR and L-NAME in groups outlined below. Twenty-four hrs later the superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte/endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

Group 2: I/R alone. C57BL/6J (n=7) and eNOS-/- (n=6) mice in this group were treated as described for Group 1 above except that I/R was induced 24 hrs

after the i.p. injection of saline vehicle on Day 1. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion following 45 min of ischemia on Day 2.

Group 3: Late AICAR PC + I/R. To determine whether AMPK activation with AICAR could initiate late phase preconditioning and prevent postischemic leukocyte rolling and adhesion on exposure to I/R 24 hrs later, wild-type (n=6) or eNOS-/- (n=6) mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml, i.p. injection) on Day 1. Twenty-four hrs later (Day 2) the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 4: Early AICAR PC + I/R. The aim of the studies outlined for this group was to determine whether administration of AICAR (Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml, i.p. injection) 30 min before I/R, would induce an early phase of preconditioning in wild-type C57BL/6J (n=9) and eNOS-/- (n=6) mice. Thirty min after AICAR administration the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 5: L-NAME + Late AICAR PC + I/R. To further substantiate whether direct activation of AMPK with AICAR produces late preconditioning via an NO-dependent mechanism, a specific NOS inhibitor L-NAME (Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml, i.p. injection) was administered 10 min prior to AICAR on day 1 to wild-type C57BL/6J (n=9) mice. Twenty-four hrs later the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Statistical Analysis

The data were analyzed with standard statistical analysis, i.e., ANOVA with Scheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significances were defined at $P < 0.05$.

RESULTS

Figure 3 illustrates the effects of early and late phase preconditioning with AICAR on postischemic leukocyte rolling and adhesion determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. I/R markedly increased leukocyte rolling (Figure 3A) and adhesion (Figure 3B) compared to sham (no ischemia) control mice. While AICAR administration 24 hrs prior to induction of I/R (late AICAR PC) prevented postischemic leukocyte rolling and adhesion. Early phase preconditioning with this AMPK activator (early AICAR PC) at the same dose was only effective in preventing I/R-induced leukocyte adhesion although there was a tendency for postischemic leukocyte rolling to be reduced after early AICAR PC.

The data depicted in Figure 4 show the effects of preconditioning with AICAR on I/R-induced leukocyte rolling and adhesion after 30 and 60 min of reperfusion in eNOS-deficient mice. I/R increased leukocyte rolling and adhesion in eNOS-/- mice (Figure 4A and 4B, respectively) to comparable levels to those noted in WT animals (Figure 3A and 3B, respectively). Early and late phase preconditioning with the AMPK activator (early and late AICAR PC, respectively) failed to limit postischemic rolling (Figure 4A) in eNOS-/- mice results which support the concept that AMPK-dependent eNOS activation is essential to the beneficial effect of late phase AICAR preconditioning to prevent postischemic leukocyte rolling. However, the ability of early and late AICAR preconditioning to prevent postischemic leukocyte adhesion that was noted in WT animals (Figure 3B) persisted in mice genetically deficient in eNOS (Figure 4B). Thus, the effect

of preconditioning with the AMPK activator to prevent postischemic leukocyte adhesion appears to occur by an eNOS-independent mechanism.

Further substantiation of the role for NOS-derived NO in the beneficial action of AMPK activation 24 hrs prior to induction of I/R (late AICAR PC) to prevent postischemic leukocyte rolling was obtained by treating WT mice with L-NAME prior to AICAR administration on Day 1 (Figure 3). Pharmacologic NOS inhibition abrogated the effects of late AICAR preconditioning to limit postischemic leukocyte rolling but did not prevent the reduction in leukocyte adhesion induced by antecedent AMPK activation. There were no significant differences in mean arterial blood pressures between treatment groups and their respective controls (data not shown).

DISCUSSION

AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric serine/threonine kinase that is composed of an alpha, a beta, and a gamma subunit (3, 155). The alpha subunit exists as either the α1 or α2 isoform and is responsible for the catalytic activity of the enzyme. The regulatory beta and gamma subunits occur as the β1, β2 or γ1, γ2, or γ3 isoforms. AMPK is often referred to as a metabolic "master switch" due to its high sensitivity to the AMP:ATP ratio and its centralized role in both short and long term metabolic signalling pathways (17, 21, 51, 67). When cellular energy decreases as ATP is converted to AMP in response to stressful stimuli such as exercise or hypoxia, AMPK is activated by binding AMP via its gamma subunit (67, 155). The ability of AMPK activation to protect cellular energy levels and maintain the integrity of the mitochondrial membrane potential makes it an essential pro-survival signalling mediator in protecting cells and tissues from I/R injury (60).

Recent observations support the possibility that AMPK activation induces development of preconditioned states that render tissues resistant to the deleterious effects of I/R. For example, treatment with AICAR or anti-diabetic drugs of the biguanide class (e.g., metformin) has been shown to activate AMPK, prevent the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α and decrease adhesion molecule expression, effects that were abrogated by addition of a siRNA directed towards AMPK α1 (53). AMPK also phosphorylates and activates eNOS and the cystic fibrosis transmembrane regulator (CFTR), both of which have been implicated as

essential triggering elements in the development of ischemic preconditioning (24, 49). Furthermore, antecedent ethanol ingestion, which induces late phase preconditioning and prevents postischemic leukocyte rolling and adhesion by an eNOS-dependent mechanism, also activates AMPK (65). These observations led us to postulate that direct pharmacologic activation of AMPK should induce development of an anti-inflammatory state similar to that seen with ethanol or ischemic preconditioning. In addition, we sought to determine if AMPK agonist preconditioning is eNOS-dependent.

These hypotheses were tested by examining the effects of an AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), as a preconditioning stimulus. AICAR is first metabolized to ZMP, an AMP analogue, that initially activates AMPK by binding to its gamma subunit (112). This allosteric activation increases both AMPK activity and its affinity for upstream AMPK kinases, which in turn, phosphorylate AMPK at threonine-172 on the alpha subunit to further activate the enzyme. This latter process is responsible for the majority of AMPK activity and is known to be catalyzed by at least two kinases LKB1 and Ca^{2+}-calmodulin-dependent kinase kinase β (58, 120, 125, 143, 144). The binding of AMP or ZMP also increase AMPK activity by increasing the duration of the active state, which is accomplished by decreasing the affinity of phosphorylated AMPK for deactivating phosphatases such as protein phosphatase 2C (30).

Our results indicate that early and late phase preconditioning with an AMPK activator (early and late AICAR PC, respectively) produce an anti-

inflammatory phenotype in postcapillary venules. However, whereas late AICAR PC prevented both postischemic leukocyte rolling and adhesion, only I/R-induced leukocyte adhesion was abrogated during the early phase (early AICAR PC). These observations suggest that late AICAR PC may prevent expression of adhesion molecules that mediate leukocyte rolling (e.g., P-selectin) and adhesion (e.g., ICAM-1) and the signalling mechanisms activated by early AICAR PC may selectively target adhesive structures that specifically mediate stationary adhesion, without influencing P-selectin expression. Alternatively, it is possible that early and late phase AMPK activation may exert differential effects on the production of inflammatory mediators that preferentially influence leukocyte rolling versus adhesion.

The aforementioned results clearly establish that AMPK activation induces early and late preconditioning, but the identity of downstream effectors that contribute to the development of the anti-inflammatory phenotype are unknown. However, a myriad of downstream signaling molecules are phosphorylated secondary to AMPK activation and include: glycogen synthase, 6-phosphofructo-2-kinase, insulin receptor substrate-1, transcription factor ChREBP, acetyl-CoA carboxylase-1/α and 2/β, HMG-CoA reductase, hormone-sensitive lipase, transcription factor NRF1, UCP3, co-activator PGC-1α, CFTR, eNOS, TSC2, TOR, and co-activator p300 (67). Of the effectors listed here, we were most interested in initially testing for a role for eNOS as a downstream triggering event because: 1) it is well-established that AMPK phosphorylates eNOS at Ser1177, resulting in activation and increased NO production (24, 91) and 2) activation of

eNOS has been implicated as a major triggering event for the development of late phase preconditioning in response to adenosine A2 receptor agonist treatment, antecedent exposure to short bouts of ischemia, and ethanol ingestion (152). Thus, we sought to evaluate the role of eNOS in the beneficial anti-inflammatory effects of antecedent AICAR by employing a pharmacologic inhibitor approach in wild-type mice and molecular genetic evidence obtained using an eNOS knockout model.

Given that the pattern of postischemic leukocyte/endothelial cell adhesive interactions in response to early AICAR preconditioning were comparable in WT and eNOS-/- mice, it does not appear that eNOS activation plays a role in the anti-adhesive effect noted in this phase. However, the ability of AICAR treatment 24 hrs prior to I/R (late phase) to prevent postischemic leukocyte rolling was completely absent in eNOS-/- mice, whereas the abrogation of stationary leukocyte adhesion remained effective in these mice. A comparable pattern was noted in wild-type animals pretreated with the NOS inhibitor L-NAME at the time of AICAR preconditioning 24 hrs prior to I/R. These observations suggest that the ability of late phase AICAR preconditioning to prevent postischemic leukocyte rolling was triggered by eNOS-derived NO that was formed during the period of AMPK activation 24 hrs earlier, since L-NAME has been shown to lose its effect over hrs not days. Interestingly, late phase AMPK activation prevented leukocyte adhesion by an NO-independent mechanism.

These results are intriguing due to their divergence from the canonical view that NO release prevents both leukocyte rolling and adhesion (119) and that

preconditioning stimuli such as antecedent ethanol ingestion, treatment with exogenous adenosine or adenosine A2 receptor agonists, calcitonin gene-related peptide, bradykinin, or NO donors, that utilize NO to trigger entrance into an inflammatory state prevent both types of adhesive interactions (12,21,22,33,43). Kubes et al. (77) have presented evidence that adherent leukocytes are almost exclusively recruited from the rolling cell population and that reductions in rolling by greater than 90% are required before a significant effect on leukocyte adhesion is noted. Thus, it may be that the eNOS-dependent effects of late AICAR preconditioning to reduce postischemic leukocyte rolling, despite being quite dramatic, are not sufficient to elicit a significant change in leukocyte adhesion. Such a scenario implies that other AICAR-induced effects contribute to the anti-adhesive responses of this AMPK activator. Indeed, it is well-established that in addition to activating AMPK, AICAR administration also increases tissue levels of the potent anti-adhesive purine nucleoside, adenosine, that has been implicated as a trigger for the development of other forms of preconditioning (48). However, this hypothesis seems unlikely in view of the fact that late phase preconditioning with adenosine or adenosine receptor agonists prevents both leukocyte rolling and stationary leukocyte adhesion (33, 152). Clearly, substantial additional work will be required to elucidate the mechanisms underlying the differential role of AICAR-induced eNOS activation to prevent leukocyte rolling without influencing postischemic leukocyte adhesion.

Our results may have important implications regarding the cardioprotective actions of therapeutic agents that activate AMPK such as the glucose-lowering

agent, metformin. That is, in addition to its salutary metabolic actions in diabetes, metformin may induce significant anti-inflammatory effects in afflicted patients. AMPK activation has been shown to augment ischemic preconditioning, suggesting that AICAR might represent a useful adjunctive therapy when administered with other agents that produce a preconditioned phenotype (15, 124). As another example, Xenos et al. (148) demonstrated that the HMG-CoA reductase inhibitor fluvastatin increases AMPK expression and activation in addition to increasing eNOS expression and phosphorylation. They also showed that fluvastatin was able to decrease ICAM-1 and PECAM-1 expression in an NO-dependent manner. An earlier study showed that fluvastatin also decreased expression of E-selectin and ICAM-1 in addition to increasing eNOS activity (92). Taken together with our work in the present study these intriguing findings suggest that anti-inflammatory phenotype induced by AMPK activation contributes to the well-known cardioprotective effects of widely prescribed statin drugs and metformin, in addition to their actions to inhibit HMG-CoA reductase and reduce plasma glucose, respectively.

Studies conducted to date showing that AICAR is cardioprotective in the setting of I/R have utilized protocols that involve treatment coincident with the onset of reperfusion or throughout ischemia and reperfusion. Our study is the first to demonstrate that preconditioning with this AMPK activator induces development of early and late phase protection in the microcirculation such that postcapillary venules fail to support leukocyte rolling and adhesion following subsequent exposure to I/R. However, important mechanistic differences exist

as late phase AICAR preconditioning prevents both leukocyte rolling and adhesion induced by I/R, whereas early phase AICAR limits only postischemic leukocyte adhesion. Additionally, the ability of late AICAR preconditioning to prevent leukocyte rolling involves eNOS-derived NO while the salutary effects on leukocyte adhesion occur by an eNOS-independent mechanism. A role for eNOS in the early phase appears unlikely because the pattern of response to AICAR preconditioning 30 min prior to I/R was identical in both WT and eNOS-deficient mice.

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Kazuhiro Kamada is currently with the Department of Inflammation and Immunology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto 602-8566, Japan

GRANTS

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Figure Legends

Figure 2: Schematic illustration of the experimental protocols assigned to each group. The numbers at the top of the diagram refer to min in the time line for the protocol on Day 1 and Day 2 (24 hrs between both 0s). Hatched bars indicate when the 10 min video recordings were obtained in the protocol. Solid bars depict the 45 min period of ischemia. Triangles illustrate when administration of saline vehicle or drugs was accomplished in the protocol timeline. I/R = ischemia and reperfusion. See the text for further details.

Figure 3A and 3B: Effects of preconditioning with AICAR 30 min (Early AICAR PC + IR) or 24 hrs (Late AICAR PC + IR) prior to ischemia/reperfusion on postischemic leukocyte rolling (Panel A) and adhesion (Panel B) determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. L-NAME + Late AICAR PC +IR refers to experiments in which the NO synthase inhibitor was administered 10 min prior to preconditioning with AICAR in animals subsequently exposed to I/R 24 hrs later. Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion respectively. * denotes values statistically different from IR ($p<.05$), # denotes values that are statistically different compared to sham control ($p<.05$).

