Mechanisms of autophagy activation in endothelial cell and their targeting during normothermic machine liver perfusion

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Abstract

Ischaemia-reperfusion injury (IRI) is the leading cause of injury seen in the liver following transplantation. IRI also causes injury following liver surgery and haemodynamic shock. The first cells within the liver to be injured by IRI are the liver sinusoidal endothelial cells (LSEC). Recent evidence suggests that LSEC co-ordinate and regulates the livers response to a variety of injuries. It is becoming increasingly apparent that the cyto-protective cellular process of autophagy is a key regulator of IRI. In particular LSEC autophagy may be an essential gatekeeper to the development of IRI. The recent availability of liver perfusion devices has allowed for the therapeutic targeting of autophagy to reduce IRI. In particular normothermic machine liver perfusion (NMP-L) allow the delivery of pharmacological agents to donor livers whilst maintaining physiological temperature and hepatic flow rates. In this review we summarise the current understanding of endothelial autophagy and how this may be manipulated during NMP-L to reduce liver IRI.

Key words: Autophagy; Liver transplant; Ischaemia-reperfusion injury; Normothermic machine liver perfusion

Core tip: Liver sinusoidal endothelial cells autophagy regulates liver ischaemia reperfusion injury and this process can be targeted for therapeutic benefit using normothermic machine liver perfusion.

INTRODUCTION

The term autophagy is derived from the Greek meaning "eating of self" and the precise cellular role of autophagy has been controversial[1]. However research over the past decade has demonstrated that the evolutionarily conserved process of autophagy is primarily a cell survival mechanism allowing cells and tissues to maintain homeostasis during periods of stress such as starvation and ischaemia[2]. Specifically autophagy eliminates damaged organelles, long-lived proteins or intracellular pathogens through the co-ordinated engulfment of the targeted cargo in a double membrane cytoplasmic structure known as an autophagosome[3,4]. Autophagosomes then fuse with lysosomes to form autophagolysosomes that degrade the engulfed cargo allowing their reuse and thus potentially negating periods of cellular stress[2,4-6]. Hence unsurprisingly autophagy is involved in number of cellular processes such as metabolism, protein synthesis and cellular transportation[4,7]. Dysregulated or uncoordinated autophagy is linked to cell injury and a number of disease processes such as neurodegenerative diseases and cancer[2,4,6,7].

Three distinct types of autophagy have been characterised. The mostly widely studied is macro-autophagy (referred to as autophagy hereafter) and is the primary focus of this review[4,5,8]. Micro-autophagy is characterized by the invagination of the target by the lysosomal membrane itself[5,9]. Chaperone-mediated autophagy targets proteins with the KFERQ motif to the lysosome via interaction with lysosomal-associated membrane protein (LAMP)[9,10]. More recently distinct macroautophagy signalling pathways have been characterised that are activated to specifically eliminate portions of the cell and/or cytoplasm leading to the characterisation of mitophagy (mitochondria), ERphagy (endoplasmic reticulum), xenophagy (microorganisms), lipophagy (lipids) and ribophagy (ribosomes). It is becoming increasingly apparent that specific forms of autophagy are important in the development and pathophysiology of different disease processes. The precise regulation of each type of autophagy is beyond the scope of this review but the reader is referred to recent excellent reviews on the subject[2,4,6,7].

The therapeutic targeting of autophagy has gathered momentum since the publication of the first clinical trials using autophagy inhibitors in treatment of cancers[11-13]. For instance autophagy inhibitors (*e.g.,* hydroxychloroquine) were used in combination with chemotherapy in patients with advanced pancreatic cancer and in those patients with increased levels of the autophagy marker LC3 in peripheral blood mononuclear cells there was an improvement in disease free and patient survival[11]. However in other trials using hydroxychloroquine in patients with glioblastoma the optimal therapeutic dose was not found due to the marked side effects with the investigators concluding that drugs with less toxicity should be awaited[12]. Although these studies have provided the impetus to manipulate autophagy it still remains to be established whether autophagy should be activated or inhibited to derive therapeutic benefit in many disease processes. Moreover many groups working on the therapeutic manipulation of autophagy are now suggesting that the targeting of autophagy during pathophysiological processes needs to tissue and possibly even cell specific[14].

