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Freezing Activity:

The Target To Induce Cold-Ischemic Tolerance?

'The Role of AMPK Agonists in Cold Preservation Related Organ Injury as Compared to Ischemic Preconditioning in Transplantation Medicine'

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Content list:

Abstract:
Currently, in transplantation medicine an important risk factor for long-term graft rejection is cold-ischemia derived tissue-injury, occurring during prolonged organ preservation. The main consequences of cold-ischemia are ATP-depletion, inflammation and endothelial dysfunction. In recent decades growing attention has been paid to ischemic preconditioning (IP) as a phenomenon to induce cold-ischemia tolerance in organs. By exposing organs to a brief ischemic period before the prolonged cold-ischemic period, several protecting mechanisms are induced on the cellular level. The adenosine-monophosphate kinase (AMPK) has recently been discovered to play a central role in the IP mechanism. Therefore, in this review I outlined the similarities of the IP and AMPK effector mechanisms, thereby presenting a possible common way of action, but also outlining the AMPK specific role as regulator of metabolism. As several studies reveal an energy-saving, anti-inflammatory and indirect endothelium protecting effect of AMPK, targeting this kinase could be a promising strategy in future pharmacological preconditioning. Moreover, in this review I propose the AMPK-agonists AICAR and 5'AMP as possible candidates in the protection of organs against prolonged cold-ischemic preservation.

List of abbreviations:

AICAR:	5-AminoImidazole-4-Carboxamide Ribonucleoside
5'AMP:	5'AdenosineMonophosphate
AMPK:	AMP-activated kinase
NFkB:	Nuclear Factor kappaB
NO:	Nitric Oxide
eNOS:	endothelial NO synthase
iNOS:	inducible NO synthase
IP:	Ischemic Preconditioning
PKC:	Phosphokinase C
ZMP:	5-AminoImidazole-4-Carboxamide Ribonucleotide /AICA-Monophosphate

1. Introduction

To date, organ transplantation has become common practice in the clinic as a final treatment method for organ failure in patients. Since its start at the beginning of the 20th century, the results of organ transplantation have been strongly improved and in recent decades thousands of recipients have substantially benefited from this treatment.[1] Due to –among others- (HLA) matching of donor and recipient, the hyperacute and acute graft rejection can nowadays strongly be reduced, although graft immunogenicity is not completely dependent on HLA differences between donor and recipient.[2] Moreover, allocation of donor organs has been made possible by success of organ preservation, of which a main principle is hypothermia. This cold preservation is responsible for suppressing cell metabolism and delaying acute injury provoked by oxygen-deficiency during the transplantation procedure.[2, 3]

Nowadays, chronic rejection is the most prominent factor for graft loss after transplantation and is characterized by a relative slow rate of decline in organ function[2, 4] Since in organs surviving more than five years only a 10% difference between best and worst matched HLA pairs has been found, it was recently hypothesized by Lu et al.(1995)[5] that not HLA compatibility, but tissue injury is an important determining factor for long-term allograft survival. In their study Lu et al. namely showed that the more severe the initial injury was, the greater the incidence of allograft rejection. Several sets of further observations by others support this hypothesis, for example the observation of Terasaki et al (1995)[6] that transplant survival of renal allografts from living donors is superior to that of brain death donors. These studies show that it is essential to adapt proceedings that reduce tissue injury and therefore will increase chronic graft survival.

Factors influencing tissue injury can be divided in donor-related and graft preservation-related: donor dependent factors are for example the age, previous diseases, cause of death of the donor and whether the donor is still living or brain death. Important graft-preservation related parameters are the duration of cold storage and the ischemic time of the preserved organ.[7] Although-as mentioned above-hypothermic organ preservation is common practice in transplantation of solid organs, as it has metabolic benefits, prolonged cold ischemic storage may lead to tissue damage and may thereby have adverse effects on long-term graft survival. [7, 8] Attempts should thus be undertaken to limit cold preservation related tissue injury of the graft before organ implantation.

Therefore, recent studies have been focusing on preconditioning of the donor or allograft in several ways to positively influence long-term organ survival by offering protection of the organ to the cold-ischemic period. For example ischemic preconditioning (IP) has been found to protect against prolonged cold ischemic injury. The principle of this method is exposing the organ to brief episodes of ischemia, which protects the allograft against functional damage and vascular endothelium cell death in subsequent prolonged ischemia.[9] The mechanisms of IP are complex, and involve several mediators, but it is believed that IP mainly preserves energy metabolism and reduces inflammation during cold ischemic actions.[10] This can be caused via induction of Adenosine Monophosphate Activated Protein Kinase (AMPK), which acts as a low-fuel warning system that is switched on by ATP-depletion or AMP rises, thereby preventing lactate accumulation and cell injury.[10] Furthermore, the Nitric Oxide (NO) pathway has been described as an important IP-mediator, since increase of endothelial NO synthase and NO levels was measured in several studies

conducting ischemic preconditioning experiments, positively influencing endothelium function.[11, 12] For practical reasons IP cannot easily be accomplished in transplantation medicine, and therefore pharmacological agents are being developed for treating donor or allograft, to accomplish the same beneficial effects on the consequences of cold-ischemic organ preservation. Most of these agents are directed either to prevent local immune activation (i.e. inhibiting adhesion molecules on endothelium) or directed to prevent systemic organ damage (i.e. catecholamines).[13-15] More interesting, however, might be pharmacological agents directed to known downstream mediators of mentioned IP, such as AMPK and eNOS, for these can possibly mimic the beneficial effects of IP more efficient.

Therefore, in this review I will discuss the possibility of AMPK agonists in cold preservation-related organ injury protection, compare this to ischemic preconditioning, and propose a connection between both preconditioning treatments via underlying, conceivable mechanisms. The main focus thereby is the influence of organ pretreatment with the AMPK agonists 5-Amino-4-Imidazole Carboxamide Riboside (AICAR) and 5'AdenosineMonoPhoshate (5'AMP) on cold ischemia related tissue injury, as been investigated in *in vivo* and *in vitro* models.

