

■ H Y P O T H E S I S

# Ubiquitin–proteasome system inhibitors and AMPK regulation in hepatic cold ischaemia and reperfusion injury: possible mechanisms

Susagna PADRISSA-ALTÉS\*, Mohamed Amine ZAOUALI\*, Ramon BARTRONS† and Joan ROSELLÓ-CATAFAU\*‡

\*Experimental Hepatic Ischemia-Reperfusion Unit, Institut d'Investigacions Biomèdiques de Barcelona-Consejo Superior de Investigaciones Científicas, Barcelona, Spain, †Unitat de Bioquímica i Biologia Molecular, Departament de Ciències Fisiològiques, Campus de Bellvitge, Universitat de Barcelona, L'Hospitalet, Spain, and ‡Unitat de Transplantament de fetge i viabilitat de l'empelt, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Consejo Superior de Investigaciones Científicas, Barcelona, Spain

## A B S T R A C T

In the present Hypothesis article, we summarize and present data from the literature that support our hypothesis on the potential mechanisms by which UPS (ubiquitin–proteasome system) inhibitors reduce I/R (ischaemia/reperfusion) injury in the liver. I/R is the main cause of primary liver failure and, consequently, minimizing the detrimental effects of this process could increase the number of suitable transplantation grafts and also enhance the survival rate of patients after liver transplantation. A potential strategy to reduce I/R injury is the use of UPS inhibitors either as additives to preservation solutions or as drugs administered to patients. However, there is still controversy over whether the use of UPS inhibitors is beneficial or deleterious with regard to liver injury. From our experience and the few studies that have investigated the role of UPS in hepatic I/R, we believe that the use of UPS inhibitors is a potential strategy to reduce I/R injury in liver transplantation and graft preservation. We hypothesize that one of the main mechanisms of action of UPS inhibitors may be the up-regulation of AMPK (AMP-activated protein kinase) activity and the consequent down-regulation of mTOR (mammalian target of rapamycin), which may finally influence autophagy and preserve the energy state of the cell.

## INTRODUCTION

I/R (ischaemia/reperfusion) injury, inherent in LT (liver transplantation), is the main cause of initial deficiencies and primary non-function of liver allografts [1]. Therefore minimizing the adverse effects of I/R injury could increase the number of both suitable transplantation grafts and patients who successfully recover from LT. The mechanisms involved in the

pathophysiology of I/R injury have been the focus of previous extended reviews [2]. In essence, during the ischaemic phase, blood flow and oxygen and nutrient supply to the organ are inhibited, which stops energetic metabolism, depletes ATP levels and renders the organ more susceptible to blood reflow in the reperfusion phase. In this last phase, a ROS (reactive oxygen species) burst, as well as activation of pro-inflammatory cells and mediators, takes place, enhancing organ injury

**Key words:** AMP-activated protein kinase (AMPK), autophagy, ischaemia/reperfusion, liver, transplantation, ubiquitin–proteasome system.

**Abbreviations:** AMPK, AMP-activated protein kinase; ER, endoplasmic reticulum; HIF-1, hypoxia-inducible factor-1; I/R, ischaemia/reperfusion; LT, liver transplantation; mTOR, mammalian target of rapamycin; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NOS, NO synthase; eNOS, endothelial NOS; ROS, reactive oxygen species; UPS, ubiquitin–proteasome system.

**Correspondence:** Professor Joan Roselló-Catafau (email joan.rosello@iibb.csic.es).

even more [2]. A strategy to reduce I/R injury is the use of UPS (ubiquitin–proteasome system) inhibitors either as additives to preservation solutions or as drugs administered to patients.