The use of autophagy as a target in treating liver diseases have been the focus of intense recent research[15]. Indeed the manipulation of autophagy may be useful in treating many liver diseases. For instance induction of autophagy/lipophagy may reduce steatosis in fatty liver disease[16] and inhibiting autophagy in hepatobiliary cancers may promote cancer cell death[11,17]. The manipulation of the autophagy signalling pathway also holds significant promise in attempting to reduce the liver ischaemia-reperfusion injury (IRI). IRI is an antigen independent pro-inflammatory process that mediates liver injury following transplantation, liver surgery and haemorrhagic shock[18]. The injury occurs in two distinct phases. In the ischaemia phase blood flow to the liver is interrupted leading to tissue hypoxia and the generation of reactive oxygen species (ROS). During the reperfusion phase although blood flow to the liver is restored there is a concomitant increase in pro-inflammatory mediators, ROS and inflammatory cells that amplifies the liver injury[18]. It is well established that IRI targets and injuries the liver parenchymal cells such as hepatocytes and liver sinusoidal endothelial cells (LSEC)[19]. However early IRI is characterised by LSEC injury and dysfunction[19]. Indeed recent studies demonstrate the key role of LSEC in co-ordinating the livers response to injury whilst also mediating the recovery from liver injury[20]. Thus the targeting of autophagy in the LSEC during IRI is an attractive method with which to reduce liver IRI.