2. Rational of cold preservation and mechanisms of cold ischemic tissue injury

In most transplantations the graft derives from non-living donors, necessarily leading to a period of time wherein the organ is preserved. A main problem thereby is lack of oxygenation of the graft i.e. the ischemic period, appearing as organ injury after reperfusion. Ischemia initiates complex injury processes such as ATP loss, hypoxanthine accumulation, loss of the Na^+-K^+ pump-activity, cell swelling, and cytosolic calcium increase, among others.[16] Therefore, currently, a rapid in situ flushing of the organ with a preservation solution and rapid cooling to 4°C remains the main strategy to minimize ischemic injury and increase organ viability. Thus storage of organs at a lower temperature plays a very important role in organ transplantation. However, prolonged cold storage itself may give rise to side effects and –as it allows extended ischemic time- it can lead to prolonged ischemic preservation damage.[16]

As Salahudeen et al. (2004)[16] recently described there are at least four components involved in cold ischemic tissue injury: the coupled effect of ischemia and hypothermia during cold storage and the coupled effect of reperfusion and rewarming after transplantation. The effects of ischemia and reperfusion are widely studied, but the contribution of hypothermia and rewarming to them is difficult to separate and are therefore rarely studied apart. For these reasons, in this review, I will define cold-ischemia derived damage as the netto-end effect of cold-ischemia as a whole, as measured on organ damage after reperfusing the organ (*in vivo*) or rewarming the cells (*in vitro studies*).

The known mechanisms of cold ischemic injury are related to perturbations in osmoregulation, energetics and aerobic metabolism.[17] Namely, during hypothermic preservation, intracellular ATP concentrations rapidly decline, as low temperature induces a decrease in overall cellular metabolism and the lack of oxygen switches the cell to anaerobic metabolism. Consequently, energy depletion leads to dissipation of ion gradients across the cell membrane as a result of impaired function of the Na-K ATPase, permitting intracellular accumulation of sodium and water, resulting in cell swelling. Furthermore, during cold ischemia, lactic acid generated from the anaerobic metabolism of glucose contributes to intracellular acidosis. This leads to

lysosomal and mitochondrial instability, the latter further contributing to cellular energy depletion.[16, 17]

Another characteristic of cold ischemic tissue injury is the accumulation of intracellular reactive oxygen species (ROS), among others as a result of the abolishment of cellular ROS-scavengers and mitochondrial disbalance, due to the ATP depletion.[1, 16] Reactive oxygen may eventually result in endothelial damage, another prominent feature of cold ischemia.[18] Moreover, at the organ level, cold storage -due to a reduction in interstitial pressure- allows interstitial expansion and edema, leading to capillary compression, tissue injury, and poor organ function after transplantation.[1, 16]

Furthermore, other studies have demonstrated that prolonged cold ischemia is associated with more inflammation, as among others measured on the expression of allograft inflammation markers after transplantation, such as endothelial adhesion molecules ICAM, VCAM and selectins.[19] Moreover, recipients of grafts with long cold ischemia experience early acute rejection more often than those with minimal cold ischemia and are at higher risk for delayed graft function.[20-22]

As elaborated, several mechanisms are related to tissue damage due to prolonged cold preservation that may form the base to intervene in long-term graft survival.

3. Modes and mechanisms of organ preconditioning

Since donor and graft factors are important parameters of short-term and especially long-term allograft survival, much research has been done in the last decades in order to positively influence these determinants. In this respect, the value of organ preconditioning has been elaborated and many different approaches to achieve this objective were succesfull.[23]
Based on the concept being used, preconditioning can be divided into ischemic preconditioning (IP) and pharmacological preconditioning.

3.1 Ischemic preconditioning -Introduction
Principally, ischemic preconditioning is applied to reduce damage deriving from warm and cold ischemia by exposing the organ -still in the body- to brief episodes of ischemia. IP episodes have especially been found to protect vascular endothelium of organs against functional damage and cell death, thereby positively influencing whole organ viability in experimental models. Preconditioning can be induced by periods of ischemia as short as 3 to 5 minutes followed by 5 minutes of reperfusion. Hereafter the organ shows less damage caused by the following longer ischemic period. A single episode of transient ischemia is sufficient to induce preconditioning, although laboratories often use repetitive episodes of brief ischemia to induce the phenomenon.[9]

Furthermore, both an early and late phase of protection has been identified in IP, whereby the early (acute) phase of protection occurs when the extended ischemic period follows preconditioning within the first 5 minutes. When this period of time is extended to 30 to 60 minutes, preconditioning will not work anymore. However, if the duration between the ischemic preconditioning episodes and the long-duration cold ischemic period is extended to 24 to 96 hours, then the protective effect returns, although to a smaller extent as when the long occlusion occurs shortly after ischemic preconditioning.[9] This effect at 24 to 96 hours is called the delayed or late preconditioning protection. Although the exact explanation for this phenomenon is still elusive, it is believed that in the early phase IP preserves energy metabolism on a more direct way, for example via phosphorylation and direct receptor targeting mechanisms, having an immediate protective effect in following low-oxygen period.[10] Whereas late IP is thought to influence cellular homeostasis

via indirect regulation of translation and gene transcription, thereby –among others- influencing long-term NO and apoptosis pathways.[9] Good evidence for the first is the activation of AMPK by ATP-sensing and phosphorylation, found in among studies of Peralta et al. (2001)[10] on early ischemic pretreatment, using radio-labelled phosphate. Moreover, in studies on delayed IP, novel protein synthesis has been measured, for example several NO synthases, having a more long-term effect. In the period after the first critical minutes and before the later 24 hours, direct energy saving actions are then thought to be returned to basic level again, as the direct stressor has been disappeared. And on the other hand, products of gene-transcription are not yet present on a sufficient level to offer protection.[24-26]