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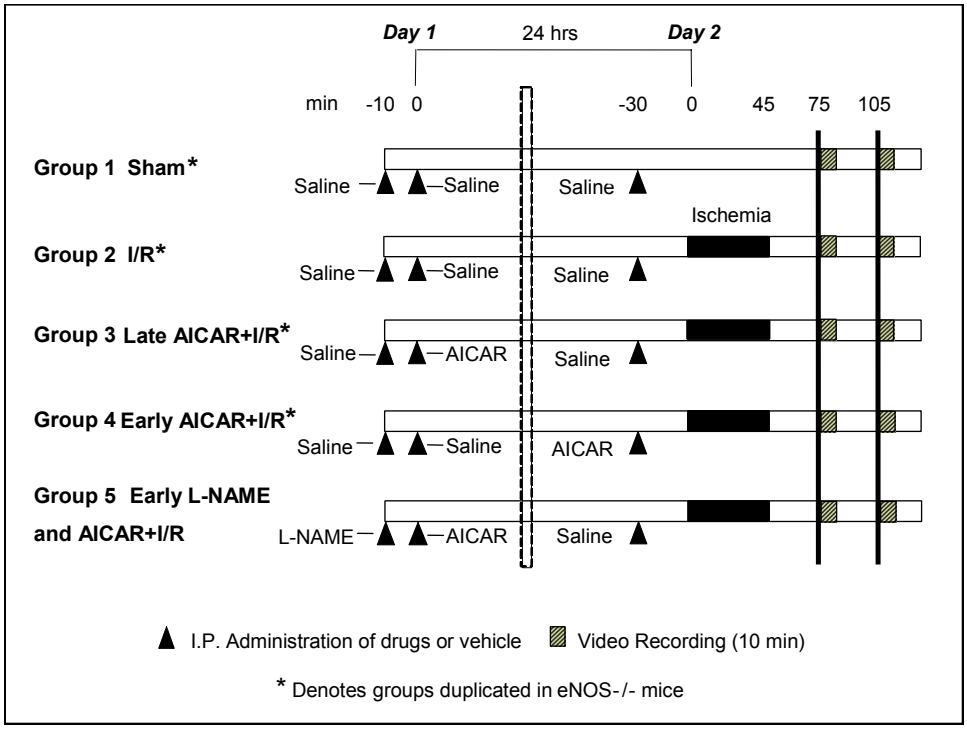
FIGURES



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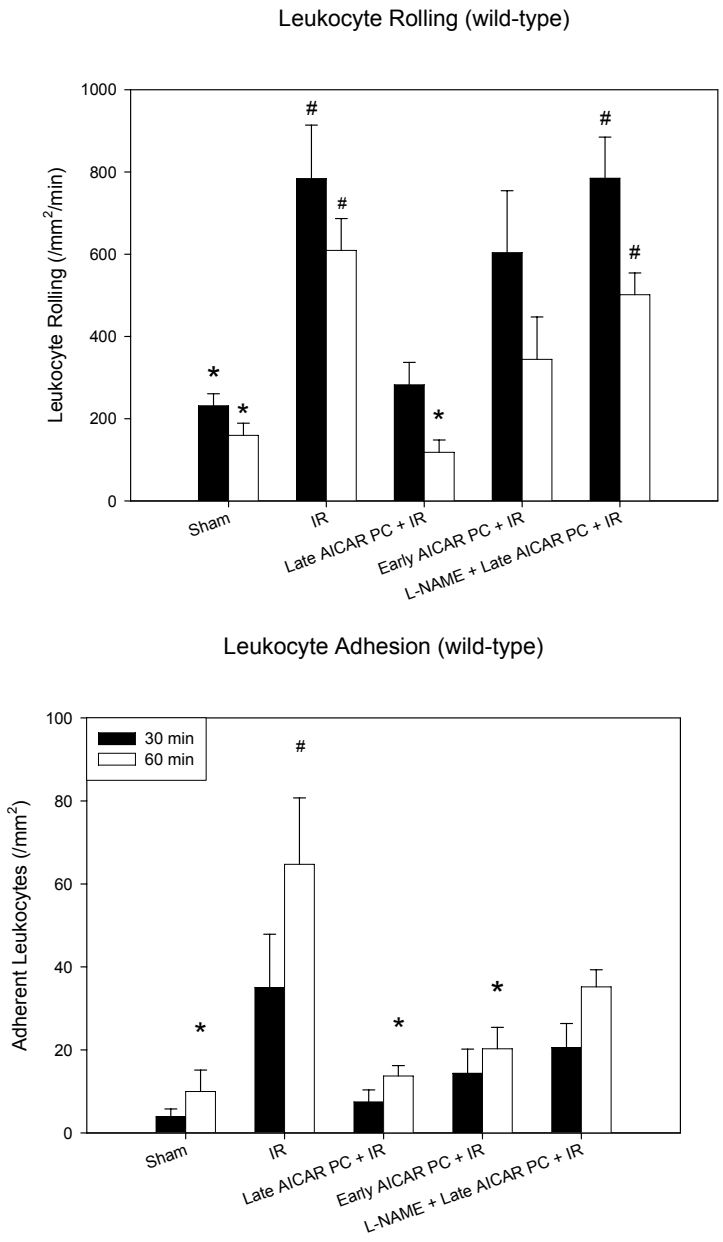

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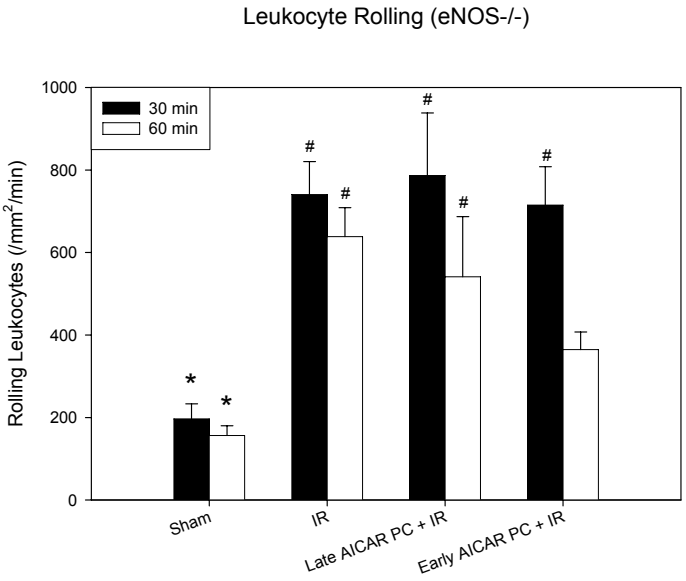

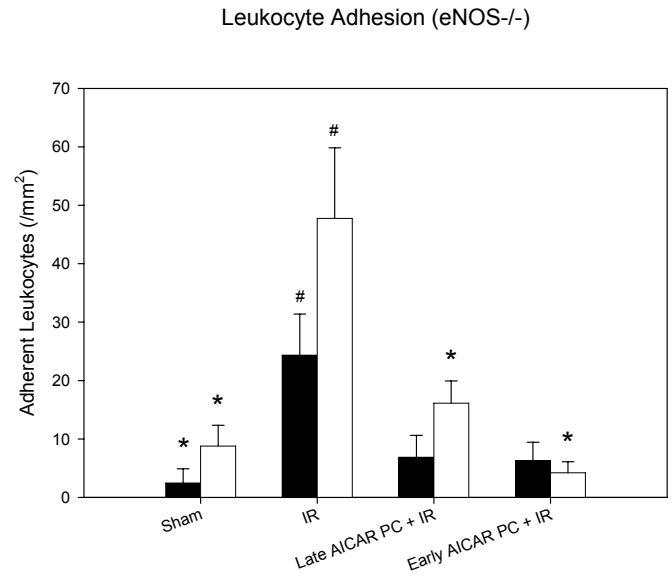

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ETHANOL PRECONDITIONING IS TRIGGERED BY 5'-AMP-ACTIVATED PROTEIN KINASE-DEPENDENT MECHANISM TO PREVENT POSTISCHEMIC LEUKOCYTE-ENDOTHELIAL CELL ADHESIVE INTERACTIONS

F. Spencer Gaskin[1], Kazuhiro Kamada[1], Mozow Yusof[1],

Leona J. Rubin[3], and Ronald J. Korthuis[1,2]

[1] Department of Medical Pharmacology and Physiology, [2] Dalton Cardiovascular Research Center, and the [3] Department of Biomedical Sciences, University of Missouri, Columbia, MO 65212

Running Title: ETHANOL AND AMPK IN PRECONDITIONING

Address for correspondence:

Ronald J. Korthuis, PhD
Department of Medical Pharmacology and Physiology
University of Missouri-Columbia
One Hospital Drive
Columbia, MO 65212

Telephone number: (573) 882-8059
Fax number: (573) 884-4276
E-mail address: korthuisr@health.missouri.edu

ABSTRACT

We previously demonstrated that preconditioning induced by ethanol consumption at low levels (ethanol preconditioning or EPC) or with 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR-PC) 24 hrs prior to ischemia/reperfusion prevent postischemic leukocyte-endothelial cell adhesive interactions (LEI) by a mechanism that is initiated by nitric oxide (NO) formed by endothelial NO synthase (eNOS). Recent work indicates that: 1) ethanol increases the activity of 5'-AMP-activated protein kinase (AMPK) and 2) AMPK phosphorylates eNOS at the same activation site seen following EPC (Ser1177). In light of these observations, we postulated that the heterotrimeric serine/threonine kinase, AMPK may play a role in triggering development of the anti-inflammatory phenotype induced by EPC. Ethanol was administered to C57BL/6J mice by gavage in the presence or absence of AMPK inhibition. Twenty-four hrs later the numbers of rolling and adherent leukocytes in postcapillary venules of the small intestine were recorded using an intravital microscopic approach. Following 45 min of ischemia LEI were recorded after 30 and 60 min of reperfusion (I/R) or at equivalent time points in control animals. I/R induced a marked increase in LEI relative to sham control mice. The increase in LEI was prevented by EPC, an effect that was lost with AMPK inhibition during the period of ethanol exposure. Studies conducted in AMPK α1 and α2 knockout mice suggest that the anti-inflammatory effects of AICAR are not dependent on the isoform of the catalytic alpha subunit present because deficiency of either

isoform resulted in loss of protection. In contrast, preconditioning with ethanol appears to be triggered by an AMPK α2 isoform-dependent mechanism.

Key Words: ischemia, reperfusion, leukocyte rolling and adhesion, ethanol, AMPK, preconditioning

INTRODUCTION

Prior to the discovery of the infarct-sparing effects of ischemic preconditioning (IPC) by Murry et al (94), the only effective mean of reducing the inimical effects of ischemia/reperfusion (I/R) was by decreasing the duration of ischemia prior to re-establishing blood flow. These studies demonstrated that subjecting the myocardium to brief periods of vascular occlusion followed by reperfusion just prior to induction of a prolonged ischemic insult (index ischemia) significantly reduced myocardial infarct size. Subsequent work demonstrated that increasing the reperfusion time interval between the preconditioning stimuli and the onset of index ischemia resulted in a progressive decline and eventual loss of the infarct-sparing effects of IPC. However, a second less powerful phase of protection emerged 24 hrs after preconditioning, an observation that gave rise to the concept that IPC induced biphasic (early versus late phase) preconditioned responses (4, 129, 132). Due to the impracticality of inducing IPC in the clinical arena, an intensive research effort has been directed at identifying other interventions that trigger the development of innate preconditioning mechanisms. As a result of this work, several pharmacologic agents are now known to induce a protective phenotype similar to that seen with IPC, such as preconditioning with: ethanol, adenosine receptor agonists, bradykinin, nitric oxide (NO) donors, and exogenous calcitonin gene-related peptide (CGRP) (14, 22, 81, 131, 140). Indeed, our own work has established that these agents induced the development of a protective anti-inflammatory phenotype in postcapillary venules, such that these vessels fail to support adhesion molecule expression, leukocyte

rolling and adhesion, and increased vascular permeability when the small bowel is subsequently exposed to prolonged I/R (32, 33, 68, 69, 121, 122, 152).

Most of the agents that induce development of preconditioned states do so by a triggering mechanism that involves the formation of NO by endothelial nitric oxide synthase (eNOS). Indeed, we previously demonstrated that direct pharmacologic activation of AMP-activated protein kinase (AMPK) a ubiquitously expressed heterotrimeric serine/threonine kinase that regulates a diverse array of enzymes and substrates, induced development of an eNOS-dependent anti-inflammatory phenotype in I/R (42). In view of recent work indicating that ethanol increases AMPK activity (56) and AMPK activation, like EPC, has been shown to increase endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 and its activity (24, 55, 91, 150, 151), we postulated that this heterotrimeric serine/threonine kinase may play a role in triggering the development of the anti-inflammatory phenotype induced by EPC. Interestingly the glucose lowering agent metformin has been shown to activate AMPK and prevent the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α (53). This study also demonstrated that adhesion molecule expression was reduced by metformin or the AMPK activator, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), protective effects that were abrogated by the addition of a siRNA directed towards AMPK α1. Thus, we also sought to evaluate the relative roles of two different AMPK catalytic α-subunit isoforms in preconditioning by evaluating the effectiveness of ethanol and AICAR

preconditioning in mice that were genetically deficient in AMPK α1 versus AMPK α2 (AMPK α1-/- versus AMPK α2-/- mice).

MATERIALS AND METHODS

Animals: Wild-type male C57BL/6J and AMPK α-subunit-/- mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME) and Dr. Leona Rubin at the University of Missouri, respectively. All mice were maintained on standard mouse chow and water ad libitum with 12 hr light and dark cycles, and used at 8-10 weeks of age. The experimental procedures described have been described previously (69), and were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee.

Ethanol Preconditioning, Surgical Procedures, and Induction of I/R: Mice were preconditioned 24 hrs prior to I/R with ethanol instilled into the stomach as a bolus by gavage. The alcohol is given at a dose that produces a peak plasma ethanol concentration of 45 mg/dl 30 min after administration, by using the following equation: [EtOH] = ((0.6 x body weight in grams) + 0.3) µl, diluted in 0.3 ml deionized H_2O. Preconditioned mice were subsequently anesthetized initially with a mixture of ketamine (150 mg/kg body wt, i.m.) and xylazine (7.5 mg/kg body wt, i.m.). After attaining a surgical plane of anesthesia, a midline abdominal incision was performed and the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After these procedures, the right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould)

connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital converter (MP 100; Biopac Systems). The left jugular vein was also cannulated for administration of carboxyfluorescein diacetate, succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR, USA), a fluorescent dye that labels leukocytes. CFDASE was dissolved in DMSO at a concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until use. During the preparation and storage of CFDASE, care was taken to minimize light exposure. After the 45 min ischemic period, the clip was gently removed and leukocytes were labeled with CFDASE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at a rate of 20 µl/min, for 5 min. The sham group had an equivalent 45 min period without occlusion of the SMA prior to CFDASE administration. Leukocyte/endothelial cell adhesive interactions were observed over min 30-40 and 60-70 of reperfusion via intravital fluorescence microscopy.

Intravital Fluorescence Microscopy: The mice were positioned on a 20 x 30 cm Plexiglas™ board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 x 3 cm hole centered in the Plexiglas™. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to

minimize tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lump. The Plexiglas^{TM} board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20x objective lens. Fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on DVD using a DVD video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over min 30-40 and 60-70 of reperfusion or at equivalent time points in the sham control groups, as described below. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm in diameter, 100 μm in length) were observed for at least 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent

leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move or detach from the venular wall for at least 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per mm^2 vessel area.

Experimental Protocol: Figure 5 illustrates the general design of the experimental protocols for the study. The number of mice used in each group is described below, with the protocols for Groups 1 through 4 being conducted in : wild-type C57BL/6J, AMPK α1-/-, AMPK α2-/-, and their wild-type littermate mice. Drug doses were selected based on previous experiments in our laboratory and reports in the literature (20, 104, 122).

Group 1: Sham. As a time control for the effects of experimental duration, wild-type C57BL/6J (n=6), AMPK α1-/- (n=3), AMPK α1+/+ (n=3), AMPK α2-/- (n=5), or AMPK α2+/+ (n=5) mice in this group received an intraperitoneal (i.p.) injection of 0.5 ml saline, which was used as a vehicle for AICAR and other pharmacologic agents in groups outlined below. The AMPK α1+/+ and AMPK α2+/+ wild-type littermate controls were used to ensure there were no confounding effects as a result of the different sources of mice, though they are on a C57BL/6J background. Twenty-four hrs later, the superior mesenteric artery

was exposed but not subjected to occlusion, with leukocyte/endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

Group 2: I/R alone. Wild-type (n=6), AMPK α1-/- (n=3), AMPK α1+/+ (n=3), AMPK α2-/- (n=5), or AMPK α2+/+ (n=5) mice in this group were treated as described for Group 1 above except that I/R was induced 24 hrs after the i.p. injection of saline vehicle on Day 1. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion following 45 min of ischemia on Day 2.