The emerging technology of normothermic machine perfusion of the liver (NMP-L) provides an
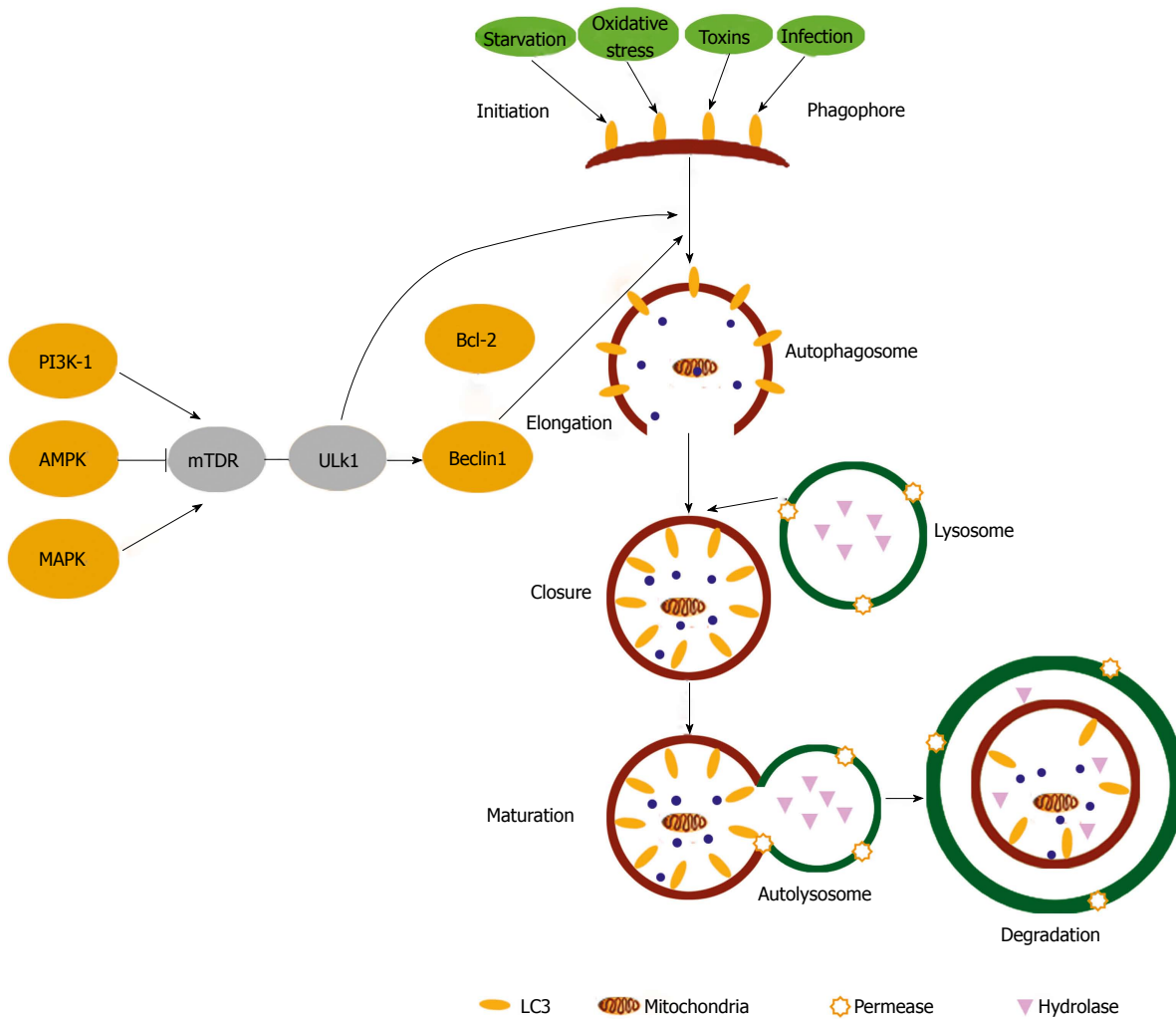
Figure 1 Autophagy signalling pathways. Upstream autophagy activation is regulated by the integration of signalling from a number of pathways including AMPK, PI-3K and the Mitogen-Activated Protein Kinases. Phagophore initiation is directly regulated by the serine/threonine protein kinases ULK1 that forms a complex with Beclin 1. Upon initiation, cytoplasmic constituents are enclosed in a double membraned isolation structure known as an autophagosome that is elongated mainly through the action of two ubiquitin-like conjugation systems. Autophagosomes fuse with lysosomes to form autophagolysosomes, where breakdown of the vesicle contents/cargo takes place along with the autophagosome inner membrane. Autophagy can be activated by many stimuli including starvation, toxins, oxidative stress and infections. (Taken from Shan NN *et al* Targeting autophagy as a potential therapeutic approach for immune thrombocytopenia therapy. *Crit Rev Oncol Hematol* 2016; **100**: 11-15. DOI: 10.1016/j.critrevonc.2016.01.011).