3.2 Ischemic preconditioning –Overview of mechanisms

The exact mechanisms underlying IP are complex and have not yet all been defined and connected, although various potential mediators have been investigated and involve a variety of triggers and second messengers, acting via a receptor-targeting mechanism.[24] From studies on the preconditioned myocardium several molecules are known to be released during short ischemia, attach to cellular receptors and contribute to the preconditioning response.[25, 26] Important examples of mediators are adenosine, protein kinase C, ATP dependent potassium channels and (paradoxically) oxygen radicals. Furthermore several cytosolic kinases, nuclear factor kappa-B and heat shock proteins are described to play a significant role in the preconditioning cascade.[24-26] Recently, Koti et al. (2003)[24] have proposed a schematic overview of possible interconnections between the several found mediators, as illustrated in figure 1. Here can be seen that adenosine via its membrane receptors induces a cascade of kinase and enzyme activity, such as phosphokinase C (PKC) and endothelial NO synthase. Via –among others- NO production and NF-kB inhibition, the cell may turn to a protected state of low inflammation, finally leading to increased ischemic tolerance. Since insight to these mechanisms growths and connections between several mediators are more well-known, pharmacological agents are being developed for intervening and inducement of similar preconditioning effects as IP.

3.3 Ischemic preconditioning –Specific mediators

Of the known mediators in the proposed IP mechanism I will here elaborate the ones who are possible candidates for recent and future pharmacological intervention.

Adenosine is an extracellular nucleotide proposed as both a triggering factor and a mediator of IP. During cold ischemia adenosine triphosphate is degraded to adenosine, as a consequence of oxygen-shortage. The consequently high extracellular adenosine levels are found to inhibit leukocyte adhesion, to decrease expression of adhesion molecules (as ICAM, VCAM and selectins) and inhibit neutrophil and platelet function.[27] Furthermore, adenosine may directly and indirectly inhibit free radical production. Direct inhibition acts via reducing neutrophil activation –which are immunological producers of ROS- and indirect inhibition by adenosine acts via preventing mitochondrial disbalance by its activated intracellular kinases (fig. 1).[24, 27, 28] Adenosine thus acts as an important inflammation-inhibitor. Recently it was found that the adenosine receptors A1 and A2 are the most important ones in this mechanism, strongly connected to intracellular cAMP levels.[27-30] The above suggests adenosine not only to be protective against ischemia-reperfusion injury via inhibition of inflammation, but also offers via cAMP a possible connection to cellular metabolism, as cAMP is strongly connected to ATP. Furthermore, Peralta et al. have postulated activation of adenosine A2 receptors with subsequent

formation of NO to play a role in mediating IP against ischemia-reperfusion injury.[31] In this study adenosine administration increased cellular NO levels, thereby reproducing the protective effect of IP on hepatic parenchymal cells. On the other hand, administration of adenosine in the presence of an eNOS inhibitor abolished these effects. As these studies show that the effects of adenosine in IP are likely to be multifactorial, they may offer a good opportunity for investigations to application in pharmacological preconditioning.

Secondly, phosphokinase C (PKC) has been suggested to play a role in the beneficial effect of IP. The PKC-mediated signalling pathway of myocardial preconditioning was proposed by Downey and colleagues.[26, 32] The hypothesis proposes that during ischemia preconditioning G protein activation following G protein coupling with adenosine receptors leads to PKC activation and subsequent translocation from the cytosol to the membrane where it phosphorylates substrate proteins to induce tolerance to subsequent ischemia.[26] However, as also Koti et al (2003)[24] put forward, most of this evidence is not consistent in several different species models, at least not in small animals (rodents). Moreover, evidence is mostly indirectly based on a pharmacological approach, using PKC activators and inhibitors, some of which have been showed not to be purely specific for PKC. Yet, recently, Ricciardi et al. (2001)[33,34] have extended support for involvement of PKC in liver IP in larger animals. In their studies, tolerance of ischemically preconditioned pig livers to cold ischemia was abolished by pretreatment with the PKC-specific inhibitor chelerythrine.[34] Furthermore, a good support of these findings offer several other studies using PKC specific inhibitors, which clearly abolished the protective effect of IP in cold ischemia.[33] Although this still is indirect evidence, these studies at least support a role for PKC in IP, as well do studies to downstream effectors of the PKC kinase cascade. In the heart for example, it has been found that the kinase cascade activated during preconditioning leads to the opening of mitochondrial K-ATP channels, as a signal transduction step.[35] Furthermore, results have been found pointing to phosphorylation of Heat Shock Proteins, changes in activity of NF-kB and upregulation of inducible NO synthase.[36-38] As showed in figure 1, these mediators may contribute to the clarification of the IP induced anti-ischemic state.

A third molecule found to have a role in IP protection is nitric oxide. NO is a colorless, odorless, free radical gas which has been identified as an important signalling molecule in almost every tissue in the body. NO is produced from *L*arginine by the enzyme NO synthase.[24] It has been proposed that NO plays a key role in both initiating and mediating IP. Functional evidence in the heart indicates that NO modulates both acute and delayed preconditioning, whereby-as mentioned earlier- the acute effect mostly is regulated via downregulation of direct cellular energy metabolism.[39] A recent study in rat hearts by Lochner et al. revealed that NO through elevation of cGMP acts as a trigger of acute preconditioning, which in turn could reduce energy demand by limiting myocardial cAMP levels via stimulation of cGMP-sensitive cAMP phosphodiesterase enzymes.[40] Also here it appears that whereas the acute phase of preconditioning is protein synthesis independent, the late phase requires new protein synthesis. It has been found that eNOS-derived NO leads to activation of discussed PKC and other kinases, which in turn through NF-kB and other transcription factors leads to an increase in transcription of iNOS.[41] The end effector of IP in the supposed NO pathway is less clear and cGMP-dependent mechanisms and ATP-sensitive potassium channel have been proposed as factors modulating cellular metabolic and inflammatory state. Via