Group 3: EPC + I/R. The aim of this group was to determine whether EPC could initiate preconditioning and prevent postischemic leukocyte rolling and adhesion on exposure to I/R 24 hrs later, wild-type (n=6), AMPK α1-/- (n=3), AMPK α1+/+ (n=3), AMPK α2-/- (n=5), or AMPK α2+/+ (n=5) mice were given ethanol by gavage on Day 1. Twenty-four hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 4: AICAR-PC + I/R. The purpose of this experimental group was to determine whether AMPK activation with AICAR could initiate preconditioning and prevent postischemic leukocyte rolling and adhesion on exposure to I/R 24 hrs later in mice lacking specific AMPK alpha subunit isoforms, AMPK α1-/- (n=3), AMPK α1+/+ (n=3), AMPK α2-/- (n=5), or AMPK α2+/+ (n=5) mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml, i.p. injection) on

Day 1. Twenty-four hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 5: EPC+ Compound C + I/R. The aim of the studies outlined for this group was to determine whether administration of an AMPK inhibitor 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (compound C, Sigma, St. Louis, MO, USA) (3 mg/kg, 0.5 ml, i.p. injection) 10 min before EPC, would prevent the protective effects previously seen with EPC in wild-type C57BL/6J (n=6) mice. Twenty-four hrs later the intestines were exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 6: Compound C + I/R. To ensure that compound C itself did not affect LEI, wild-type C57BL/6J (n=6) mice were treated identically to group 5 with the exception that they did not receive EPC. Twenty-four hrs later, the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Statistical Analysis

The data were analyzed with standard statistical analysis, i.e., ANOVA with Scheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significances were defined at $P < 0.05$.

RESULTS

Figure 6 illustrates the effects of AMPK inhibition on the ability of EPC to reduce postischemic leukocyte rolling and adhesion determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. I/R markedly increased leukocyte rolling (Figure 6A) and adhesion (Figure 6B) compared to sham (no ischemia) control mice. Ethanol administration 24 hrs prior to induction of I/R prevented the increased postischemic leukocyte rolling and adhesion, but coincident inhibition of AMPK with compound C blocked this protective effect. To ensure that compound C did not have an effect on LEI in the absence of EPC, this agent was administered by itself on Day 1, with LEI assessed during I/R 24 hrs later. This treatment protocol did not modify the proadhesive effects of I/R alone.

The compound C data presented in Figure 6 lends support to our hypothesis that EPC is triggered by an AMPK-dependent mechanism though it is interesting to note that initial studies with another putative AMPK inhibitor, adenine 9-β-D-arabinofuranoside (Ara-A) failed to modify the effect of preconditioning with ethanol. Ara-A was also ineffective in preventing the anti-adhesive effects of preconditioning with the AMPK activator AICAR (data not shown), suggesting that this agent exerts little AMPK inhibitory activity in our model despite using doses previously reported to be effective in this regard. This finding is supported by the recent observation that Ara-A fails to modify AMPK activity in vascular tissue (111). Nevertheless, these disparate observations with pharmacologic inhibitors led to us to further evaluate the roles of AMPK in EPC

and AICAR-PC by examining their effects as preconditioning stimuli in AMPK knockout animals.

The data depicted in Figure 7 compare the effects of preconditioning with ethanol versus the AMPK activator AICAR on I/R-induced leukocyte rolling and adhesion after 30 and 60 min reperfusion in AMPK α1-subunit isoform-deficient mice and their wild-type littermates. I/R increased leukocyte rolling and adhesion in AMPK α1+/+ and AMPK α1-/- mice (Figures 7A+B and 7C+D, respectively) to levels that were similar to that noted in WT animals (Figure 6A and 6B, respectively). In addition, preconditioning with either ethanol or AICAR remained effective in WT littermates. However, the ability of AICAR-PC to prevent postischemic leukocyte rolling and adhesion was absent in AMPKα1-/- mice. In stark contrast, EPC retained its ability to induce an anti-inflammatory phenotype in mice lacking this isoform.

Figure 8 shows the effects of preconditioning with ethanol and AICAR on I/R-induced leukocyte rolling and adhesion after 30 and 60 min reperfusion in AMPK α2-subunit isoform-deficient mice and their wild-type littermates. I/R increased leukocyte rolling and adhesion in AMPK α2+/+ and AMPK α2-/- mice (Figures 8A+B and 8C+D, respectively) to levels that were comparable to that noted in WT animals (Figure 6A and 6B, respectively) and AMPK α1-/- mice (Figures 7A+B and 7C+D, respectively). Although LEI in sham animals appeared to be somewhat elevated in AMPK α2 knockout mice relative to that seen in the other sham groups, this difference was not statistically significant. Preconditioning with either ethanol or AICAR reduced both leukocyte rolling and

adhesion in the AMPK α2+/+ wild-type littermates as seen in figures 8A+B. However, both preconditioning stimuli failed to limit postischemic leukocyte rolling and adhesion (Figure 8C+D) in AMPK α2-/- mice. Taken together with the results presented in Figure 7C+7D, these observations indicate that the anti-inflammatory effects, though modest, of EPC occur by an AMPK α2-dependent mechanism though a deficit in expression of either AMPK α1 or AMPK α2 subunits is sufficient to prevent AICAR-PC.

DISCUSSION

AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric serine/threonine kinase composed of an alpha, a beta, and a gamma subunit (3, 155). The alpha subunit, which exists as either the α1 or α2 isoform, is responsible for the catalytic activity of the enzyme. The regulatory beta and gamma subunits occur in the β1 or β2 and the γ1, γ2, or γ3 isoforms. AMPK is often referred to as a metabolic "master switch" due to its high sensitivity for the AMP:ATP ratio and its centralized role in both short and long term metabolic signalling pathways (17, 21, 51, 67). When cellular energy levels decrease as ATP is converted to AMP in response to stressful stimuli such as exercise or hypoxia, AMPK is activated by binding AMP via its gamma subunit (67, 155). The ability of AMPK activation to protect cellular energy levels and maintain the integrity of the mitochondrial membrane potential makes it an essential pro-survival signalling mediator in protecting cells and tissues from I/R injury (60).

Recent observations support the possibility that AMPK activation induces development of preconditioned states that render tissues resistant to the deleterious effects of I/R. For example, treatment with AICAR or anti-diabetic drugs of the biguanide or thiazolidinedione class (e.g., metformin and rosiglitazone, respectively) have been shown to activate AMPK (40), prevent the signalling of inflammatory cytokines through nuclear factor kappa β and tumor necrosis factor α, and decrease adhesion molecule expression, effects that were abrogated by the addition of a siRNA directed towards AMPK α1 (53). AMPK

also phosphorylates and activates eNOS, actions that have been implicated as essential triggering elements in the development of ischemic preconditioning (24, 49). Furthermore, antecedent ethanol ingestion, which induces late phase preconditioning and prevents postischemic leukocyte rolling and adhesion by an eNOS-dependent mechanism, also activates AMPK (65). These observations led us to postulate that ethanol preconditioning may be triggered by an AMPK-dependent mechanism.

The ongoing hypothesis was tested by evaluating the effects of pharmacologic AMPK inhibition coincident with ethanol ingestion on leukocyte rolling and adhesion during I/R 24 hrs after treatment/ingestion. Compound C completely abrogated the ability of antecedent ethanol to prevent the increased leukocyte rolling and adhesion induced by I/R 24 hrs later. A third line of evidence supporting the notion that antecedent ethanol triggers entrance into a preconditioned state by an AMPK-dependent mechanism was provided by our studies conducted in AMPK knockout animals. Ethanol ingestion failed to elicit an anti-inflammatory phenotype in AMPK α2-/- mice but remained effective as a preconditioning stimulus in mice genetically deficient in AMPK α1. These latter observations suggest that ethanol differential activates AMPK in an α-subunit-dependent manner. However, the mechanism for this selective effect remains obscure and should be the focus of future investigations. Likely possibilities include differential ethanol binding in water-filled pockets of the different AMPK α subunits, inhibition of select protein phosphatases that target specific

phosphorylated sites in AMPK α1 versus AMPK α2, and/or unique alterations in upstream kinases that allow singular activation of the different isoforms.

Given our demonstration of a role for AMPK in EPC one would expect that other agents that activate AMPK would produce an anti-inflammatory state, perhaps by an AMPK α2-dependent mechanism. Indeed, we previously reported that direct pharmacologic AMPK activation with 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) was as effective as antecedent ethanol ingestion in producing a preconditioned anti-inflammatory state. AICAR is first metabolized to ZMP, an AMP analogue, which initially activates AMPK by binding to its gamma subunit (112). This allosteric activation increases both AMPK activity and its affinity for upstream AMPK kinases, which phosphorylate AMPK at threonine-172 on the alpha subunit to further activate the enzyme. This latter process is responsible for the majority of AMPK activity, and is known to be catalyzed by at least two kinases: LKB1 and Ca^{2+}-calmodulin-dependent kinase kinase β (58, 120, 125, 143, 144). The binding of AMP or ZMP also increase AMPK activity by increasing the duration of the active state, which is accomplished by decreasing the affinity of phosphorylated AMPK for deactivating phosphatases such as protein phosphatase 2C (30).

Substantiating our earlier findings (42), we again demonstrated that preconditioning with the AMPK activator AICAR produces an anti-inflammatory phenotype in postcapillary venules in tissues subsequently exposed to I/R 24 hrs later, preventing postischemic leukocyte rolling and adhesion. As we observed with EPC, the anti-adhesive effects of antecedent AICAR treatment were

completely prevented by AMPK inhibition with compound C. However, in contrast to a selective role of AMPK α2 in ethanol preconditioning, the ability of AICAR to prevent postischemic LEI was absent in mice genetically deficient in either the AMPK α1 or AMPK α2 isoforms. These observations suggest that both isoforms participate in development of the preconditioned phenotype in response to antecedent AICAR but that the absence of either is sufficient to prevent the anti-adhesive effects of preconditioning with this agent.

It is interesting to note that initial studies with the putative AMPK inhibitor adenine 9-β-D-arabinofuranoside (Ara-A) failed to prevent the anti-adhesive effects of preconditioning with ethanol while treatment with another structurally unrelated AMPK inhibitor, compound C, was effective in this regard. Much of the previous work regarding the role of AMPK in physiologic processes has relied on data obtained using Ara-A as an inhibitor of the enzyme. Although this agent inhibits AICAR-induced AMPK activity in a variety of tissues (1, 20, 23, 72), it does seem to be more effective at blocking the α1 isoform in response to AICAR or metabolic challenge than in contraction-induced AMPK activation of either isoform in skeletal muscle where it had no effect (96). In addition, Ara-A has recently been shown to be ineffective in inhibiting AMPK activity in vascular tissues (111). Thus, it was not surprising that Ara-A failed to prevent the anti-adhesive effects noted in postcapillary venules after ethanol preconditioning in our model, even at doses 5-10 fold greater than previously used. Interestingly, we also demonstrated that Ara-A fails to prevent AICAR-PC, which supports the notion that this agent may not effectively inhibit AMPK in vascular tissues. In

view of our observations with compound C and in AMPK knockout mice, where both EPC and AICAR PC were ineffective in producing an anti-inflammatory phenotype, this conclusion seems likely.

The aforementioned results clearly establish that AMPK activation induces preconditioning in AICAR- and ethanol-treated animals but the identities of downstream effectors that contribute to the development of the anti-inflammatory phenotype are largely unknown. However, a myriad of downstream signaling molecules are phosphorylated secondary to AMPK activation and include: glycogen synthase, 6-phosphofructo-2-kinase, insulin receptor substrate-1, transcription factor ChREBP, acetyl-CoA carboxylase-1/α and 2/β, HMG-CoA reductase, hormone-sensitive lipase, transcription factor NRF1, UCP3, co-activator PGC-1α, CFTR, eNOS, TSC2, TOR, and co-activator p300 (67). Of the effectors listed here, a role for eNOS as a downstream triggering event is highly likely because: 1) it is well-established that AMPK phosphorylates eNOS at Ser1177, resulting in activation and increased NO production (24, 91) and 2) activation of eNOS has been implicated as a major triggering event for the development of late phase preconditioning in response to adenosine A_2 receptor agonist treatment, antecedent exposure to short bouts of ischemia, and ethanol ingestion (152). Indeed, we previously demonstrated that eNOS plays an essential role in the beneficial anti-inflammatory effects of both antecedent ethanol and AICAR-PC, suggesting a mechanistic link between ethanol-induced increases in eNOS/AMPK activity (42, 154).

Our results may have important implications regarding the cardioprotective actions of therapeutic agents that activate AMPK such as the glucose-lowering agent, metformin. That is, in addition to its salutary metabolic actions in diabetes, metformin may induce significant anti-inflammatory effects in afflicted patients. AMPK activation has been shown to mediate ischemic preconditioning, suggesting that AICAR might represent a useful adjunctive therapy when administered with other agents that produce a preconditioned phenotype (15, 124). As another example, Xenos et al. (148) demonstrated that the HMG-CoA reductase inhibitor fluvastatin increases AMPK expression and activation in addition to increasing eNOS expression and phosphorylation. They also showed that fluvastatin decreased ICAM-1 and PECAM-1 expression in an NO-dependent manner. An earlier study showed that fluvastatin decreased expression of E-selectin and ICAM-1 in addition to increasing eNOS activity (92). Taken together with our work in the present study, these intriguing findings suggest that anti-inflammatory phenotype induced by AMPK activation may contribute to the well-known cardioprotective effects of widely prescribed statin drugs and metformin, in addition to their actions to inhibit HMG-CoA reductase and reduce plasma glucose, respectively.

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Figure Legends

Figure 5: Schematic illustration of the experimental protocols assigned to each group. The numbers at the top of the diagram refer to min in the time line for the protocol on Day 1 and Day 2 (24 hrs between both 0s). Hatched bars indicate when the 10 min video recordings were obtained in the protocol. Solid bars depict the 45 min period of ischemia. Triangles illustrate when administration of saline vehicle or drugs was accomplished in the protocol timeline. I/R = ischemia and reperfusion. See the text for further details.

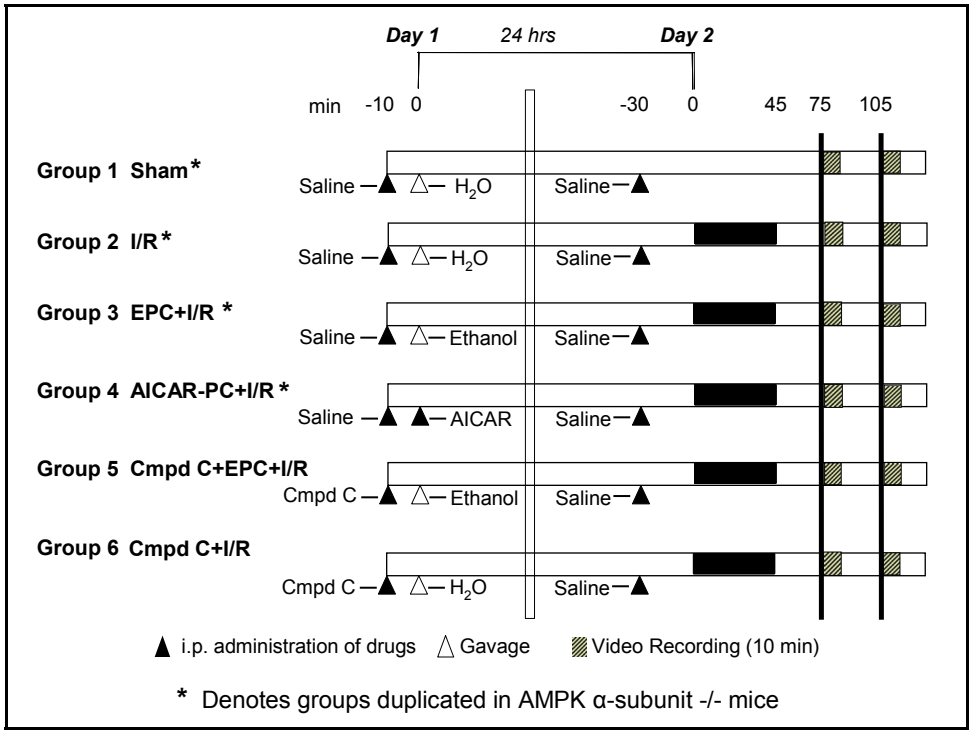
Figure 6: Effects of ethanol preconditioning 24 hrs prior to ischemia/reperfusion (EPC) on postischemic leukocyte rolling (Panel A) and adhesion (Panel B) determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. Cmpd C+EPC+IR refers to experiments in which the AMPK inhibitor was administered 10 min prior to EPC in animals subsequently exposed to I/R 24 hrs later. Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion respectively. * denotes values statistically different from control ($p<.05$).