exciting modality with which to target autophagy in LSEC whilst simultaneously allowing an assessment of liver function[21]. There are different NMP-L devices available allowing either selective hepatic artery perfusion or dual hepatic perfusion of donor livers. Using primarily blood based perfusion fluids, oxygen is delivered to donor livers whilst maintaining a normal temperature (37 ℃). NMP-L is typically performed for 6 h and the sequential sampling of perfusates and liver tissue allows a dynamic assessment of liver function. One of the many potential benefits of NMP-L is the reduction in the liver IRI. Therefore, the manipulation of autophagy in LSEC during NMP-L is an attractive therapeutic target with which to improve donor liver organ function prior to transplantation.

AUTOPHAGY SIGNALLING PATHWAY

The autophagy signalling pathway is regulated by

specific and dedicated cellular machinery. These proteins were first isolated in yeast two-hybrid screens and are now known as the Autophagy-related proteins (ATGs). To date 30 ATGs have been characterised that are essential for autophagy induction. Recent reviews by Stork *et al*[21] provide an in-depth review of the pathway whilst a brief overview is given here. In general the autophagy signalling pathway can be divided into distinct phases including the initiation, elongation, autophagosome formation, fusion and autophagolysosomal formation (Figure 1).

The formation of autophagosomes commences upon omegasomes and is known as the initiation phase[22]. This process is regulated by the ATG proteins unc-51 like autophagy activation kinase (ULK1) and WIPI1-4/ ATG18[22,23]. Starvation is a classical activator of autophagy and in nutrient rich conditions the activation of autophagy is inhibited by Mammalian Target of Rapamycin (mTOR)[24]. However when autophagy is
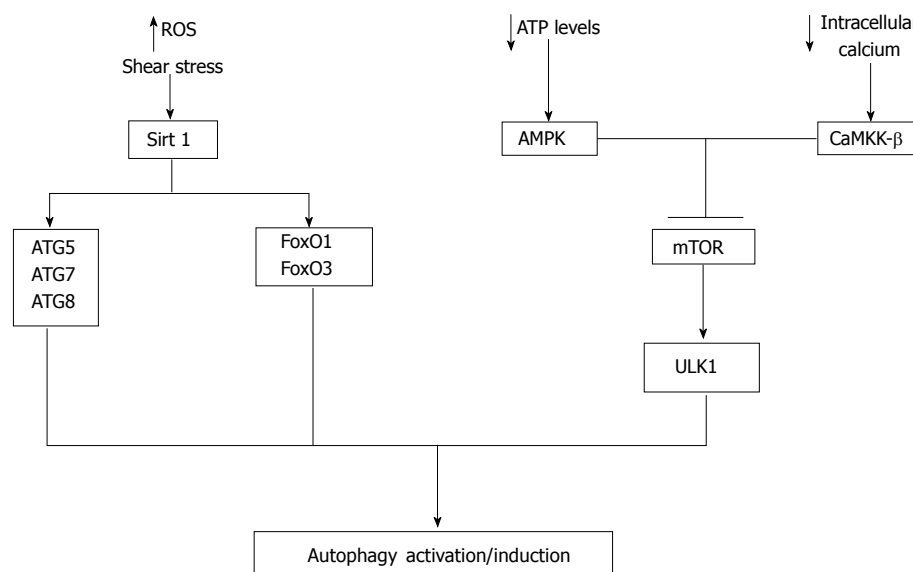
Figure 2 Autophagy activation in endothelial cells. A number of mechanisms potentially regulate autophagy activation in endothelial cells. A decrease in cellular ATP or reduction in growth factors availability leads to the activation of AMP-activated protein kinase (AMPK). Once activated AMPK can inhibit mTOR leading to the activation of ULK1 and hence autophagy activation. In addition decreases in intracellular calcium can activate CaMKK-β leading to mTOR inhibition and autophagy activation. Moreover, Sirt1 can activate autophagy *via* deacetylation of ATG5, ATG7, ATG8 and increased transcription of FoxO1 and FoxO3 that then regulate the expression ATGs *via* deacetylation of Akt. Reactive oxygen species (ROS) and shear stress are important regulators of Sirt1 activity.

activated, the inhibitory effect of mTOR is lost allowing for the activation of ULK1 kinase complex during the initiation phase[9]. In addition autophagy can also be initiated by AMP-activated protein kinase (AMPK)[25] (Figure 1). Subsequently there is recruitment of class Ⅲ phosphatidylinositol 3-kinase (PI3-K), Vps34 and Beclin-1/ATG6 to the developing autophagosome[5,9]. ULK1 is then able to activate the PI3-K complex leading to the activation of Beclin-1 and the later phases of autophagy. This pathway details the canonical autophagy signalling pathway but recent evidence suggests that autophagy can be activated by Beclin-1 independent mechanisms/non-canonical pathways too[9,26].

The elongation phase of autophagy involves two separate ATG conjugation systems. Firstly ATG12 conjugates ATG5 *via* ATG7/ATG10. The ATG12-ATG5 complex is then able to interact with ATG16 resulting in the formation of the ATG12-ATG5-ATG16L complex[5,27]. The second conjugation system involves LC3-I/ATG8 that is cleaved by ATG4 in a process requiring ATG7 and ATG3. This culminates in the formation of LC3-Ⅱ [5,28]. Both these conjugation systems are integral to autophagosome membrane expansion[4,7,9]. At the point at which appropriate membrane expansion has been reached there is closure of double layered membrane that is regulated by amongst other proteins the docking protein p62 which is located on the outer membrane of the autophagosome and is responsible for docking with the lysosome *via* a dynein-dependent mechanism[5]. Thereafter, lysosomal acid hydrolases degrade the engulfed content/cargo and lysosomal efflux permeases release the final products to the cytosol for anabolic processes (Figure 1).

AUTOPHAGY AND ENDOTHELIAL CELLS

Regulation of autophagy activation in endothelial cells

Much of our understanding of the role of autophagy in endothelial cells, such as LSEC, has come from the study of endothelial cell biology in cardiovascular disease. Recent studies have demonstrated how autophagy dysfunction in endothelial cells contributes to the pathogenesis of atherosclerosis by regulating angiogenesis, haemostasis and nitric oxide pro-duction[9].