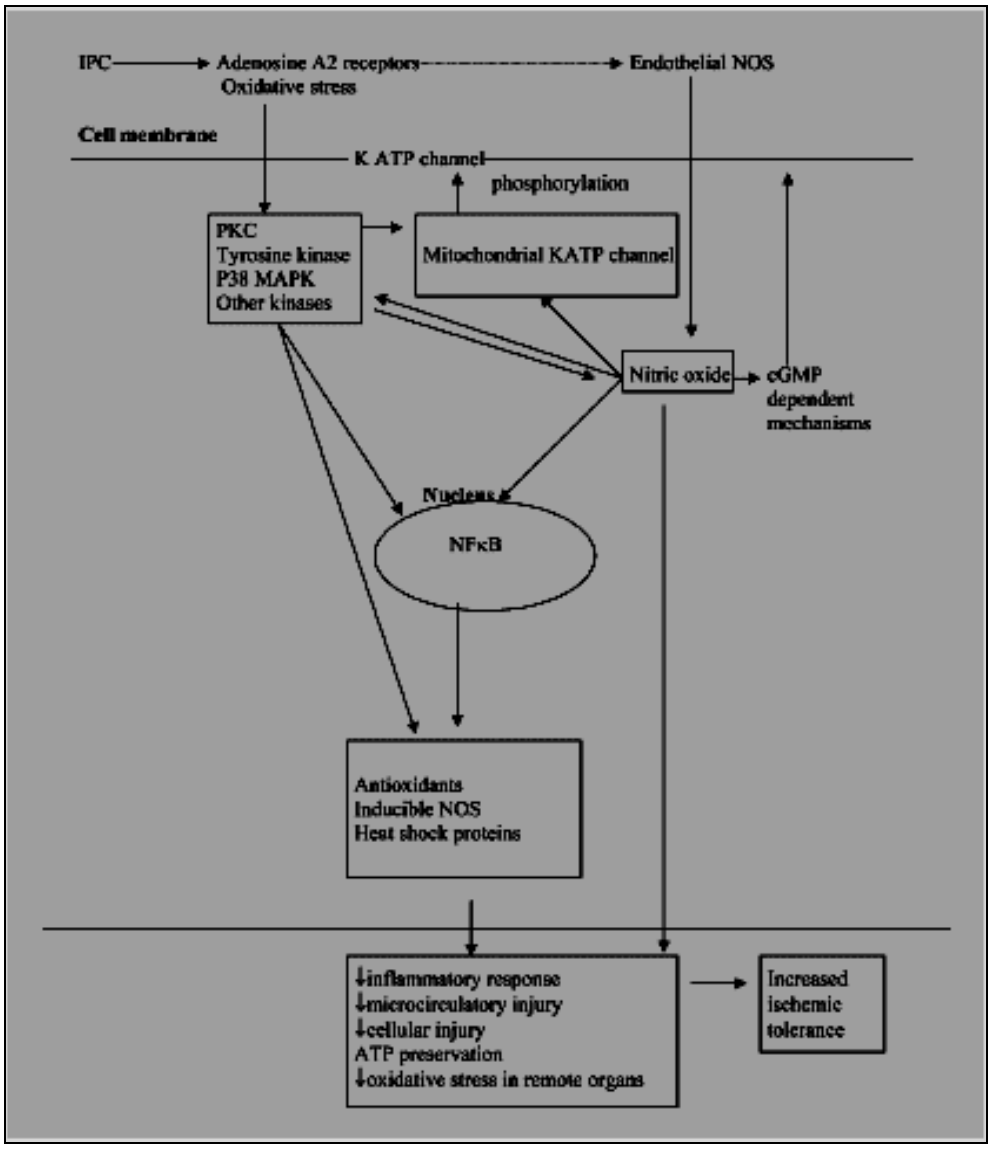
these mediators NO is also thought to influence cellular apoptosis cascade.[39-41]

3.4 Ischemic preconditioning – Pharmacological targeting

The partly clarified mechanism through which IP accomplishes its protective action in cold ischemia in animal models, form the basis to pharmacologically mimic important mediators by specific agonists and antagonists. Already mentioned is adenosine, for which preconditioning of hepatic cells give similar effects as IP.[31] However, in most cold ischemia models, intervention is directed to inhibition of cold-ischemia induced inflammation, few studies can be found to specifically address IP mediators.[1, 4] Examples of inflammatory intervention are the preconditioning of allografts with inhibitors of the expression of adhesion molecules. The use of anti-sense ICAM constructs, soluble ligands or antibodies against adhesion molecules have been shown to be successful in cold-ischemia injury models.[42, 43]

In addition, other studies have shown that donor-pretreatment with steroids and catecholamines significantly decreases tissue and serum expression of proinflammatory cytokines.[44,45]

Figure 1: *Mechanism of Ischemic Preconditioning, as proposed by Koti et al.* Via induction of adenosine, several kinases (i.e. PKC) and enzymes (i.e. eNOS) are activated in IP. By influencing NO and NFkB levels, these mediators protect the cell against mitochondrial energy disbalance and ROS-induced inflammation, finally leading to increased ischemic tolerance. *Koti et al. (2003)*[24]



More recently, however, growing attention is paid to the possibilities of the IP-mechanism, as shown by studies that make use of IP-mediator related agents. Examples are studies which show that the pretreatment of organs with NF-kB oligodeoxy-nucleotides prior to transplantation, resulted in a decrease of tissue-damage.[46]. Furthermore, the use of gaseous agents for donor-pretreatment has gained more interest. For example the pretreatment of allografts with eNOS-inducers showed significantly effect, as cellular NO levels increases.[47] Promising also are the preconditioning experiments with Carbon Monoxide (CO), that show a cytoprotective effect in cold-ischemia and lead to diminished graft rejection.[48] Both molecules share a common mechanism of

action, namely they both stimulate guanyl cyclase. Via production of cGMP this has a similar effect on energy metabolism as IP accomplishes; namely, by saving cAMP the cellular ATP-depletion can be reduced.