Figure 7: Effects of preconditioning with ethanol and AICAR 24 hrs prior to ischemia/reperfusion (EPC and AICAR PC + IR) on postischemic leukocyte rolling (top right) and adhesion (bottom right) determined after 30 and 60 min of reperfusion in AMPK alpha-1 subunit deficient mice and their wild-type littermates (top left and bottom left). Open and solid bars represent data obtained at min

30–40 and 60–70 of reperfusion, respectively. * denotes values statistically different from control ($p<.05$).

Figure 8: Effects of preconditioning with ethanol and AICAR 24 hrs prior to ischemia/reperfusion (EPC and AICAR PC + IR) on postischemic leukocyte rolling (C) and adhesion (D) determined after 30 and 60 min of reperfusion in AMPK alpha-2 subunit deficient mice and their wild-type littermates (A and B). Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion, respectively. * denotes values statistically different from control ($p<.05$).

FIGURES



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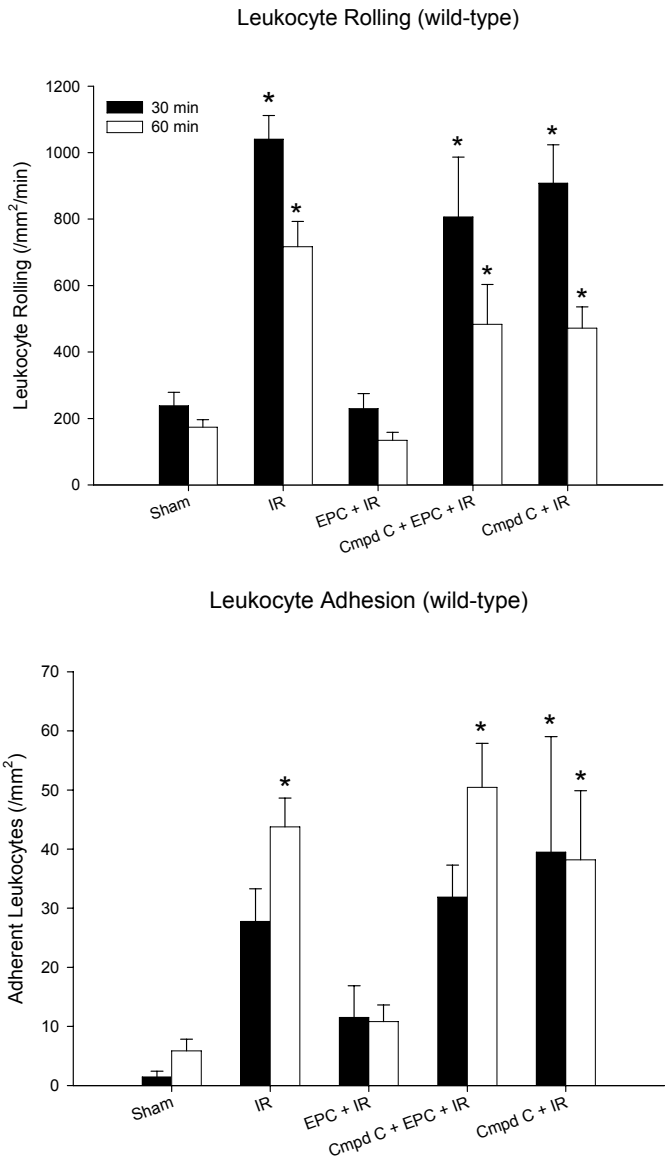

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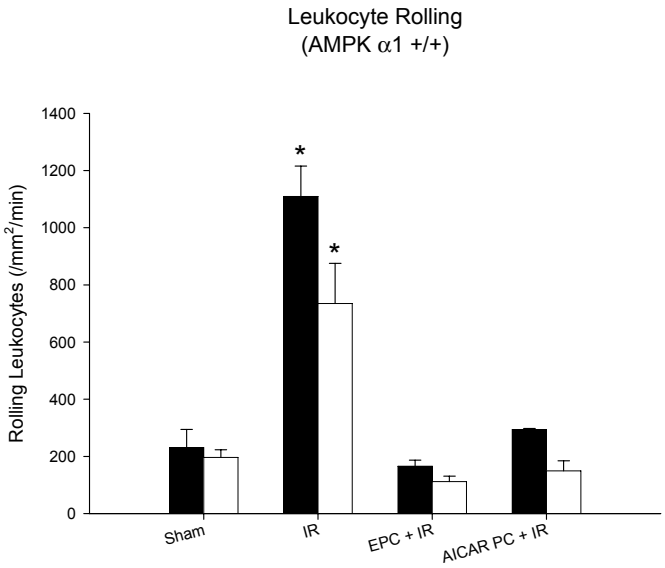
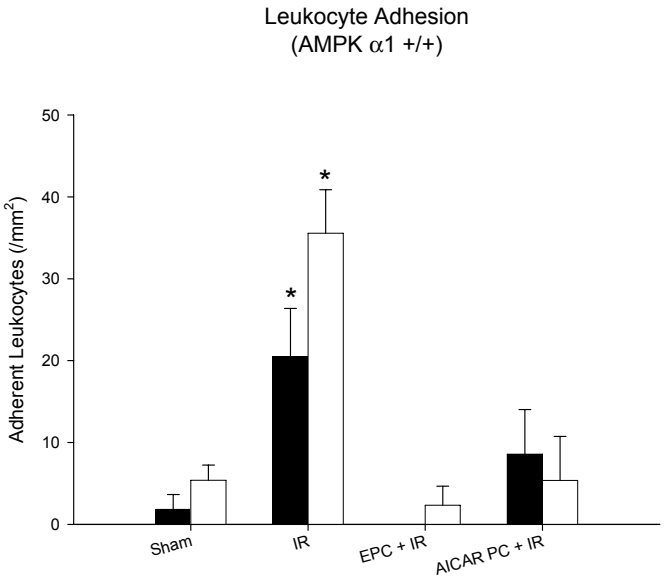

Leukocyte Rolling
(AMPK α1 +/+)
Rolling Leukocytes (/mm²/min)
1400
1200
1000
800
600
400
200
0
*
*
Sham
IR
EPC + IR
AICAR PC + IR


Leukocyte Adhesion
(AMPK α1 +/+)
Adherent Leukocytes (/mm²)
50
40
30
20
10
0
*
*
Sham
IR
EPC + IR
AICAR PC + IR

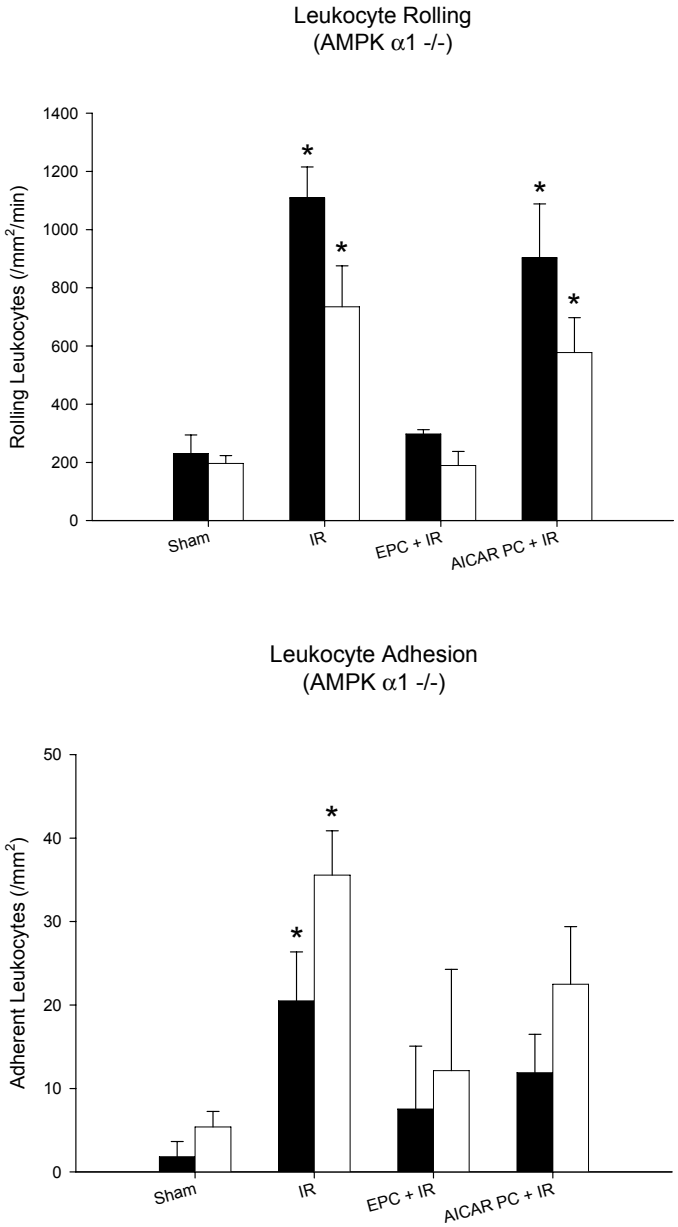
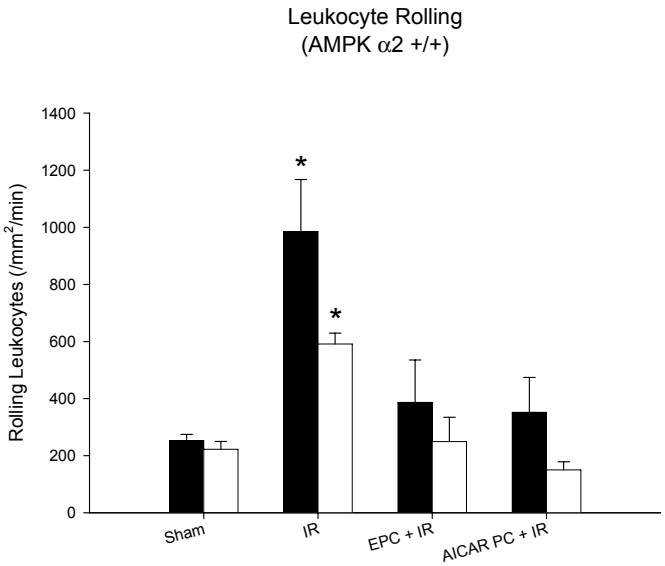


Figure 7: Effects of preconditioning with ethanol and AICAR 24 hrs prior to ischemia/reperfusion (EPC and AICAR PC + IR) on postischemic leukocyte rolling (top right) and adhesion (bottom right) determined after 30 and 60 min of reperfusion in AMPK alpha-1 subunit deficient mice and their wild-type littermates (top left and bottom left). Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion, respectively. * denotes values statistically different from control ($p<.05$).


Leukocyte Rolling
(AMPK α2 +/+)
Rolling Leukocytes (/mm²/min)
1400
1200
1000
800
600
400
200
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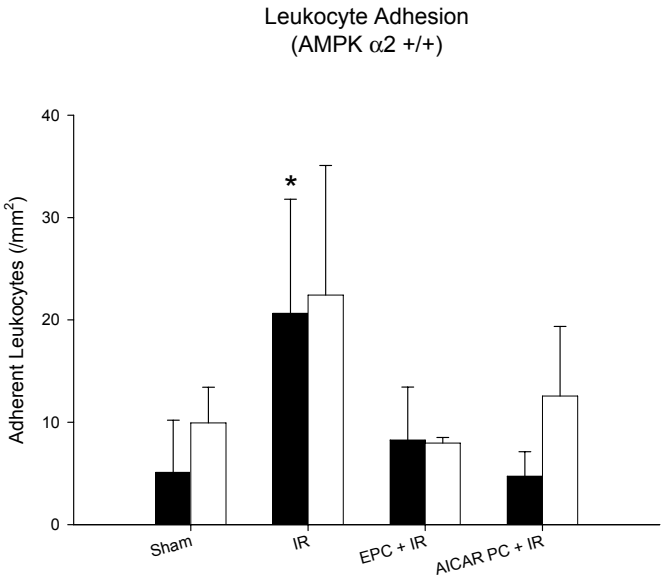
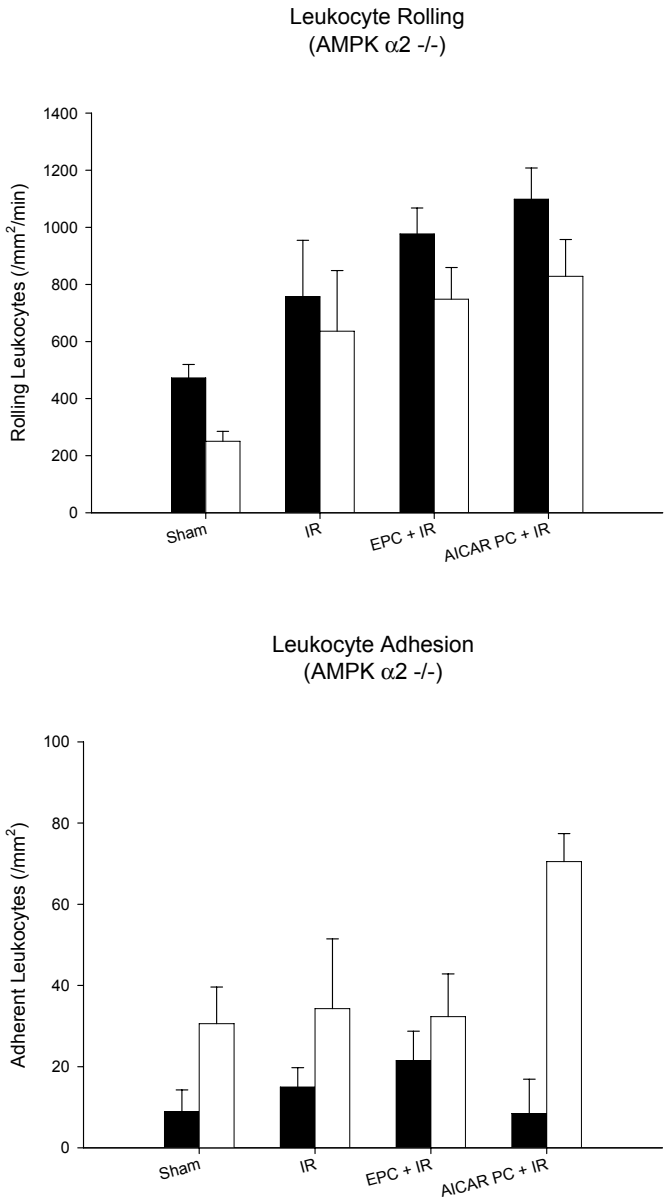
Sham
IR
EPC + IR
AICAR PC + IR


Leukocyte Adhesion
(AMPK α2 +/+)
Adherent Leukocytes (/mm²)
40
30
20
10
0
*
Sham
IR
EPC + IR
AICAR PC + IR



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AICAR PRECONDITIONING PREVENTS POSTISCHEMIC LEUKOCYTE ROLLING AND ADHESION: ROLE OF K_{ATP} CHANNELS AND HEME OXYGENASE

F. Spencer Gaskin[1], Kazuhiro Kamada[1], Mozow Yusof[1],

William Durante[1], Garrett Gross[3], and Ronald J. Korthuis[1,2]