In endothelial cells upstream activation of the au-tophagy is regulated by the integration of signalling from the AMPK and mTOR-ULK1 pathways[9] (Figure 1). AMPK can detect decreases in cellular ATP levels and activate autophagy primarily to provide substrates for energy used for essential cellular processes[9,29] (Figure 2). Specifically AMPK phosphorylates the Tuberous Sclerosis 2 (TSC2) protein that inhibits mTOR and activates autophagy in an analogous mechanism to that seen during starvation[9,29-33]. In HeLa cells starvation increases intracellular calcium levels and can activate AMPK through Calmodulin-dependent protein kinase kinase-β (CaMKK-β) that then subsequently activates autophagy[9,34,35]. In endothelial cells CaMKK-β mediated AMPK activation inhibits mTOR leading to ULK1 upregulation and autophagy activation[34]. Thus in endothelial cells the activation of autophagy is a dynamic interplay between AMPK, intracellular calcium, mTOR and ULK-1.

The deacylation protein Sirt1 has attracted recent attention as an important regulator of autophagy activation. Specifically Sirt1 can deacetylate ATG5, ATG7, and ATG8[9,36] whilst activating the transcription

factors Forkhead box O (FoxO) FoxO1 and FoxO3. Both FoxO1 and FoxO3 can regulate the expression of ATGs *via* deacetylation of Akt[9,35,37-39]. Indeed during liver IRI ROS can regulate the activation of Sirt1, which may have important implications for endothelial cell autophagy during NMP-L[9,40-43]. Specifically perfusion fluid used during NMP-L induces tangential force across the endothelium causing shear stress that is associated with autophagy activation *via* the Sirt1-FoxO pathway[43]. Low shear stress is associated with reduced levels of autophagy suggesting an important relationship between shear stress and autophagy[44]. Therefore autophagy activation in endothelial cell can be regulated by multiple signalling pathways and at present is remains to be established which of these pathways is important during NMP-L.

Effects of autophagy on endothelial cell function

Autophagy activation has recently been demonstrated to have a number of important effects upon endothelial cell function. Following endothelial injury exposure of the sub-intimal layer promotes glycoproteins, expressed on platelets, to bind to subendothelial von Willebrand Factors (vWF) leading to the formation of a platelet thrombus and production of thromboxane A2, serotonin and adenosine diphosphate[34]. Recent studies demonstrate that vWF is found in close proximity to autophagosomes in endothelial cells[35]. The inhibition of autophagy impairs the secretion of vWF from endothelial cells suggesting that endothelial cell autophagy may have an anti-thrombotic function[45].

In endothelial cells, endothelial nitric oxide synthase (eNOS) regulates nitric oxide (NO) production that in turn regulates vascular tone, platelet aggregation and leukocyte adhesion[46]. Indeed this may be a central function of endothelial cells during liver IRI and a potential protective mechanism induced within the liver by NMP-L. Furthermore eNOS can regulate autophagy induction[47,48]. The exact mechanisms involved in eNOS/NO-autophagy axis are not yet understood although impaired autophagy does result in reduced eNOS function reduced NO production[49].

As eluded to earlier blood flow within the liver generates shear stress across the endothelium that is associated with induction of eNOS and NO production[50]. This is in turn associated with a reduction in oxidative stress and inflammation in endothelial cells. Disturbed shear stress generated by non-laminar flow is associated with endothelial dysfunction that can be implicated in disease processes such as arthrosclerosis[50]. Hence low shear induces autophagy dysfunction[48] which is associated with insufficient autophagy activation in endothelial cells and thus endothelial dysfunction[43]. NMP-L may aid endothelial cell autophagy by ensuring laminar flow within the hepatic vasculature thereby prompting autophagy leading to increased eNOS transcription and NO production. In summary, physiologic shear stress is an essential mechanism for the maintenance of endothelial cells function and is in part mediated by autophagy activation[47].

Autophagy activation is also associated with angiogenesis in endothelial cells particularly within ischaemic microenvironments[51]. The relationship between redox signalling and autophagy is complex and out of the scope of this review although it has been recently reviewed[42]. Autophagy regulation of angiogenesis appears to be dependent upon the activation of the Akt signalling pathway in endothelial cells although the precise mechanism remains to be determined[9,51,52].

Therapeutic targeting of autophagy in endothelial cells

The evolving understanding of the autophagy signalling pathway has lead to the targeting of the pathway for potential therapeutic benefit in many clinical scenarios. However what is becoming evident from these studies is that for the successful therapeutic targeting of autophagy the timing of the intervention, the part of the autophagy pathway targeted, the cell type targeted and whether autophagy should be inhibited or activated are all critical factors. The targeting of autophagy within endothelial cells remains a nascent field with few studies investigating this area.