Very recently Gaskin and RJ Korthuis et al (2007)[49] postulated an interesting pursuement on this subject. First, it was repeatedly found that preconditioning with NO donors or agents that increase endothelial NO synthase (eNOS) activity resulted in reduction of allograft damage due to cold ischemia. At the same time studies signified that 5'-AMP-activated protein kinase (AMPK) phosphorylates eNOS at Ser1177, resulting in its activation. Therefore, they postulated that AMPK activation may trigger the development of a preconditioned phenotype similar to that induced by NO donors. Indeed, in their study it turned out that activation of AMPK induced a preconditioned phenotype, although this was only partly mediated via the NO-pathway. Apparently, AMPK activation affects via a broader signal transduction pathway organ preconditioning than previous pharmacological preconditioners. Moreover, in a parallel study of Peralta et al. (2006)[10] an extension of the IP mechanisms was found, i.e. they showed that during Ischemic Preconditioning itself the AMP kinase is activated, contributing to reduced ATP degradation and lactate accumulation, thereby protecting against tissue injury. A continuation on these findings are the results of Americh et al. (2006)[50], as they show in their study that extracellular adenosine- a previous mentioned key mediator in the protecting IP-mechanism- is able to activate AMPK. Together with its eNOS connected mechanism, these findings promise AMPK to be an interesting novel mode of action in cold-ischemic preconditioning. Therefore, in the next part of this review, firstly AMPK and its mechanisms as a kinase will be elucidated, whereafter an overview of the connection between IP and AMPK is presented, together with a proposed common way of action. Finally, possible agonists of this kinase will be introduced as a promising method in future cold-ischemic preconditioning.

4. AMPK as a main enzyme in cold preservation protection:

4.1 AMPK -Introduction

AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade that plays a key role in the regulation of energy control (Figure 2). AMPK is activated in response to increase in the ratio of AMP:ATP within the cell, as a consequence of ATP-depletion, which also is one of the phenomenons in cold-ischemia injury. Once activated, AMPK initiates a series of responses that are aimed at restoring the energy balance within the cell. ATP-consuming, anabolic pathways, such as fatty acid synthesis and protein synthesis are switched-off, whereas ATP-generating, catabolic pathways, such as fatty acid oxidation and glycolysis, are switched-on.[51] This kinase further not only acts on the cellular level, it also plays a role on the whole body energy regulation, as AMPK is strongly connected with food-intake regulators as leptin and ghrelin.[52, 53] However these effects are out of scope of this review.

AMPK is a heterotrimeric complex consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK is activated allosterically by binding of AMP to the γ subunit and by phosphorylation. The binding of AMP induces a conformational change of AMPK, such that it can be phosphorylated at the α subunit by upstream kinases. High levels of ATP antagonize AMP binding by inhibiting the binding place on AMPK.[53] Via this way AMPK is a sensible sensor for cellular energy levels, as little changes in AMP-ATP ratio changes AMPK activity. After binding of AMP, AMPK is phosphorylated at the thyrosine 172 (T172) residue of the α catalytic subunit by the

recently identified upstream kinase LKB1. This kinase has strong similarities with the mammalian Ca2+/calmodulin dependent protein kinase kinase (CAMKK) subgroup, pointing to a role within Ca2+ cellular 'danger' pathways. Much less is known about the β -subunit, but studies are pointing to a role in cellular glycogen-energy pathways, as radio-labelled glycogen has been found to bind the β-unit.[51]