[1] Department of Medical Pharmacology and Physiology,
[2] Dalton Cardiovascular Research Center,
University of Missouri-Columbia
Columbia, MO 65212

and

[3] Department of Pharmacology and Toxicology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226

Running Title: MEDIATORS OF AICAR PRECONDITIONING

Address for mailing proofs:

Ronald J. Korthuis, PhD
Department of Medical Pharmacology and Physiology
University of Missouri-Columbia
One Hospital Drive
Columbia, MO 65212

Telephone number: (573) 882-8059
Fax number: (573) 884-4276
E-mail address: korthuisr@health.missouri.edu

ABSTRACT

We previously demonstrated that eNOS activation plays a critical role as a trigger for the development of a preconditioned anti-inflammatory state that is induced by activation of 5'-adenosine monophosphate-activated protein kinase (AMPK) with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), such that postcapillary venules fail to support increased leukocyte-endothelial cell adhesive interactions (LEI) on exposure to ischemia/reperfusion (I/R) 24 hrs later. One aim of this study was to examine the role of ATP-sensitive potassium (K_{ATP}) channels and heme oxygenase as effectors or mediators of AICAR-PC. Because K_{ATP} channels are thought to play dual roles in many forms of preconditioning, by acting to trigger and mediate cardioprotection in I/R, we also sought to determine the role of K_{ATP} channels as triggers of AICAR-PC. To address these issues, C57BL/6J mice were treated with either 5-hydroxydecanoate (5-HD) or tin protoporphyrin (SnPP) (inhibit mitochondrial K_{ATP} (mK_{ATP}) channels or heme oxygenase, respectively) during I/R 24 hrs after AICAR-PC. I/R induced a marked increase in LEI relative to sham control mice, pro-adhesive responses that were prevented by AICAR-PC 24 hrs prior to I/R. The effects of AICAR-PC to prevent postischemic LEI were abolished by 5-HD or SnPP treatment during I/R though administration of an inactive protoporphyrin was ineffective. On the other hand, hemin treatment (which induces the expression of heme oxygenase) 24 hrs prior to I/R mimicked the anti-inflammatory actions of AICAR-PC. Interestingly, K_{ATP} channel inhibition (with either: glibenclamide, 5-HD, or HMR-1098) coincident with AICAR failed to

prevent its anti-adhesive actions during I/R, suggesting that these channels to not participate as triggers for AICAR-PC. Our results indicate that AMPK agonists produce an anti-inflammatory phenotype in postcapillary venules by a mK_{ATP}- and heme oxygenase-dependent mechanism during I/R.

Key Words: ischemia, reperfusion, leukocyte rolling and adhesion, AMPK, preconditioning, K_{ATP} channels, heme oxygenase

INTRODUCTION

A wide variety of diverse agents and stimuli (e.g., short bouts of ischemia, ethanol ingestion at low to moderate levels, or treatment with adenosine receptor agonists, opioids, exogenous calcitonin gene-related peptide (CGRP) or bradykinin, or nitric oxide (NO) donors) have been shown to induce the development of a protective anti-inflammatory phenotype in postcapillary venules, such that these vessels fail to support adhesion molecule expression, leukocyte rolling and adhesion, and increased vascular permeability when the small bowel is subsequently exposed to prolonged I/R (32, 33, 68, 69, 121, 122, 152). Most of the agents that induce development of preconditioned states do so by a triggering mechanism that involves the formation of NO by endothelial nitric oxide synthase (eNOS).

We recently demonstrated that activators (e.g., AICAR) of the ubiquitously expressed heterotrimeric serine/threonine kinase AMP-activated protein kinase (AMPK) trigger entrance into a preconditioned anti-inflammatory phenotype by an eNOS-dependent mechanism. Interestingly, the glucose lowering agent metformin has been shown to activate AMPK and prevent the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α (53). This study also demonstrated that adhesion molecule expression was reduced by metformin or AICAR administration, a protective effect that was abrogated by the addition of a siRNA directed towards AMPKα1. These activities have previously been attributed to eNOS by others (10, 148). However, little is known about the effector mechanisms that are activated during I/R 24 hrs after

AMPK-dependent eNOS activation to mediate the reductions in postischemic leukocyte rolling and adhesion.

Many of the different preconditioning stimuli that have been studied to date such as short bouts of ischemia, ingestion of ethanol at low to moderate levels, or treatment with adenosine receptor agonists, opioids, or NO donors appear to induce protection by K_{ATP} channel-dependent mechanisms. Most of this work is consistent with the concept that such preconditioning stimuli trigger entrance into a preconditioned state by activating surface (plasmalemmal) K_{ATP} (sK_{ATP}) channels while mitochondrial K_{ATP} (mK_{ATP}) channels serve as effectors that mediate protection during I/R 24 hrs after the preconditioning stimulus was applied (101, 102). In light of these observations, we examined the role of K_{ATP} channels as triggers versus effectors in AICAR-PC as the first goal of this study. Additionally, because the catalytic activity of heme oxygenase produces metabolites that exhibit powerful anti-adhesive and antioxidant effects, we hypothesized that this heat shock protein may serve as an end-effector of AICAR-PC that mediates the anti-adhesive actions of AICAR-PC during I/R 24 hrs later.

MATERIALS AND METHODS

Animals: Wild-type male C57BL/6J mice (6-7 weeks of age) were obtained from the Jackson Laboratories (Bar Harbor, ME). All mice were maintained on standard mouse chow and water ad libitum with 12 hr light and dark cycles, and used at 8-10 weeks of age. The experimental procedures described have been described previously (69), and were performed according to the criteria outlined in the National Institutes of Health guidelines and were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee.

AICAR Preconditioning, Surgical Procedures, and Induction of I/R: Mice were preconditioned with the AMPK agonist 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, Sigma, St. Louis, MO, USA) (100mg/kg, 0.5 ml) by intraperitoneal injection 24 hrs prior to induction of I/R in C57BL/6J mice. Mice were subsequently anesthetized initially with a mixture of ketamine (150 mg/kg body wt, intramuscular injection (i.m.)) and xylazine (7.5 mg/kg body wt, i.m.). After attaining a surgical plane of anesthesia, a midline abdominal incision was performed and the superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 45 min. After these procedures, the right carotid artery was cannulated and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould) connected to the carotid artery catheter. Systemic blood pressure was recorded continuously with a personal computer (Power Macintosh 8600; Apple) equipped with an analog-to-digital

converter (MP 100; Biopac Systems). The left jugular vein was cannulated for administration of carboxyfluorescein diacetate, succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR, USA), a fluorescent dye that labels leukocytes. CFDASE was dissolved in DMSO at a concentration of 5 mg/ml, divided into 25 µl aliquots, and stored in light-tight containers at -20 °C until use. During the preparation and storage of CFDASE, care was taken to minimize light exposure. After the 45 min ischemic period, the clip was gently removed and leukocytes were labeled with CFDASE by intravenous administration of the fluorochrome solution (250 µg/ml saline) at a rate of 20 µl/min, for 5 min. The sham group had an equivalent 45 min period without occlusion of the SMA prior to CFDASE administration. Leukocyte/endothelial cell adhesive interactions were observed over min 30-40 and 60-70 of reperfusion via intravital fluorescence microscopy.

Intravital Fluorescence Microscopy: The mice were positioned on a 20 x 30 cm Plexiglas™ board in a manner that allowed a selected section of small intestine to be exteriorized and placed carefully and gently over a glass slide covering a 4 x 3 cm hole centered in the Plexiglas™. The exposed small intestine was superfused with warmed (37 °C) bicarbonate-buffered saline (BBS, pH 7.4) at 1.5 ml/min using a peristaltic pump (Model M312; Gilson). The exteriorized region of the small bowel was covered with BBS-soaked gauze to minimize tissue dehydration, temperature changes, and the influence of respiratory movements. The superfusate was maintained at 37 ± 0.5 °C by pumping the solution through a heat exchanger warmed by a constant

temperature circulator (Model 1130; VWR). Body temperature of the mouse was maintained between 36.5 and 37.5 °C by use of a thermostatically controlled heat lamp. The Plexiglas^{TM} board was mounted on the stage of an inverted microscope (Diaphot TMD-EF; Nikon) and the intestinal microcirculation was observed through a 20x objective lens. Fluorescence images of the microcirculation (excitation wavelength, 420-490 nm; emission wavelength, 520 nm) were detected with a charge-coupled device (CCD) camera (XC-77; Hamamatsu Photonics), a CCD camera control unit (C2400; Hamamatsu Photonics) and an intensifier head (M4314; Hamamatsu Photonics) attached to the camera. Microfluorographs were projected on a television monitor (PVM-1953MD; Sony) and recorded on DVD using a DVD video recorder (DMR-E50; Panasonic) for off-line quantification of measured variables during playback of the recorded image. A video time-date generator (WJ810; Panasonic) displayed the stopwatch function onto the monitor.

The intravital microscopic measurements described below were obtained over min 30-40 and 60-70 of reperfusion or at equivalent time points in the sham control groups, as described below. The intestinal segment was scanned from the oral to aboral section and 10 single, unbranched venules (20-50 μm in diameter, 100 μm in length) were observed for at least 30 sec. Leukocyte-endothelial cell interactions (the numbers of rolling and firmly adherent leukocytes) were quantified in each of the 10 venules, followed by calculation of the mean value, which was used in the statistical analysis of the data. Circulating leukocytes were considered to be firmly adherent if they did not move

or detach from the venular wall for at least 30 sec. Rolling cells are defined as cells crossing an imaginary line in the microvessel at a velocity that is significantly lower than centerline velocity; their numbers were expressed as rolling cells per minute. The numbers of rolling or adherent leukocytes were normalized by expressing each as the number of cells per mm^2 vessel area.

Experimental Protocol: Figure 9 illustrates the general design of the experimental protocols for the study. Six animals were used in each group, with all protocols being conducted in wild-type C57BL/6J mice. Drug doses were selected based on previous experiments in our laboratory and reports in the literature (20, 38, 42, 46, 104, 122).

Group 1: Sham. As a time control for the effects of experimental duration, mice in this group received an intraperitoneal (i.p.) injection of 0.5 ml saline, which was used as a vehicle for AICAR and other pharmacologic agents in groups outlined below. Twenty-four hrs later, the superior mesenteric artery was exposed but not subjected to occlusion, with leukocyte/endothelial cell adhesive interactions quantified at time points comparable to those described for mice subjected to 45 min of intestinal ischemia followed by 70 min reperfusion (Group 2, below).

Group 2: I/R alone. Mice in this group were treated as described for Group 1 above except that I/R was induced 24 hrs after the i.p. injection of saline vehicle on Day 1. Leukocyte rolling and adhesion were quantified during min 30-40 and 60-70 of reperfusion following 45 min of ischemia on Day 2.

Group 3: Glibenclamide + AICAR-PC + I/R. To determine if AMPK activation with AICAR-PC requires K_{ATP} channels, the non-specific K_{ATP} channel inhibitor Glibenclamide was given prior to AICAR-PC. Mice were treated with Glibenclamide (Sigma, St. Louis, MO, USA) (6 mg/kg, 0.3 ml, i.p. injection) on Day 1, 10 min prior to AICAR-PC (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 4: 5-HD + AICAR-PC + I/R. To determine if AMPK activation with AICAR-PC requires mK_{ATP} channels as an initiator on Day 1, the specific mK_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD) was given prior to AICAR-PC. Mice were treated with 5-HD (Sigma, St. Louis, MO, USA) (10 mg/kg, 0.3 ml, i.p. injection) on Day 1, 10 min prior to AICAR-PC (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 5: HMR-1098 + AICAR-PC + I/R. To determine if AMPK activation with AICAR-PC requires sK_{ATP} channels as an initiator on Day 1, the specific sK_{ATP} channel inhibitor HMR-1098 was given prior to AICAR-PC. Mice were treated with HMR-1098 (Garrett Gross, Milwaukee, WI, USA) (6 mg/kg, 0.3 ml, i.p. injection) on Day 1, 10 min prior to AICAR-PC (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 6: AICAR-PC + 5-HD + I/R. To determine if AMPK activation with AICAR-PC requires mK_{ATP} channels as an effector on Day 2, the specific mK_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD) was given just prior to I/R. Mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection) on Day 1. Twenty-four hrs later (Day 2), 5-HD (Sigma, St. Louis, MO, USA) (10 mg/kg, 0.3 ml, i.p. injection) was administered to mice 10 min prior to the induction of I/R and leukocyte rolling and adhesion were quantified as described above.

Group 7: AICAR-PC + HMR-1098 + I/R. To determine if AMPK activation with AICAR-PC requires sK_{ATP} channels as an effector on Day 2, the specific sK_{ATP} channel inhibitor HMR-1098 was given just prior to I/R. Mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection) on Day 1. Twenty-four hrs later (Day 2), HMR-1098 (Garrett Gross, Milwaukee, WI, USA) (6 mg/kg, 0.3 ml, i.p. injection) was administered to mice 10 min prior to the induction of I/R and leukocyte rolling and adhesion were quantified as described above.

Group 8: SnPP + AICAR-PC + I/R. To determine if AMPK activation with AICAR-PC requires heme oxygenase (HO) as an initiator on Day 1, the specific HO inhibitor tin-protoporphyrin-IX (SnPP) was given prior to AICAR-PC. Mice were treated with SnPP (Porphyrin Products, Logan, UT, USA) (50 mg/kg, 0.3 ml, i.p. injection, as described by Tulis et al. (136)) on Day 1, 10 min prior to AICAR-PC (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four

hrs later (Day 2), the intestine was exposed to I/R and leukocyte rolling and adhesion were quantified as described above.

Group 9: AICAR-PC + SnPP + I/R. To determine if AMPK activation with AICAR-PC requires heme oxygenase (HO) as an effector on Day 2, the specific HO inhibitor tin-protoporphyrin-IX (SnPP) was given prior to I/R. On Day 1, mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four hrs later (Day 2), SnPP (Porphyrin Products, Logan, UT, USA) (50 mg/kg, 0.3 ml, i.p. injection, as described by Tulis et al. (136)) was administered 1 hr prior to I/R, followed by leukocyte rolling and adhesion quantification as described above.

Group 10: AICAR-PC + CuPP + I/R. To control for any non-specific effects of protoporphyrins, the inactive copper protoporphyrin-IX (CuPP) was used on Day 2, 24 hrs after AICAR-PC. On Day 1, mice were treated with AICAR (Sigma, St. Louis, MO, USA) (100 mg/kg, 0.5 ml, i.p. injection). Twenty-four hrs later (Day 2), CuPP (Porphyrin Products, Logan, UT, USA) (50 mg/kg, 0.3 ml, i.p. injection, as described by Tulis et al. (136)) was administered 1 hr prior to I/R, followed by leukocyte rolling and adhesion quantification as described above.

Group 11: Hemin-PC + I/R. To determine if HO-1 induction with Hemin-PC is a sufficient stimulus to cause late phase protection against I/R, mice were treated with Hemin (Sigma, St. Louis, MO, USA) (50 mg/kg, 0.5 ml, i.p. injection) on Day 1. Twenty-four hrs later (Day 2), intestinal I/R was induced and leukocyte rolling and adhesion were quantified as described above.

Statistical Analysis

The data were analyzed with standard statistical analysis, i.e., ANOVA with Scheffe's (post hoc) test for multiple comparisons. All values are expressed as means ± SEM. Statistical significances were defined at $P < 0.05$.