The multicatalytic proteasome is the ubiquitous proteinase found in cells throughout the plant and animal kingdoms that is responsible for the degradation of intracellular proteins. The proteasome exerts multiple intracellular functions, namely the degradation of damaged proteins and the modulation of many regulatory proteins that are involved in inflammatory processes, cell cycle, metabolism, growth and differentiation among others [3]. Several studies have proposed that UPS inhibition is protective against I/R injury in different organs. Majetschak et al. [4] proposed that proteasome inhibitors may be useful in maintaining the physiological ubiquitin–protein conjugate pool during cold ischaemia in a model of murine heart transplantation, and thus may prolong organ preservation. Other studies have in fact demonstrated that proteasome inhibition can reduce injury in models of isolated perfused rat heart through a decrease in polymorphonuclear leucocyte adherence to the endothelium [5]. On the other hand, other studies have reported contradictory results. For instance, a study on endothelial cells submitted to hypothermia showed that the UPS pathway was activated during cold preservation of endothelial cells, but proteasome inhibition could not prevent cell damage [6]. Other studies have reported a decrease in proteasome activity in cerebral ischaemia [7]. A possible explanation for this effect could be the ATP depletion observed in ischaemia [7], since the UPS is an ATP-dependent system. Interestingly, a study by Divald and Powell [8] demonstrated that the UPS is able to degrade oxidized proteins in an ATP- and ubiquitin-independent manner in a model of myocardial ischaemia. This indicates that, even though proteasome activity is decreased in ischaemia and reperfusion, the remnant pool of active proteasomes is able to maintain proteolysis even if the cell is depleted from ATP. In addition, Geng et al. [9] have also shown that a subset of 26S proteasomes is activated at low ATP concentrations and that this contributed to myocardial injury during cold ischaemia. Thus a subset of the 26S proteasomes acts as a cell-destructive protease that is activated when the cellular energy supply declines. In that study, the administration of a proteasome inhibitor resulted in preservation of the ultrastructural integrity of the cardiomyocyte. Furthermore, a subsequent study by the same group [10] revealed that proteasome inhibition during cold ischaemia of hearts prolonged myocardial viability and reduced reperfusion injury. Regarding the methods used for the measurement of the activity of the proteasome in all of these studies, analysis of Suc-LLVY-MCA (succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide)-hydrolysing activities in the presence of ATP, at a similar concentration, was used. Moreover, the latter

two studies used epoxomicin to differentiate between peptidase and proteasome activities. In addition to all of the above, UPS inhibitors have already been used in models of organ transplantation and have shown profound beneficial effects [4]. Finally, taking into account their well-established immunosuppressive effects [11], UPS inhibitors seem to be very promising candidates for the preservation of organ integrity and function during transplantation.

Concerning liver injury, the UPS system is still an almost unwalked path, particularly in the hepatic I/R field. Hence only a few studies have investigated the UPS in the conditions mentioned above. The majority of studies have investigated the effect of proteasome inhibitors on liver injury and have shown that UPS inhibitors were able to reduce injury, oxidative stress and apoptosis in different models of hepatic injury [12,13]. On the other hand, some studies have suggested that proteasome inhibitors may not be beneficial, but injurious [14]. Hence there remains a controversy over whether the use of UPS inhibitors is beneficial or injurious against liver injury, and the mechanisms are still not clear.

There are several natural and synthetic compounds that act as proteasome inhibitors. The use of all of these inhibitors and their potential for the treatment of human diseases other than hepatic I/R injury have been considered in a previous review [15]. Some of these compounds are far advanced in clinical trials for their administration in humans. For example, synthetic analogues of the bacterial metabolite lactacystin, which inhibits proteasome activity, have been developed [16]. Among these, PS-519 (a small analogue of lactacystin) is under clinical evaluation for inhibiting reperfusion injury after ischaemic central nervous system injury [16]. Another compound that has been approved for clinical trials is bortezomib, a tripeptide consisting of pyrazinoic acid, phenylalanine and leucine with boronic acid instead of a carboxylic acid (Pyz-Phe-boroLeu). Bortezomib has proven its therapeutic potential for intervention of the UPS in cancer (Velcade; Millennium Pharmaceuticals) and was approved by the US Food and Drug Administration in 2003 [17].