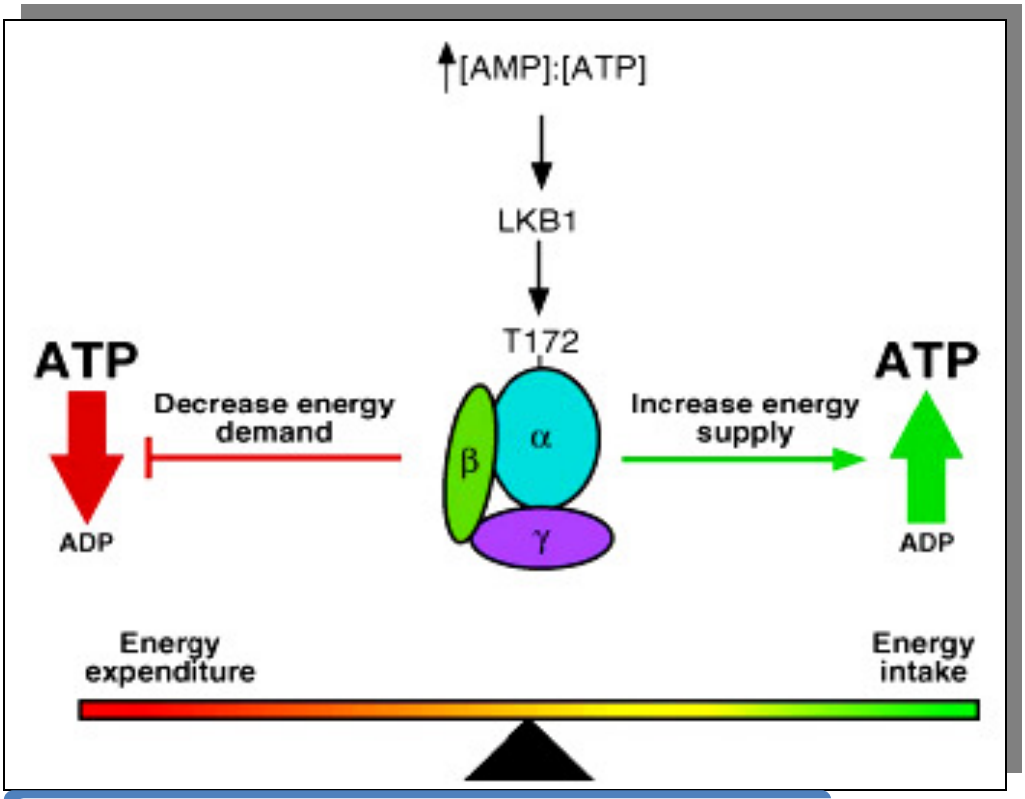


Figure 2: *AMPK: balancing the cellular energy scale*
AMPK consists of three subunits and is activated by a rise in the AMP:ATP ratio. Activation of AMPK requires phosphorylation of threonine 172 within the α subunit, catalysed by LKB1. Once activated, AMPK increases energy supply by switching-on ATP-generating pathways and decreasing energy demand by switching-off ATP using pathways. These combined roles of AMPK ensure that the energy status of the cell is finely balanced. *Carling (2005)*[51]

4.2 AMPK –Mechanisms of action

Even more interesting and under continuing investment are the downstream mediators of AMPK, as these may offer further signs to the application of AMPK-agonists in preconditioning, for reasons of energy saving and of similarity with IP-mechanisms. Generally it has been found that AMPK realizes its effects on cellular processes (i.e. energy metabolism) by direct phosphorylation of regulatory proteins involved in the process, and by indirect effects on gene expression. Furthermore, on translation level AMPK is able to inhibit *de novo* protein synthesis – which is energy consuming- by inhibiting the elongation step in translation and by downregulating the rapamycin pathway, responsible for translation initiation.[52, 54]

More specific, the energy saving effect of AMPK-activation is mostly accomplished via phosphorylation of important anabolic (energy consuming) and catabolic (energy-producing) enzymes. Examples of the former include acute inhibition of lipid biosynthesis by phosphorylation and

inactivation of key metabolic enzymes such as ACC1 (fatty acid synthesis), glycerol phosphate acyl transferase (triacylglycerol synthesis), and HMGCoA reductase (cholesterol/isoprenoid biosynthesis). Examples of the manner in which AMPK activation can generate ATP by stimulating catabolism include its stimulation of fatty acid oxidation via phosphorylation of ACC2, and its stimulation of glucose uptake via activation of both glucose transporter (GLUT) 1 and GLUT4.[52, 53] It is clear that AMPK is a kinase strongly connected to energy metabolism. Moreover, as being a main characteristic of cold-ischemia damage, loss of cellular energy levels could be one target to which AMPK can be directed.

Beside these effector mechanisms on energy balance, AMPK has several other downstream targets, more similar to the IP mechanism. Examples of mediators that were recently discovered are eNOS, NO and NFkB.[49, 55-57] As mentioned elsewhere in this review, AMPK has been found to phosphorylate eNOS (endothelial NO synthase) at Ser-1177, resulting in its activation.[51] Morrow et al (2003)[57] subsequently tried to discover whether the activation of AMPK, mediated via eNOS activation, could work protective on damage signs of human aortic endothelial cells, as dysfunction of endothelium strongly effects organ function as a whole. In their study indeed an eNOS mediated increase of NO levels was found to occur via AMPK activation, as they saw a concomitant increase in eNOS Ser-1177 phosphorylation after AMPK activation, followed by NO production. From NO it is already known that it has positive effects on endothelial function, via among others vasodilatation, and a reducing effect on injury-related local inflammation via inhibition of platelet aggregation and leukocyte adhesion.[57] The positive effect of eNOS activation in IP and of preconditioning with NO donors was already mentioned, and here it is shown that the same applies via activation of AMPK. Therefore, the anti-inflammatory and endothelium protecting roles of NO could clearly contribute to the possible beneficial role of AMPK in cold-ischemia damage, in which these two factors are importantly disturbed. Furthermore, that this mediating role of eNOS is mainly effective on the long term, is illustrated by the results of Gaskin and Korthuis et al. (2007)[49], as they found especially an eNOS mediated effect of AMPK in delayed graft preconditioning.

The other recently discovered effector mechanism of AMPK is its role in NFkB signalling. Both Cacicedo et al. (2004)[55] and Hattori et al. (2006)[56] namely found in their *in vitro* studies of human vascular endothelial cells NFkB to be inhibited by activation of AMPK. In the first study Cacidedo et al. measured a 4-fold increase in this pro-inflammatory transcription factor when cells were stimulated with TNFa, an effect almost completely inhibited after AMPK activation by its agonist AICAR. Moreover, the latter study of Hattori et al. expanded this anti-inflammatory capacity of AMPK. By activating AMPK they not only confirmed the inhibition of NFkB, they also proved significantly decrease in the pro-inflammatory molecules that are activated in the NFkB pathway. The expression of the endothelial adhesion molecules VCAM-1, E-selectin, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1 in HUVECs was namely strongly reduced after AMPK-activation.[55, 56]

4.3 AMPK – A main enzyme in cold-ischemia protection

As elaborated until now, AMP kinase comprises a broad range of effects in cellular functioning. For being a kinase involved in regulating cellular homeostasis and in responding on (metabolic) stress, its discovered downstream effector mechanisms in energy metabolism might not come as a surprise. However, the role

of AMPK in endothelial functioning via eNOS and its anti-inflammatory role via NFkB are entirely novel and make this kinase an even more interesting target in cold-ischemia preconditioning. Namely, taking together, the main characteristics of cold-ischemia tissue injury are, firstly, ATP-depletion due to anaerobic metabolism, causing osmoregulation problems and cell death. Furthermore, inflammation occurs, due to ROS accumulation, which is also connected to cellular energy disbalance via mitochondrial problems and depletion of ATP-consuming scavengers. Moreover, inflammation strongly contributes to endothelial dysfunction, a third factor severely contributing to organ dysfunction due to prolonged cold-ischemia. [16-18]