RESULTS

Figure 10 illustrates the effects of coincident administration of AICAR with the various K_{ATP} channel inhibitors 24 hrs prior to I/R on postischemic leukocyte rolling and adhesion. I/R markedly increased leukocyte rolling (Figure 10A) and adhesion (Figure 10B) compared to sham (no ischemia) control mice. As we demonstrated previously (42), AICAR-PC completely prevented the postischemic increases in leukocyte rolling and adhesion. When administered coincident with AICAR, none of the K_{ATP} channel antagonists exerted significant effects on postischemic leukocyte rolling or adhesion, although there was a tendency for increased leukocyte rolling. These results suggest that in contrast to most other preconditioning stimuli, AICAR-PC is not triggered by a K_{ATP} channel-dependent mechanism. On the other hand, treatment with the mK_{ATP} channel inhibitor 5-HD during I/R 24 hrs after AICAR-PC abrogated the anti-adhesive effects of this AMPK agonist to prevent leukocyte rolling and adhesion, indicating that mK_{ATP} channels may serve as a mediator of these anti-inflammatory effects.

The data depicted in Figure 11 show the effects of heme oxygenase inhibition during I/R on AICAR-PC and demonstrate that the anti-adhesive actions of antecedent AICAR can be mimicked by preconditioning with hemin, a heme oxygenase inducer. Treatment with SnPP, a potent inhibitor of heme oxygenase, completely abolished the effects of antecedent AICAR to prevent postischemic leukocyte rolling and adhesion. Treatment with CuPP, a protoporphyrin that exhibits no inhibitory activity towards heme oxygenase, was ineffective in preventing the anti-adhesive effects of AICAR-PC. Interestingly,

preconditioning with hemin, in lieu of AICAR effectively reduced postischemic leukocyte rolling and adhesion. These observations suggest that heme oxygenase is an important end-effector of AICAR-PC.

DISCUSSION

AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric serine/threonine kinase that is composed of an alpha, a beta, and a gamma subunit (3, 155). The alpha subunit, which exists as either the α1 or α2 isoform, is responsible for the catalytic activity of the enzyme. The regulatory beta and gamma subunits occur in the β1 or β2 and the γ1, γ2, or γ3 isoforms. AMPK is often referred to as a metabolic "master switch" due to its high sensitivity to changes in the AMP:ATP ratios and its centralized role in both short and long term metabolic signalling pathways (17, 21, 51, 67). When cellular energy levels decrease as ATP is converted to AMP in response to stressful stimuli such as exercise or hypoxia, AMPK is activated by binding AMP via its gamma subunit (67, 155). The ability of AMPK activation to protect cellular energy levels and maintain the integrity of the mitochondrial membrane potential makes it an essential pro-survival signalling mediator in protecting cells and tissues from I/R injury (60).

We recently demonstrated that an anti-inflammatory state could be induced by preconditioning with the AMPK activator, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) (42, 43), such that postcapillary venules fail to support increased leukocyte rolling and adhesion during I/R 24 hrs later. AICAR is first metabolized to ZMP, an AMP analogue, which initially activates AMPK by binding to its gamma subunit (112). This allosteric activation increases both AMPK activity and its affinity for upstream AMPK kinases, which in turn, phosphorylate AMPK at threonine-172 on the activation loop of the alpha

subunit to further activate the enzyme. This latter process is responsible for the majority of AMPK activity, and is known to be catalyzed by at least two kinases: LKB1 and Ca^{2+}-calmodulin-dependent kinase kinase β (58, 120, 125, 143, 144). The binding of AMP or ZMP also increase AMPK activity by increasing the duration of the active state, which is accomplished by decreasing the affinity of phosphorylated AMPK for deactivating phosphatases such as protein phosphatase 2C (30).

Our earlier work indicated that AICAR-PC involves both the AMPK α1 and AMPK α2 isoforms and is triggered by eNOS activation (42, 43). However, little is known about the effector mechanisms that are activated during I/R 24 hrs after exposure to this preconditioning stimulus. The fact that mK_{ATP} channels have been implicated as an important mediator for most forms of preconditioning while treatment with K_{ATP} channel agonists prevent leukocyte infiltration when administered during reperfusion after prolonged ischemia (13, 89, 90, 137, 142) led us to first evaluate their role as an effector of AICAR-PC. Administration of the mK_{ATP} channel inhibitor 5-HD during I/R 24 hr after AICAR-PC effectively abolished the anti-adhesive effects that are induced by this preconditioning stimulus.

Because strong evidence exists to support the notion that K_{ATP} channels play a dual role in many forms of preconditioning, with sK_{ATP} channels subserving a trigger function in the initiation of a protected state while mK_{ATP} channels serve as a mediator during I/R 24 hrs later, we also evaluated their role as instigators for AICAR-PC. However, coincident treatment with a variety of K_{ATP} channel

inhibitors together with AICAR failed to prevent postischemic leukocyte adhesion and exerted a modest and statistically insignificant effect on I/R-induced leukocyte rolling. Our results are consistent with the observation that AICAR fails to directly modify K_{ATP} channel activity at the doses used in our study and inhibits K_{ATP} channel activity at higher doses (141). Thus, a unique finding of the present study is the apparent lack of involvement of a K_{ATP} channel-dependent triggering mechanism for AICAR-PC.

Heme oxygenase (HO) is a ubiquitously expressed protein that catalyzes the oxidative degradation of protoheme IX into equimolar quantities of biliverdin, divalent iron, and carbon monoxide (CO) (71). Biliverdin is further metabolized to bilirubin, a powerful endogenous antioxidant, by the action of biliverdin reductase (134). Three isoforms of the enzyme, HO-1, HO-2, and HO-3, have been described (87). HO-3 appears to exhibit lower activity and is less well characterized than HO-1 and HO-2. HO-2 is a constitutively expressed and non-inducible gene product. On the other hand, HO-1 is an inducible enzyme which is regarded as a heat shock protein (HSP32).

We hypothesized that heme oxygenase might serve as an effector of AICAR-PC for the following reasons. First, heme oxygenase activity is exquisitely sensitive to upregulation by NO donors (80) and NO appears to play an important role in initiating the effects of AICAR-PC (42). Second, NO induces the expression HO-1 mRNA by a mechanism that involves transcription factor NFκB (84), which is also inhibited by AMPK (53). Third, cAMP, a downstream signaling molecule that is produced in response to adenosine A_2-receptor

activation, increases HO-1 mRNA, protein, and activity (52), and produces a preconditioned phenotype in the small intestine (74). Adenosine A_2 receptor activation is another important trigger for AICAR-PC in the heart (27). Fourth, induction of HO-1 suppresses P-selectin expression and leukocyte adhesion induced by hydrogen peroxide or ischemia/reperfusion in the small intestine (54, 138), inflammatory processes which are also prevented by AICAR-PC. Fifth, HO-1-derived CO also inhibits the expression of proinflammatory cytokines, as does AMPK activation (44, 126). Sixth, hemin-induced HO-1 expression exerts infarct-sparing effects in the setting of myocardial I/R (50) while the protective effects of IPC against I/R could be inhibited by pharmacologic inhibition of HO-1 with zinc protoporphyrin (ZnPP) or siRNA (61). Finally, and perhaps most importantly, the reaction products of HO-1-catalyzed heme degradation exert powerful anti-adhesive and antioxidant effects (87, 126, 134). Moreover, HO-1 activity appears to be particularly rich in postcapillary venules of the small intestine (54). In light of these considerations, we sought to determine whether treatment with SnPP, an HO inhibitor, would prevent AICAR-PC, which it did. Importantly, administration of Cu-PP, which demonstrates no inhibitory activity towards heme oxygenase, failed to prevent the anti-inflammatory actions of antecedent AICAR. This latter result indicates that the non-specific effects due the protophorphyrin moiety do not account for the ability of SnPP to prevent AICAR-PC. Finally, treatment with hemin, an agent known to induce the expression of HO-1, mimicked the anti-adhesive actions of antecedent AICAR and prevented postischemic leukocyte rolling and adhesion. While our studies

do not shed light on the heme oxygenase isoform involved in AICAR-PC, it is likely that induced expression of HO-1 plays a major role.

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Kazuhiro Kamada has returned to Japan, and is currently with the Department of Inflammation and Immunology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto 602-8566, Japan. This work was supported by grants from the National Institutes of Health (DK-43785, AA-14945, and HL-73414).

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Figure Legends

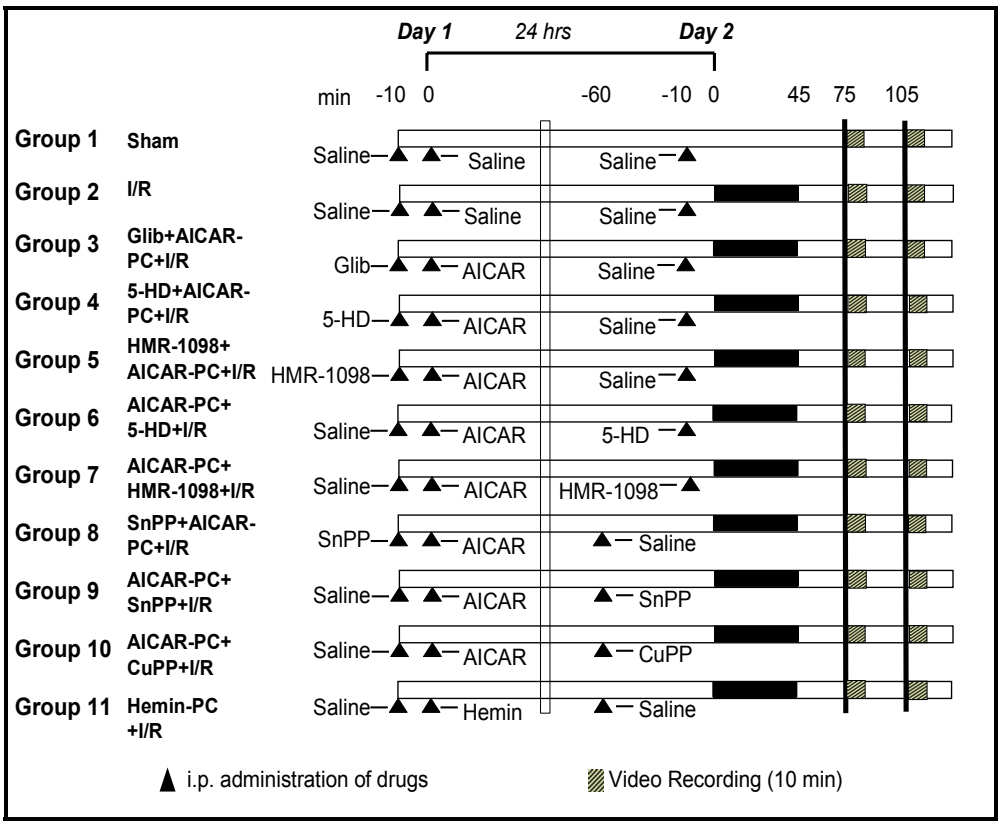
Figure 9: Schematic illustration of the experimental protocols assigned to each group. The numbers at the top of the diagram refer to min in the time line for the protocol on Day 1 and Day 2 (24 hrs between both 0s). Hatched bars indicate when the 10 min video recordings were obtained in the protocol. Solid bars depict the 45 min period of ischemia. Triangles illustrate when administration of saline vehicle or drugs was accomplished in the protocol timeline. I/R = ischemia and reperfusion. See the text for further details.

Figure 10A and 10B: Role of K_{ATP} channels in preconditioning with AICAR (AICAR-PC) 24 hrs prior to ischemia/reperfusion on postischemic leukocyte rolling (Panel A) and adhesion (Panel B) determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. The non-specific K_{ATP} channel inhibitor Glibenclamide, as well as the surface specific K_{ATP} channel inhibitor HMR-1098 and the mitochondrial K_{ATP} channel specific inhibitor 5-HD were administered either 10 min prior to preconditioning with AICAR or 10 min prior to I/R. Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion, respectively. * denotes values statistically different from control ($p<.05$).

Figure 11A and 11B: Role of HO in preconditioning with AICAR (AICAR-PC) 24 hrs prior to ischemia/reperfusion on postischemic leukocyte rolling (Panel

A) and adhesion (Panel B) determined after 30 and 60 min of reperfusion in wild-type C57BL/6J mice. The HO inhibitor SnPP, or the inactive protoporphyrin CuPP, was administered 1 hr prior to I/R on Day 2, 24 hrs after preconditioning with AICAR on Day 1. The HO-1 inducer Hemin as given on Day 1 in place of AICAR-PC to examine their ability to induce a protective phenotype during I/R 24 hrs later on Day 2. Open and solid bars represent data obtained at min 30–40 and 60–70 of reperfusion, respectively. * denotes values statistically different from control ($p<.05$).

FIGURES



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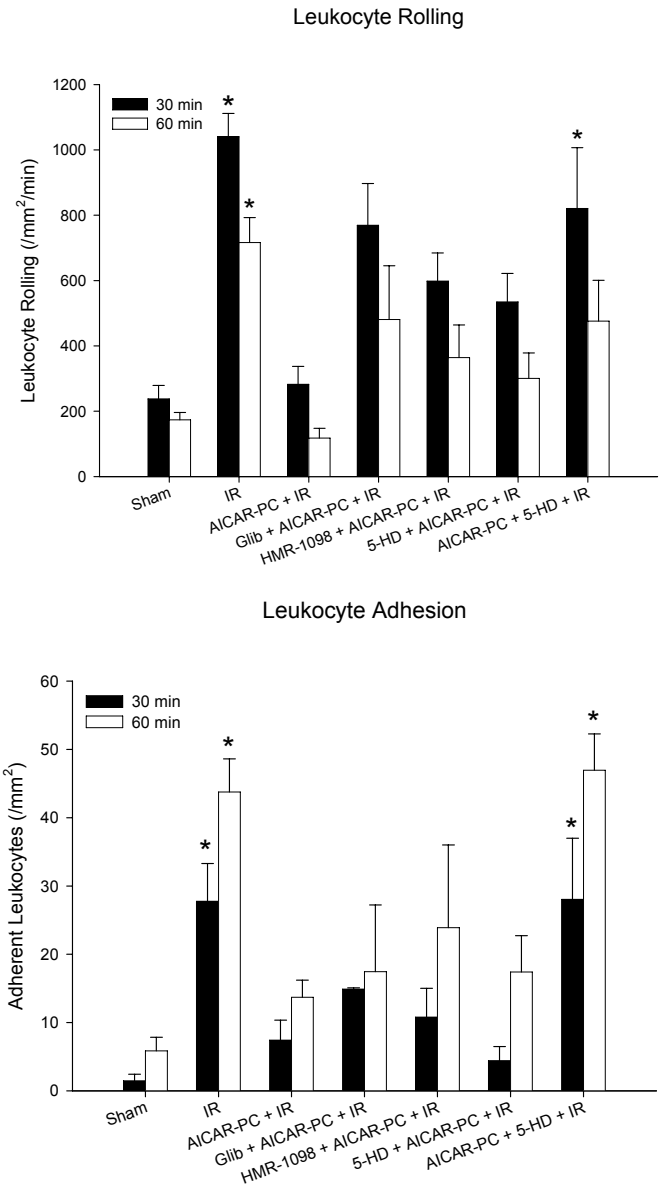