## HYPOTHESIS AND DISCUSSION

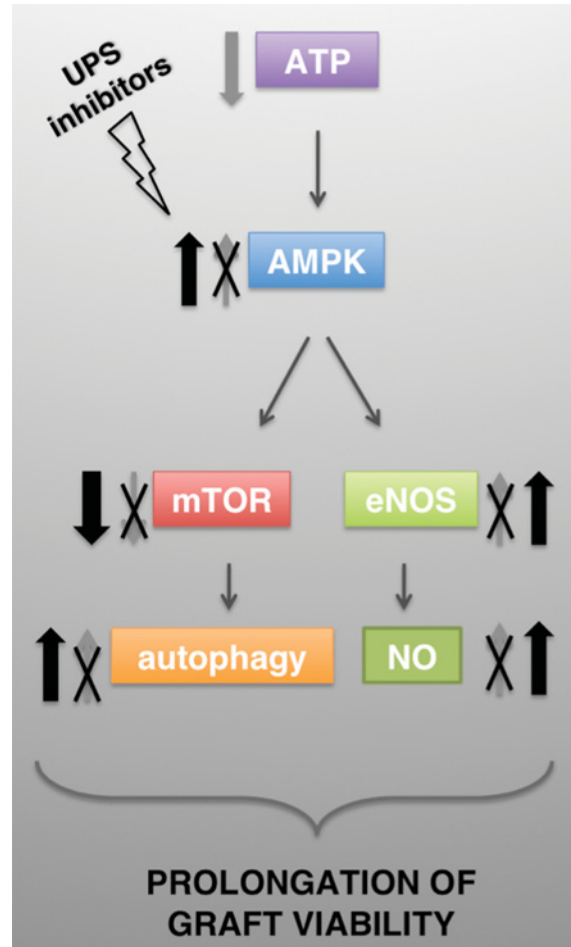
From our experience and studies that have investigated the role of the UPS in hepatic I/R, it is clear that UPS inhibitors are a potential strategy to reduce I/R injury in LT and graft preservation. Moreover, we hypothesize that UPS inhibition may improve graft preservation due to an increase in AMPK (AMP-activated protein kinase) activity and autophagy.

Previous studies from our group have reported that the beneficial effects of ischaemic preconditioning in

liver graft preservation was due to the up-regulation of AMPK, among other mechanisms [18], and that AMPK was also involved in steatotic liver preservation [18]. AMPK acts as a metabolic fuel gauge, which is activated in response to diverse stress factors to restore cellular and whole-body energy balance [19]. AMPK is allosterically regulated by the competitive binding of AMP and ATP, thereby ‘sensing’ cellular energy status and, when activated, triggers compensatory ATP-generating mechanisms while attenuating ATP-consuming processes [20]. Knowing that AMPK is basically degraded, and therefore regulated by the UPS [21,22], it appears that, when administering UPS inhibitors, AMPK cannot be degraded at the usual rate and therefore it is stabilized and its action perpetuated.

In the rat, AMPK and the mTOR (mammalian target of rapamycin) signal transduction pathway are involved in the control of autophagic proteolysis [23]. The mTOR pathway is a key regulator of cell growth and proliferation, and integrates signals regarding availability of nutrients and growth factors to regulate many cellular processes, including ribosome biogenesis and metabolism [24]. mTOR is inhibited during energy starvation and its inhibition stimulates autophagy [25]. Importantly, previous studies have demonstrated that AMPK activation inhibits mTOR in several tissues [26,27]. The lysosomal pathway, autophagy, renders complete organelles and individual proteins to be engulfed by a newly formed membrane, termed a phagophore or isolation membrane, to form a double-membrane vesicle, called the autophagosome, which is delivered to lysosomes for hydrolytic degradation [28]. Autolysosomal degradation of membrane lipids and proteins generates non-esterified ‘free’ fatty acids and amino acids, which can then be reused to maintain mitochondrial ATP production and ribosomal protein synthesis [29]. Autophagy is also activated in order to remove damaged organelles and to stimulate phagocytic clearance of apoptotic cells [30]. Previous studies have reported that AMPK activation can induce autophagic proteolysis [23]. Induction of autophagy by AMPK may contribute to the preservation of ATP content, as well as promotion of cell survival in the ischaemic heart [31]. Furthermore, activation of AMPK also enhances ATP production through other multiple mechanisms, such as increases in glucose uptake, glycolysis and fatty acid oxidation [32].