Summarizing, as illustrated in figure 3, AMPK might be able to have a protective influence in triplo. Primarily, the energy saving role of AMPK would be able to positively change the energy depletion during cold ischemia, thereby diminishing the cellular problems in homeostasis due to anaerobic metabolism and mentioned consequences of osmoregulation dysfunction and ROS-accumulation. Furthermore, acting via eNOS, AMPK can reduce endothelial dysfunction by increasing NO-levels. And together with its anti-inflammatory role via NFkB, AMPK may have a broad third protecting influence on cold-ischemia derived damage. Moreover, the latter possibilities of AMPK also strongly mimic the well-known ischemic preconditioning effect, further candidating it as a target in pharmacological preconditioning. With this knowledge and appearing connections in underlying mechanisms, the possibility of a common way of action rises. Therefore, in figure 3 I propose a schematic overview of possible interconnections between IP and AMPK in inducing a cellular ischemic- tolerant state. As shown in figure 3, IP itself is able to activate AMPK via induction of its known mediator adenosine[10], and thereby indirectly induces an AMPK mediated protection. Whereas pharmacological activation of AMPK would connect to the IP mechanism via the previous mentioned mediators of AMPK, eNOS and NFkB. The netto-end effect via both preconditioning ways will be an anti-inflammatory and metabolic energy-sparing state, offering protection in subsequent cold-ischemic period. Therefore, in the last part of this review I will introduce two agonists of AMPK, as possible future candidates in pharmacological preconditioning: the known pharmacological activator of AMPK AICAR, and the very recently studied 5'AMP.

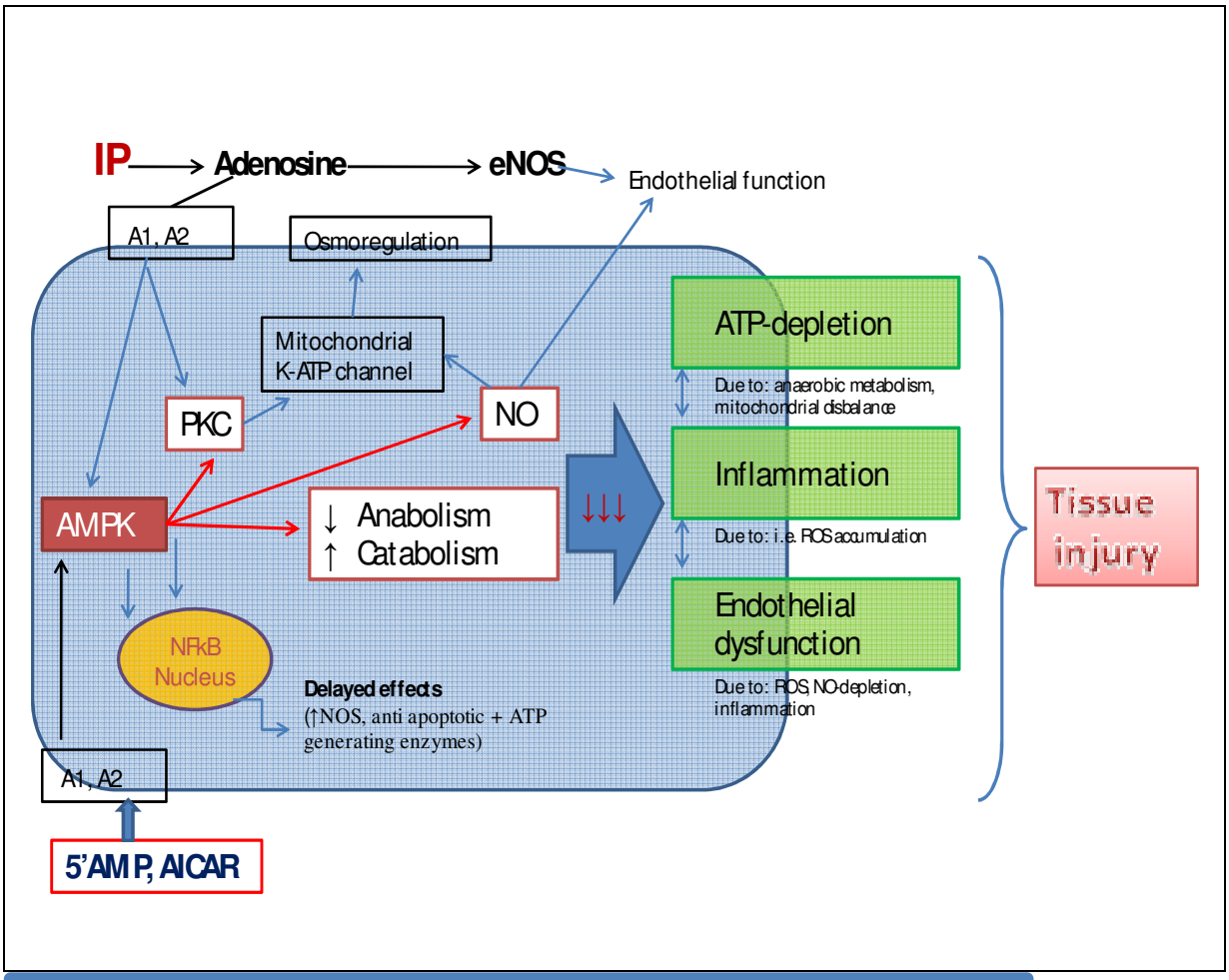


Figure 3: *Proposed common way of action of AMPK and IP*
A schematic overview is presented of the mediators via which IP and AMPK are possibly connected, and how this contributes to the protection against cold-ischemia derived tissue injury.