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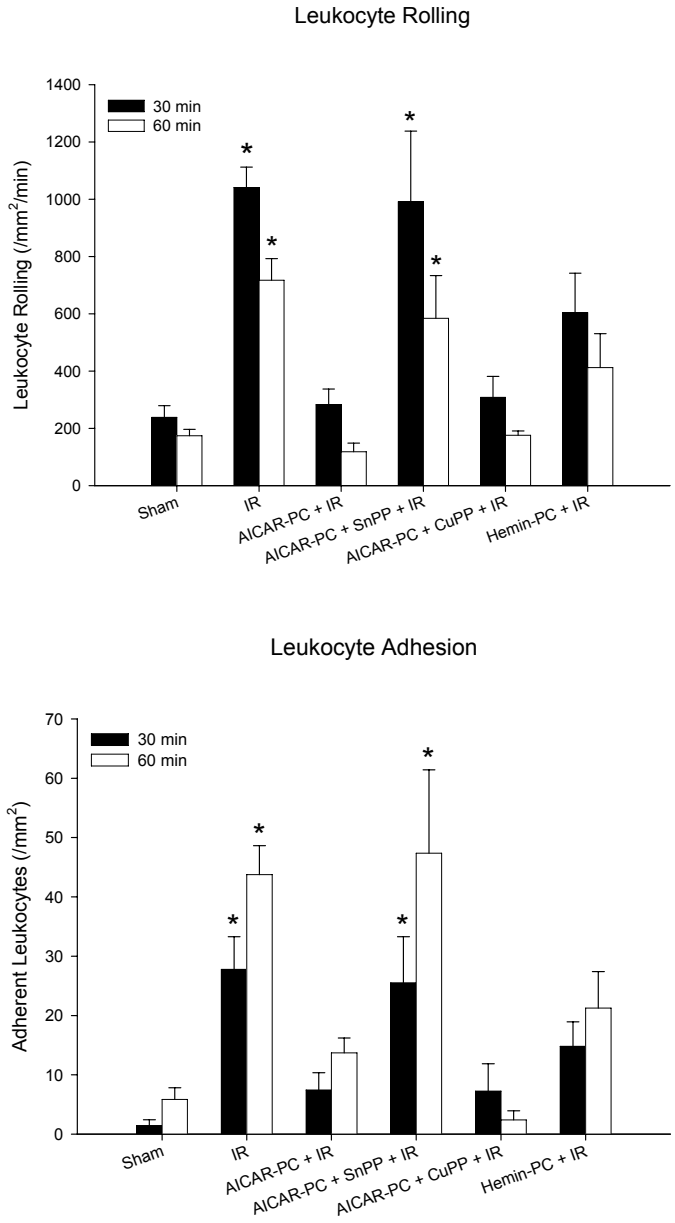

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SUMMARY

The search for a means to prevent, or at least significantly attenuate, ischemia/reperfusion (IR) injury has been the focus of intense investigation for many years and has resulted in a burgeoning body of literature. A major breakthrough in this growing field was the discovery in 1986 by Murry et al. (94) of ischemic preconditioning (IPC), which demonstrated the first significantly effective means to reduce I/R-induced cellular dysfunction and tissue injury apart from reducing the duration of ischemia. Since that time IPC has been shown to be effective in every tissue and animal model evaluated. Additionally, there is an emerging body of evidence indicating that this highly conserved form of preconditioning is operant in humans. As a consequence the elucidation of the mechanisms that activate this innate protective mechanism and its effectors has enormous therapeutic potential. However, induction of IPC in patients can be difficult and may pose significant risks that limit its clinical utility. In addition, while IPC is effective in healthy young animals, it is less effective or absent in many animal models with co-existing risk factors such as diabetes and hypertension. Thus, this work has spurred investigation into alternative means of activating the protective mechanisms elicited by IPC that demonstrate effectiveness in the presence of risk factors and do not require the application of invasive approaches. As research into the mechanisms of IPC was occurring independently, evidence was accumulating to suggest that low-to-moderate ethanol consumption reduced the risk of cardiovascular events,

reminiscent of a preconditioned phenotype, and was effective in humans at risk for ischemic syndromes.

Support for this hypothesis initially came from epidemiological data suggesting a role for alcohol consumption in cardioprotection, a phenomenon known as the French Paradox (6, 26, 41, 108). Consumption of wine in France was suggested to underlie the paradoxically low ischemic heart disease in a population that has a high dietary fat intake (26, 105). Subsequent work in animals confirmed the cardioprotective effects of ethanol. The first experimental evidence that ethanol may limit I/R injury was gathered by Kobayashi et al. (73). In these experiments addition of low to moderate ethanol concentrations to the perfusion media during an I/R protocol in isolated rat hearts reduced cell injury compared to controls. In a later study by Chen et al. (22), brief exposure to low ethanol levels immediately prior to I/R was shown to protect both isolated cardiac myocytes and Langendorff-perfused hearts from adult rats. Although these studies demonstrated potential infarct-sparing effects of ethanol, Miyamae et al. (88), were the first to demonstrate that prolonged ethanol ingestion in an animal model was cardioprotective in a setting akin to IPC. That is, guinea pigs ingested ethanol for three to twelve weeks, and eighteen hrs prior to induction of I/R, ethanol was withdrawn. Subsequent exposure to myocardial I/R resulted in significant reductions in myocardial infarct size in ethanol treated versus sham controls, a cardioprotective effect that was comparable to that induced by IPC. Later work conducted by this same group provided evidence that prolonged ethanol exposure induced preconditioning by mechanisms comparable to

acute phase ischemic preconditioning in involving adenosine A_1 receptor activation, translocation of novel protein kinase C isoforms, and stimulation of K_{ATP} channels.

More recently, EPC has been shown to have a temporally biphasic response in humans and other animal models wherein consumption of the equivalent of one to two alcoholic beverages was shown to be protective (68, 86, 109, 153, 154). This latter body of work demonstrated that entrance into a preconditioned protective phenotype was initiated by formation of reactive oxygen species (ROS) and adenosine A_2 receptor-dependent eNOS activation. It has been proposed that eNOS-derived NO interacts with superoxide generated from NADPH oxidase and xanthine oxidase to produce reactive nitrogen oxide species that trigger the release of calcitonin-gene related peptide (CGRP) (7, 9, 13, 57, 69, 101, 154). Preconditioning with ethanol (EPC) provides several advantages over IPC and the elucidation of its mechanisms may yield targets for intervention with greater therapeutic potential. These benefits include: a means of inducing a protective mechanism like IPC without the need for the prohibitively hazardous tissue or organ ischemia, the relative immunity of EPC in the presence of risk factors such as aging or diabetes, and a greater magnitude of late phase preconditioning protection (68, 103, 106, 123, 124). These results suggest that EPC is a superior means of inducing protection against I/R than IPC.

Despite these advantages EPC is not without peril. Aside from social stigmas and practices that may prevent alcohol consumption, alcohol has inimical health consequences and addictive properties that may reduce its enthusiasm for use. However, elucidation of the biochemical events that serve to trigger entrance into

EPC, serve as downstream mediators and effectors of the preconditioned phenotype might be exploited to develop therapeutic interventions that mimic the protective responses attributed to ethanol ingestion but lack its negative psychosocial stigmas and pathologic side effects.

One potential target that may be involved in ethanol preconditioning is adenosine monophosphate-activated protein kinase (AMPK). AMPK is composed of an alpha, a beta, and a gamma subunit (3, 155). The alpha subunit, which exists as either the α1 or α2 isoform, is responsible for the catalytic activity of the enzyme. The regulatory beta and gamma subunits are expressed as the β1 or β2 and the γ1, γ2, or γ3 isoforms. AMPK is a central regulator of metabolism that is activated in times of stress such as exercise, hypoxia, and ischemia, when ATP levels decrease resulting in an increase in AMP:ATP ratio, and acts to inhibit ATP consuming processes while activating ATP producing pathways (85). This notion is furthered by work demonstrating that ethanol exposure leads to increased AMP:ATP ratios and an increased AMPK phosphorylation and activity as measured by the phosphorylation state of AMPK and its downstream targets such as acetyl coenzyme A carboxylase (ACC) (65). Additionally, AMPK activation, as determined by phosphorylation at Thr172, has been shown to increase in rats fed a caloric restricted diet. A finding which corresponds to the improved left ventricular recovery following I/R in young and aged animals, suggesting that AMPK activation may be cardioprotective (124).

Although it has not been previously shown, several other lines of evidence implicate a role for AMPK in EPC. Like EPC, AMPK activation has been shown

to increase endothelial nitric oxide synthase (eNOS) phosphorylation at Ser1177 and its activity (24, 55, 91, 150, 151). The phosphorylation and activation of eNOS has been shown to have a salubrious effect, whereas a decrease in eNOS activity leads to the pathology of I/R by increasing leukocyte infiltration and decreasing vasodilatation (70, 79, 95). IPC has also been shown to involve an eNOS-dependent mechanism in numerous studies such as reported in the recent study by Xuan et al. (150) who showed that late phase IPC was ineffective at reducing infarct size in eNOS deficient mice. Based on the literature and findings in our lab, we decided to test our hypothesis for a role of AMPK in EPC and the role of eNOS.

A direct role for AMPK in preconditioning is suggested by our first study showing that pharmacologic activation of AMPK with 5-aminoimidazole-4-carboxamide 1-beta-d-furanoside (AICAR) 24 hrs prior to I/R (AICAR-PC) (42) was an effective preconditioning stimulus, preventing the increased LEI in response to I/R. Our results also show a differential protective effect of AICAR-PC on leukocyte rolling and adhesion (LR and LA), where LR but not LA was dependent on NOS, and in particular eNOS. This was demonstrated by pharmacologic inhibition of NOS with N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) prior to AICAR-PC which blocked the protective effects of AICAR-PC against leukocyte rolling, an effect that was also absent in eNOS deficient mice, although there was not a significant effect on LA. Thus the protective effect of AICAR-PC on LA appears to occur via an eNOS-independent mechanism, an interesting phenomenon that deviates from the tendentious view

that LR and LA are causally linked, and merits further examination. We hypothesize that the aberration from the canonical observations may be due to differential effects on AICAR-PC on adhesion molecule expression. Perhaps AICAR-PC suppresses P-selectin in eNOS-dependent manner, whereas its effects on intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) occur via an eNOS-independent mechanism allowing them to still function in the presence of NOS inhibition or in eNOS deficient mice to reduce LA.

Our studies involving EPC and AMPK have shown that AMPK activation is in fact required for the beneficial reduction in LR and LA. To test this hypothesis, we evaluated the effects of pharmacologic AMPK inhibition with compound C coincident with ethanol ingestion on leukocyte rolling and adhesion during I/R 24 hrs after treatment/ingestion. Compound C completely abrogated the ability of antecedent ethanol to prevent the increased leukocyte rolling and adhesion induced by I/R 24 hrs later. A third line of evidence supporting the notion that antecedent ethanol triggers entrance into a preconditioned state by an AMPK-dependent mechanism was provided by our studies conducted in AMPK knockout animals. Ethanol ingestion failed to elicit an anti-inflammatory phenotype in AMPK α2-/- mice but remained effective as a preconditioning stimulus in mice genetically deficient in AMPK α1. These results substantiate the earlier finding that IPC has been shown to activate myocardial AMPK, and hearts from transgenic mice lacking the catalytic alpha 2 subunit of AMPK could not be preconditioned (128). These latter observations suggest that ethanol

differentially activates AMPK in an α-subunit-dependent manner. However, the mechanism for this selective effect remains obscure and should be the focus of future investigation. Likely possibilities include differential ethanol binding in water-filled pockets of the different AMPK α subunits, inhibition of select protein phosphatases that target specific phosphorylated sites in AMPK α1 versus AMPK α2, and/or unique alterations in upstream kinases that allow singular activation of the different isoforms. This study also substantiated our earlier findings (42), again demonstrated that preconditioning with the AMPK activator AICAR produces an anti-inflammatory phenotype in postcapillary venules in tissues subsequently exposed to I/R 24 hrs later, preventing postischemic leukocyte rolling and adhesion.

As we observed with EPC, the anti-adhesive effects of antecedent AICAR treatment were completely prevented by AMPK inhibition with compound C. However, in contrast to a selective role of AMPK α2 in ethanol preconditioning, the ability of AICAR to prevent postischemic LEI was absent in mice genetically deficient in either the AMPK α1 or AMPK α2 isoforms. These observations suggest that both isoforms participate in the development of the preconditioned phenotype in response to antecedent AICAR, but that the absence of either is sufficient to prevent the anti-adhesive effects of preconditioning with this agent.

It is interesting to note that initial studies with the putative AMPK inhibitor adenine 9-β-D-arabinofuranoside (Ara-A) failed to prevent the anti-adhesive effects of preconditioning with ethanol while treatment with another structurally unrelated AMPK inhibitor, compound C, was effective in this regard. Much of the

previous work regarding the role of AMPK in physiologic processes has relied on data obtained using Ara-A as an inhibitor of the enzyme. Although this agent inhibits AICAR-induced AMPK activity in a variety of tissues (1, 20, 23, 72) it does seem to be more effective at blocking the α1 isoform in response to AICAR or metabolic challenge, but was without effect in contraction-induced AMPK activation of either isoform in skeletal muscle (96). In addition, Ara-A has been shown to be ineffective in inhibiting AMPK activity in vascular tissues (111). Thus, it was not surprising that Ara-A failed to prevent the anti-adhesive effects noted in postcapillary venules after ethanol preconditioning in our model, even at doses 5-10 fold greater than previously used. Interestingly, we also demonstrated that Ara-A fails to prevent AICAR-PC, which supports the notion that this agent may not effectively inhibit AMPK in vascular tissues. In view of our observations with compound C and in AMPK knockout mice, where both EPC and AICAR PC were ineffective in producing an anti-inflammatory phenotype, this conclusion seems likely.

Having established a role for AMPK in EPC, the ability of AICAR-PC to induce a protective phenotype, and the involvement of eNOS in both, our next goal was to better understand the effectors of these protective mechanisms, as little is known about the proteins activated during I/R 24 hrs after exposure to AICAR-PC. The fact that mK_{ATP} channels have been implicated as important mediators for most forms of preconditioning while treatment with K_{ATP} channel agonists prevent leukocyte infiltration when administered during reperfusion after prolonged ischemia (13, 89, 90, 137, 142) led us to first evaluate their role as an

effector of AICAR-PC. Administration of the mK_{ATP} channel inhibitor 5-HD during I/R 24 hr after AICAR-PC effectively abolished the anti-adhesive effects that are induced by this preconditioning stimulus.

Strong evidence exists to support the notion that K_{ATP} channels play a dual role in many forms of preconditioning, with sK_{ATP} channels subserving a trigger function in the initiation of a protected state while mK_{ATP} channels serve as a mediator during I/R 24 hrs later. Specifically, it has been shown that preconditioning the myocardium with short bouts of ischemia has been shown to activate AMPK, an effect that led to activation of surface (sarcolemmal or plasmalemmal) K_{ATP} (sK_{ATP}) channels and was blocked with the sK_{ATP} channel blocker HMR-1098 (128). Because of these observations we also evaluated the role of K_{ATP} channels as instigators for AICAR-PC. Coincident treatment with a variety of K_{ATP} channel inhibitors together with AICAR failed to prevent postischemic leukocyte adhesion and exerted a statistically insignificant effect on I/R-induced leukocyte rolling. Our results are consistent with the observation that AICAR fails to directly modify K_{ATP} channel activity at the doses used in our study and inhibits K_{ATP} channel activity at higher doses (141). Thus, a unique finding of the present study is the apparent lack of involvement of a K_{ATP} channel-dependent triggering mechanism for AICAR-PC.

We have also shown that heme oxygenase (HO) serves as an effector of AICAR-PC for the following reasons. First, heme oxygenase activity is exquisitely sensitive to upregulation by NO donors (80) and NO appears to play an important role in initiating the effects of AICAR-PC (42). Second, NO induces

the expression HO-1 mRNA by a mechanism that involves transcription factor NFκB (84), which is also inhibited by AMPK (53). Third, cAMP, a downstream signaling molecule that is produced in response to adenosine A_2-receptor activation, increases HO-1 mRNA, protein, and activity (52), and produces a preconditioned phenotype in the small intestine (74). Adenosine A_2 receptor activation is another important trigger for AICAR-PC in the heart (27). Fourth, induction of HO-1 suppresses P-selectin expression and leukocyte adhesion induced by hydrogen peroxide or ischemia/reperfusion in the small intestine (54, 138), inflammatory processes which are also prevented by AICAR-PC. Fifth, HO-1-derived CO also inhibits the expression of proinflammatory cytokines, as does AMPK activation (44, 126). Sixth, hemin-induced HO-1 expression exerts infarct-sparing effects in the setting of myocardial I/R (50) while the protective effects of IPC against I/R could be inhibited by pharmacologic inhibition of HO-1 with zinc protoporphyrin (ZnPP) or siRNA (61). Finally, and perhaps most importantly, the reaction products of HO-1-catalyzed heme degradation exert powerful anti-adhesive and antioxidant effects (87, 126, 134).