Nonetheless, although autophagy during energy starvation is generally protective [31], its induction by other stimuli can lead to autophagic cell death and thus can be detrimental [33]. Previous studies have reported a cross-talk between autophagy and apoptotic and necrotic cell death pathways [34], and activation of autophagy may favour cellular survival by decreasing ROS production [35] and suppressing ER (endoplasmic reticulum) stress [36]. It is thought that ER stress induced by I/R induces autophagy in the heart as an adaptive mechanism [37].



**Figure 1** Proposed mechanism for the protection exerted by UPS inhibitors in hepatic cold ischaemia in the reperfusion phase of LT

Moreover, a recent study by Esposti et al. [38] has shown that the beneficial effects of ischaemic preconditioning in steatotic livers undergoing I/R were due to the activation of autophagy, which could modulate apoptosis and necrosis and may be involved in the attenuation of ER stress. Additionally, another study has reported that ischaemic preconditioning increases autophagy in human patients and this correlated with a decrease in liver cell death [39].

Returning to our hypothesis (Figure 1), when AMPK is up-regulated under UPS inhibition, mTOR may be inhibited and therefore cannot exert its inhibitory effect on autophagy. Consequently, proteolytic autophagy would be enhanced. This would result in the preservation of cellular ATP levels and thus prevention of cell death. In addition, this induction of autophagy could help the cell to get rid of oxidized proteins that can damage the cell membrane and other cellular compounds. Enhancement of AMPK activity also induces eNOS [endothelial NOS (NO synthase)] activity and thus NO production [40], which has been widely demonstrated

to prevent endothelial cell damage [41]. Furthermore, AMPK inhibits iNOS (inducible NOS) activity, which is known for its injurious effects through the generation of NO [42]. In addition, a link between NO and autophagy has been demonstrated previously [43], and, in endothelial cells, AMPK was shown to activate eNOS and thus NO production, which promoted vasodilation [44] and reduced leucocyte adhesion [45]. Moreover, AMPK activity also acts on several downstream targets that preserve the energetic state of the cell and prevent liver I/R injury (reviewed in [46]). mTOR inhibition may also enhance the compensatory up-regulation of upstream survival kinases, such as PI3K (phosphoinositide 3-kinase) and Akt, which will also protect the cell from apoptosis [47]. All of this correlates with previous studies showing that ischaemic preconditioning can induce autophagy in the liver and thus prevent cell death [38].

In addition to what has been discussed above, the induction of autophagy is also beneficial against I/R, because when autophagy is inhibited it is not possible to remove dysfunctional mitochondria. Therefore these mitochondria laden with ROS and calcium undergo the mitochondrial permeability transition, which in turn leads to the uncoupling of oxidative phosphorylation, energetic failure, ATP depletion and ultimately cell death. Therefore it is important that autophagy is induced under ischaemic conditions and its induction can be even more protective. This hypothesis is supported by the finding that autophagy declines in aged organisms, which correlates with the decrease in tolerance of aged patients to I/R injury [48]. However, would the induction of autophagy during reperfusion also be protective against I/R injury? This question will be discussed below.