5. Finally: AICAR and 5'AMP as AMPK activators

For its role in energy metabolism, AMPK has already been the growing focus of attention in drug targeting studies to the effects of this kinase in metabolic disorders as diabetes type 2 and obesity.[58] One of the known pharmacological activators of AMPK is AICAR (Figure 4), an agent that mimics the action of physiological cAMP. Together with AMPK-inhibitors, AICAR has been used in the many mentioned studies to assess the effects of AMPK activation on cellular metabolism and function. After being taken up into cells via adenosine transporters, AICAR is converted by adenosine kinase to the monophosphorylated nucleotide, ZMP, an AMP analogue. ZMP then allosterically activates both AMPK-similar to AMP- and the upstream AMPK kinase that causes further activation of AMPK.[58] Moreover, in this study ZMP was also shown to mimic all three effects of AMP on the AMPK system, including its anti-inflammatory effect.[58] Based on these results, AICAR seems to be a perfect candidate for usage in cold-ischemic preconditioning, as Gaskin and Korthuis et al. (2007)[49] also investigated in their study. Here they used AICAR in preconditioning mice before the prolonged ischemic period of the jejunum, and found a similar preconditioned anti-inflammatory

phenotype as induced by IP. However, it needs to be mentioned that AICAR is not entirely AMPK specific, as ZMP also regulates other cAMP-sensitive enzymes such as fructose-1,6-bisphosphatase and muscle glycogen phosphorylase.[58] Whether these effects may be interfering in the protecting role of AMPK, or rightly may contribute to the positive effects of AMPK on cellular metabolism, needs to be determined.

Furthermore, a novel AMPK activating substance is the growing focus of attention in metabolism related studies: the cAMP derived molecule 5'AMP (Figure 4). This agent is actually the same as the physiological AMP, however synthetically produced. Naturally occuring AMP is a mixture of the 3'AMP and 5'AMP molecules, the pharmacological agent 5'AMP thus being a purified form of AMP.[59] [60] Therefore it makes sense that this substance may have the same beneficial role in cold-ischemia protection as AICAR. Moreover, as 5'AMP is even more similar to physiological AMP, it might activate AMPK in a more specific way then the AMPK-agonists used until this moment. The exact evidence for these ideas needs to be determined, as extensive studies on this topic are lacking, but the similarity to physiological AMP makes it a very promising molecule in future preconditioning studies. Underlining its possible role as preconditioning agent are in vivo studies at the University Medical Centre Groningen (Bouma HR. et al., in preparing). These investigators namely show a reduction in energy metabolism after injecting mice and rats with 5'AMP, thereby possibly protecting organs to cold-derived damage. Furthermore, *in vitro* investigations of the author also indicate a beneficial effect of 5'AMP on the cold-preservation and inflammatory response of human endothelial cells, as cellular ATP-levels were found to increase, and production of pro-inflammatory molecules like IL-6 and activation of VCAM were found to decrease after 5'AMP treatment.

Summarizing, in this review the mechanisms underlying ischemic and pharmacological preconditioning as methods to protect organs during prolonged cold preservation have been extensively elaborated. A central role in the mechanism of IP protection is the AMP kinase, which not only has a central role in balancing cellular energy metabolism, but also may connect strongly to downstream targets of known IP mediators. Moreover, by having the capacities of saving cellular energy, reducing local inflammation and indirectly regulating endothelium function, I propose in this review that targeting AMPK could be a novel strategy in future pharmacological preconditioning. As being AMPK agonists, both AICAR and 5'AMP are promising candidates, and future research should then be directed to expand the knowledge on this topic. Studies should thereby both focus on further unraveling underlying mechanisms, on revealing the spectrum of possible protective effects, and on the pharmacokinetic and pharmacotoxic features of their administration as an *in vivo* drug.

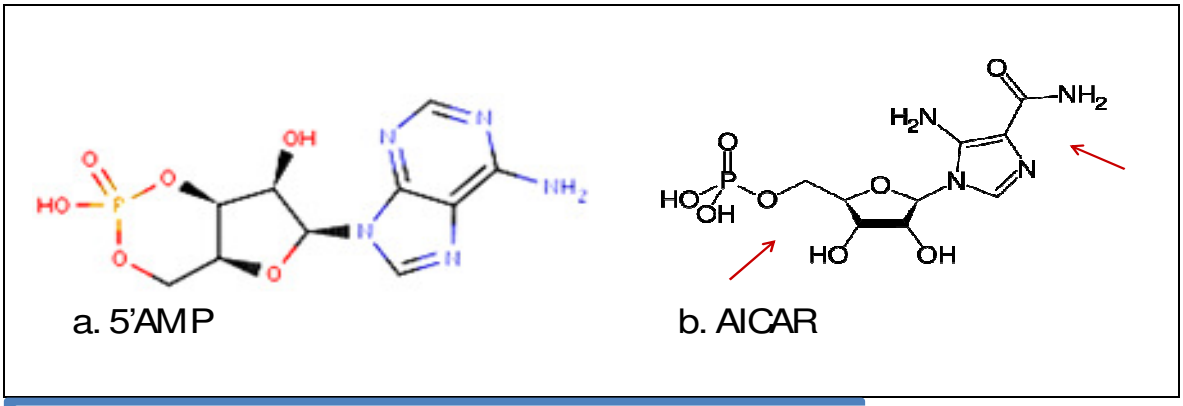


Figure 4: *Structures of the AMPK agonists 5'AMP(a) and AICAR(b)*
Arrows indicate the small differences in structure formula.

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