Moreover, HO-1 activity appears to be particularly rich in postcapillary venules of the small intestine (54). In light of these considerations, we sought to determine if treatment with SnPP, an HO inhibitor, would prevent AICAR-PC, which it did. Importantly, administration of Cu-PP, which demonstrates no inhibitory activity towards heme oxygenase, failed to prevent the anti-inflammatory actions of antecedent AICAR. This latter result indicates that the non-specific effects due the protophorphyrin moiety do not account for the ability

of SnPP to prevent AICAR-PC. Finally, treatment with hemin, an agent known to induce the expression of HO-1, mimicked the anti-adhesive actions of antecedent AICAR and prevented postischemic leukocyte rolling and adhesion. Though our studies do not definitively demonstrate which heme oxygenase isoform is involved in AICAR-PC or which cells are preconditioned, it is likely that induced expression of HO-1 in endothelial cells plays a major role. Such observations provide reasonably strong support for the notion that HO serves as an effector of AICAR-PC, but potential concerns regarding specificity of pharmacologic inhibitor studies point to the need to repeat this work in HO knockout mice and to provide biochemical evidence for HO activation and/or increased expression. Such approaches will provide important insights regarding the HO isoform involved in AICAR-PC. In this regard HO-1 is a likely candidate. The proposed future studies would provide additional credence to our data showing that HO is essential to AICAR-PC to reduce LEI in postischemic venules of the murine small intestine.

Although the precise mechanism of AMPK activation by EPC is unclear, there is evidence to support a role for ROS production as a result of the effects of ethanol on oxidative respiration in mitochondria as well as direct effects of ethanol reactivity. Ethanol has been shown to dose-dependently increase plasma malondialdehyde (MDA) and protein carbonyl levels and decrease NO, the ratio of reduced:oxidized glutathione (GSG/GSSG) and the activity of the following antioxidant enzymes: Cu/Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase (59). It has been suggested that ethanol's effect on membrane

fluidity may facilitate or impair enzyme activities, co-localization of co-factors, or interactions among signaling elements. In addition, recent work suggests that ethanol can bind to water-filled pockets in receptors and perhaps other proteins and produce conformational changes that alter their activities (66). As an example, ethanol has also been shown to increase extracellular adenosine levels by inhibiting the nucleoside transporter in plasma membranes. This limits adenosine reuptake and allows this nucleoside to reach concentrations to effectively activate adenosine A_2 receptors and induce preconditioning. There may be a calcium-dependent effect, whereby ethanol administration increases intracellular Ca^{2+} or some other stress-sensing mechanism that could in turn activate the upstream kinase LKB1 to activate AMPK. For these reasons, future work with LKB1 and CaMKK deficient mice would provide further insight into the role of these upstream kinases in EPC and AICAR-PC, and show if one or both are necessary to fully activate AMPK in addition to its activation via an increase in intercellular AMP concentration.

The studies presented here provide independent support for a role of these initiators, mediators, and effectors in preconditioning with ethanol and AICAR. Thus AMPK is involved in EPC, and can serve as a sufficient initiator of preconditioning that requires eNOS, K_{ATP} channels, and HO as illustrated in Figure 12. However, it remains unclear how these triggers, downstream mediators, and effector molecules are linked as a signaling cascade to produce EPC, which will be the next step in examining the mechanisms of EPC and AICAR-PC. This will be done with biochemical assays to validate the temporal

relationships of these mediators, as well as to confirm that the pharmacologic agents are having direct effects on their targets via immunoblot verification of phosphorylation, and the role that specific isoforms of these proteins play in the signaling pathways.

It is also essential to see which tissue or cell type is being preconditioned in these experiments. This could be determined with chimeric mice that have not been preconditioned, but have received leukocytes isolated from preconditioned mice to see if leukocytes are the preconditioned cells. And vice versa, preconditioned mice could receive a transfusion to remove leukocytes present during preconditioning, and replace them with naïve leukocytes that have not been exposed to the preconditioning stimulus. These experiments could be conducted in wild-type control and genetically deficient mice to determine if specific proteins in the leukocytes and endothelium/vascular smooth muscle cells are involved in the development of the preconditioned phenotype. This would be especially useful in mice lacking specific adhesion molecules to determine if one or several are involved in the disparate effects of AICAR-PC on LR and LA. In vitro work would also provide a means for isolating the cell type and mediators required for EPC and AICAR-PC, though several drawbacks exist to this approach. In vitro, leukocytes do not adhere to endothelial monolayers at physiologically relevant flow rates. There is also cause for concern with dedifferentiation of cultured cells, particularly if flow is absent during culture. Nonetheless, it is clear from the present results that AMPK is a valid target for pharmacologic preconditioning to protect against I/R therapeutically.

This is especially relevant in light of the demonstration that the glucose lowering agent metformin activates AMPK and prevents the signalling of inflammatory cytokines through nuclear factor kappa B and tumor necrosis factor α (53). That study also demonstrated that adhesion molecule expression was reduced by metformin or AICAR administration, a protective effect that was abrogated by the addition of a siRNA directed towards AMPK α1. These activities have been attributed to eNOS by others (10, 148). There is also evidence that AMPK can be activated by statins such as atorvastatin, which may explain the pleiotropic effects of these drugs (130). This last report is not surprising as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) was one of the first enzymes shown to be phosphorylated and inhibited by AMPK activity (18). These results are intriguing in light of the recent observation by Fisslthaler et al. (37) that fluid shear stress activates AMPK and NO production which modulate HMGR expression and activity.

The data presented within this dissertation clearly supports the hypotheses proposed in the introduction. AMPK was shown to be required for EPC, and additionally direct pharmacologic activation of AMPK with AICAR was shown to be a sufficient preconditioning stimuli. From this several downstream mediators of AICAR-PC have been identified. NO was shown to be essential in triggering the protective effect against LR on Day 1, though the effect was absent against LA. Similar results were provided by experiments in mice lacking eNOS, suggesting this effect is mediated by this specific NOS isoform. Although K_{ATP} channels were not shown to be significantly involved in triggering the protective

effect on Day 1, they did play a role. More apparent was the role of mK_{ATP} channels in mediating the protective effect on Day 2. HO is clearly a downstream effector as well, and it is likely that HO-1 is the isoform involved in this protective effect.

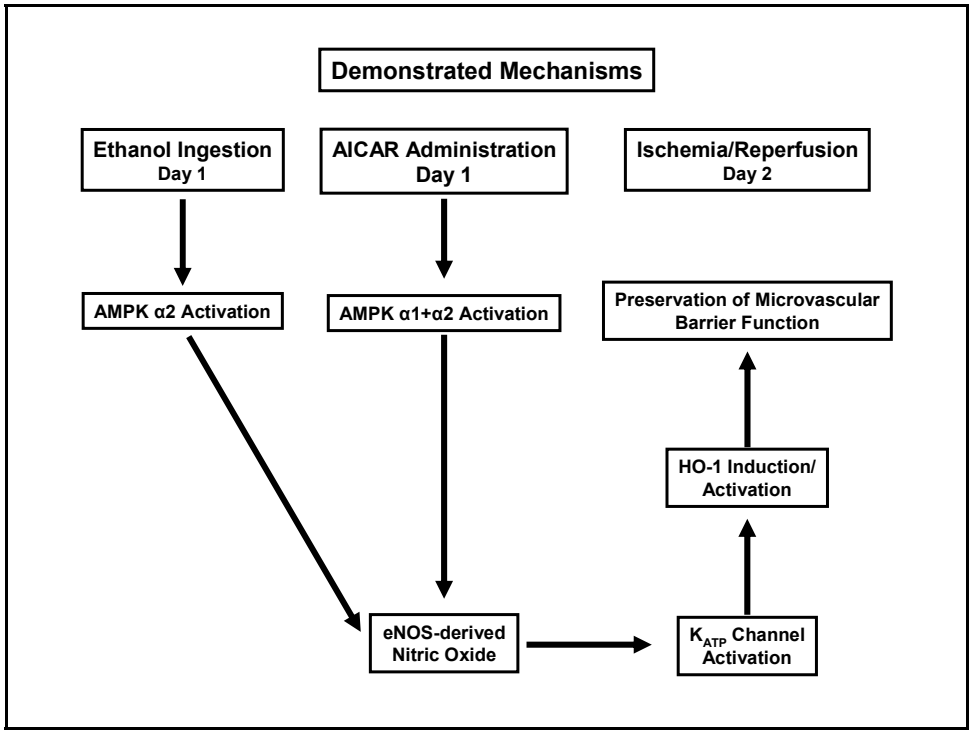

Figure 12. Proven mechanisms of EPC and AICAR-PC as demonstrated by the results of the previously discussed experiments. Though the causal relationship between some of these events is yet to be explicitly shown, the temporal relationship between events on Day 1 and Day 2 are clear.

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CURRICULUM VITAE

Frederick Spencer Gaskin

Home: 529 Huntridge Dr, Columbia, MO 65201 Phone:(314)477-7133

Office: MA415 Health Sciences Building, 1 Hospital Dr, Columbia, MO 65212

Phone: (573)882-1030 **E-mail:** fsg8wf@mizzou.edu

Research Interests

I am examining the mechanisms whereby ischemia/reperfusion induces adhesive interactions between circulating leukocytes and endothelial cells and how these adherent cells alter the functions of arterioles and venules. Intravital microscopy and isolated microvessel techniques are used to address such questions using pharmacologic approaches and a variety of mutant mouse models.

Education

BS, Biology, *St. Louis University* (2001)

John Burroughs High School, St. Louis, MO (1997)

Service Activities

Delivering food to the elderly, *Meals On Wheels*

Emergency Room Liaison, *St. Louis University Hospital*

Research Assistant, *St. Louis University Biology Department*

Professional Organizations

American Heart Association, *Member*

American Physiological Society, *Member*

American Society for Pharmacology and Experimental Therapeutics, *Member*

Microcirculatory Society, *Member*

Sigma Xi, *Member*

Society for Experimental Biology and Medicine, *Member*

Society for Neuroscience, *Member*

Student Organizations

Graduate Association of Pharmacology and Physiology Students, *President/member*

Student Chapter of ASPET, *Member*

Teaching Experience

Elements of Physiology, Fall 2003 - Fall 2004

- Pre- and Post-Laboratory Lectures
- Laboratory Setup and Clean Up
- Overseeing/assisting Experiments
- Proctoring Examinations

Physics, Fall 2000 - Winter 2001

- Pre- and Post-Laboratory Lectures
- Laboratory Setup and Clean Up

- Overseeing/assisting Experiments

- Proctoring Examinations

Grants and Fellowships

Tri-Beta Research Foundation Grant (Tri-Beta Biological Honors Society). *Funded*. January 2000

Awards

Zweifach Student Travel Award (given by Microcirculatory Society). Awarded for Research. August 2007

Molecular Biology Program Travel Award (given by Life Sciences Fellowships). Awarded for Research. May 2007

Division of Cardiovascular Pharmacology's Best Abstract Competition, runner-up (given by American Society for Pharmacology and Experimental Therapeutics). Awarded for Research. April 2007

Young Investigator Award (given by Society for Experimental Biology and Medicine). Awarded for Research. April 2007

Missouri Life Sciences Week Research Competition, second place, (given by

University of Missouri). Awarded for Research. April 2007

RCAF Health Sciences Division, third place (given by Graduate Professional Council). Awarded for Research. February 2007

Furchgott Fund Graduate Student Travel Award to the ASPET annual meeting at Experimental Biology 2006 (given by American Society for Pharmacology and Experminental Therapeutics). Awarded for Research. April 2006

Caroline tum Suden/Francis A. Hellebrandt Professional Opportunity Award (given by American Physiological Society). Awarded for Research. April 2006

Cardiovascular Day Pre-Doctoral Poster Competition, first place (given by University of Missouri). Awarded for Research. February 2006

Travel Award to the Transatlantic Microcirculatory Meeting (given by Microcirculatory Society Awards Committee). Awarded for Research. September 2005

Donald K. Anderson Graduate Teaching Assistant Award, finalist (given by University of Missouri Graduate School). Awarded for Teaching. March 2005

Presentations

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, Leona J. Rubin, and Ronald J. Korthuis *Ethanol preconditioning is dependent on the activation of 5'-AMP-activated protein kinase.* Experimental Biology. April 2007 *National/International*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, Leona J. Rubin, and Ronald J. Korthuis *Ethanol preconditioning is dependent on the activation of 5'-AMP-activated protein kinase.* Missouri Life Sciences Week. February 2007 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, Leona J. Rubin, and Ronald J. Korthuis *Ethanol preconditioning is dependent on the activation of 5'-AMP-activated protein kinase.* University of Missouri Cardiovascular Day. February 2007 *Regional*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, Leona J. Rubin, and Ronald J. Korthuis. (2007). *Ischemia/Reperfusion and Preconditioning: Role of AMPK*. RCAF Health Sciences Division, Oral Presentation. February 2007 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis

(2006). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions*. Health Sciences Research Day. November 2006 *Local*

F. Spencer Gaskin (2006). *Ischemia/Reperfusion and Preconditioning: Role of AMPK*. University of Missouri, Department of Medical Pharmacology and Physiology, Departmental Seminar. October 2006 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2006). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions*. 24th European Conference on Microcirculation. August 2006 *National/International*

F. Spencer Gaskin, Mozow Yusof, and Ronald J. Korthuis (2006). *Antecedent ethanol ingestion (EPC) prevents postischemic arteriolar endothelium-dependent dilatory (EDD) dysfunction: Role of leukocyte emigration*. Missouri Life Sciences Week. April 2006 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2006). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions: Role of ATP-sensitive potassium channels*. Experimental Biology. April 2006 *National/International*

F. Spencer Gaskin, Mozow Yusof, and Ronald J. Korthuis (2006). *Antecedent ethanol ingestion (EPC) prevents postischemic arteriolar endothelium-dependent dilatory (EDD) dysfunction: Role of leukocyte emigration*. Experimental Biology. April 2006 *National/International*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2006). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions: Role of nitric oxide*. Experimental Biology. April 2006 *National/International*

F. Spencer Gaskin, Mozow Yusof, and Ronald J. Korthuis (2006). *Antecedent ethanol ingestion (EPC) prevents postischemic arteriolar endothelium-dependent dilatory (EDD) dysfunction: Role of leukocyte emigration*. University of Missouri Cardiovascular Day. February 2006 *Regional*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2005). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions*. Health Sciences Research Day. November 2005 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2005). *5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions*. Joint Meeting of U.S. and U.K.

Microcirculatory Societies. November 2005 *National/International*

F. Spencer Gaskin (2005). *Mechanisms of Remote Preconditioning*. University of Missouri, Department of Medical Pharmacology and Physiology, Departmental Seminar. September 2005 *Local*

F. Spencer Gaskin, Kazuhiro Kamada, Mozow Yusof, and Ronald J. Korthuis (2005). *Role of 5'-AMP-activated protein kinase in the postischemic anti-inflammatory effects of antecedent ethanol ingestion*. Missouri Life Sciences Week. April 2005 *Local*

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