It should be taken into account that the induction of autophagy may be protective against apoptosis and cell injury if it is not too excessive. As mentioned above, both protective and detrimental effects of autophagy have been reported, and excessive induction of autophagy may cause cell death. Furthermore, excessive inhibition of the UPS may also result in cell death as described above. However, as alternative proteolytic pathways are active in the cell [49], it is expected that when one of the systems for proteolysis in cells is inhibited, other systems are able to degrade abnormal proteins that may trigger cell death if present in large amounts and/or for prolonged periods of time. In addition, differences in the processes of ischaemia and reperfusion may account for some of the discrepancies. Finally, even though proteasome activity declines in post-ischaemic reperfused organs, the remnant pool of active proteasomes is able to maintain proteolysis even if the cell is depleted of ATP [8]. This may explain why additional inhibition of the proteasome during reperfusion may be protective against I/R injury.

It is also noteworthy that autophagy decreases after partial hepatectomy [50], suggesting that UPS inhibition

could also be beneficial in living donor LT, as it would enhance autophagy and thus preserve ATP levels and other molecules necessary for liver regeneration. Hence UPS inhibition could also be beneficial in models of reduced-size LT by both increasing liver regeneration and protecting the liver against I/R injury.

In addition to the effect on AMPK activity described above, additional mechanisms may well contribute to the protective effects of UPS inhibitors in LT and graft preservation. For instance, Stangl et al. [51] have shown that the proteasome inhibitor MG-132 protected cardiomyocytes from hypothermic injury through the induction of HSP (heat-shock protein) 70 and 90, which enhanced their survival and functional recovery. Furthermore, others have implicated NF- $\kappa$ B (nuclear factor  $\kappa$ B) in the protective effect of proteasome inhibition. For example, Pye et al. [52] have shown that proteasome inhibition reduced reperfusion injury in myocardial I/R through a decrease in NF- $\kappa$ B activation, which in turn affected the recruitment of inflammatory cells. A study of liver injury induced by intestinal I/R showed that lactacystin inhibited NF- $\kappa$ B, and this consequently reduced liver and intestinal injury and neutrophil infiltration [53]. Alternatively, UPS inhibitors could also be protective against I/R injury through the modulation of HIF-1 (hypoxia-inducible factor-1), which is well known for its role in cell adaptation to hypoxia and its regulation by the UPS [54]. A study by Shin et al. [55] has reported that proteasome inhibition inactivates HIF-1, thereby suppressing the expression of genes essential for cellular adaptation to hypoxia. However, this could be considered a paradox as it is well established that UPS degrades the  $\alpha$  subunit under normoxia to maintain HIF-1 $\alpha$  inactivated. Thus UPS inhibition should promote HIF-1 $\alpha$  activation. In this sense, previous studies have found that HIF-1 $\alpha$  levels were increased after proteasome inhibition in xenografted tumours, although two genes which are usually up-regulated by HIF-1 were down-regulated [56]. Further studies will therefore be required to determine the specific role of HIF-1 in proteasome inhibition and I/R injury. Finally, a reduction in oxidative stress could also contribute to the protective effects of UPS inhibitors in the liver, as Bardag-Gorce et al. [13] have shown that bortezomib decreases oxidative stress in a model of rat alcoholic liver disease. In that study, bortezomib increased the expression of antioxidant enzymes and decreased the oxidative burst. Furthermore, MG-132 protected mouse hepatocytes from TNF- $\alpha$  (tumour necrosis factor- $\alpha$ )-induced apoptosis [57].

## CONCLUSIONS

In summary, we propose that the major mechanism by which UPS inhibitors reduce I/R injury in LT and graft

preservation is via the up-regulation of AMPK activity and the consequent down-regulation of mTOR during ischaemia, which may finally influence autophagy and preserve the energy state of the cell. Nevertheless, additional mechanisms need to be considered. Future studies will be required to determine the effects and mechanisms of action of UPS inhibitors during cold ischaemia in LT.

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