Epigallocatechin gallate, found in green tea, can induce the specific form of autophagy known as lipophagy in endothelial cells. Lipophagy causes the specific elimination of lipid droplets. In artheroscelosis the degradation of lipid droplets in vascular endothelial cells can potentially modulate the disease process by allowing endothelial cells to resist the effects of lipotoxicity[35,53]. In human umbilical vein endothelial cells (HUVEC) exposed to oxidative stress, vitamin D dependent autophagy activation prevents cell death by activating Beclin1 that prevents mitochondrial depolarisation and caspase activation[54]. Pterostilbene can activate AMPK to stimulate autophagy in HUVEC promoting the elimination of excess lipids and thus reducing apoptosis[53] whilst other groups have suggest that Advanced glycation end-products (AGEs) may regulate the activation of autophagy in this scenario[55]. Furthermore the autophagy activator rapamycin can increase the viability of HUVEC during starvation[56]. These limited studies do suggest that selective activation of autophagy in cells of endothelial lineage promotes cell survival.

Very few studies have addressed the role of autophagy in endothelial cells *in vivo*. Torisu *et al*[45] reported, using a Cre-lox conditional ATG7 endothelial knockdown mice, that inhibition of autophagy leads a reduction in the secretion of vWF suggesting that autophagy may have a role in preventing thrombosis. However the role of endothelial cell autophagy *in vivo* need much more investigation.
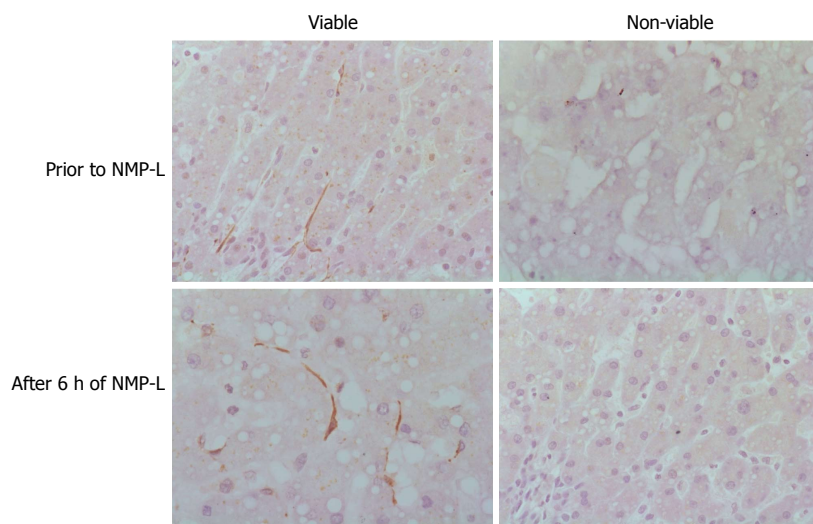
Figure 3 Autophagy and liver viability following normothermic machine liver perfusion. Immunohistochemical analysis was performed for the autophagy protein LC3B in liver tissue prior to normothermic machine liver perfusion (NMP-L) and after 6 h of NMP-L. Livers deemed viable after NMP-L demonstrated LC3B immunostaining in the hepatic sinusoids prior to commencing NMP-L and at the end of perfusion. Donor livers not fulfilling viability criteria demonstrated no LC3B Immunostaining.

THERAPEUTIC MANIPULATION OF ENDOTHELIAL CELL AUTOPHAGY DURING NMP-L

NMP-L is a novel technique that can be employed to assess and recondition donor livers prior to transplantation. One of the potential benefits of NMP-L is the potential reduction in liver IRI especially when compared to traditional static cold storage[57,58]. Recent studies have established haemodynamic and biochemical parameters that allow donor livers to be classified as viable and non-viable where viable denotes a liver that can be transplanted following NMP-L. Indeed donor livers represent conditions were autophagy is expected to be activated; relative tissue ischaemia and reduced availability of nutrients. Recent data from our laboratory has shown that donor livers that demonstrate these viability criteria shows increased levels of autophagy within hepatic sinusoids as assessed by immunohistochemical analysis of the specific autophagy marker LC3B (Figure 3). This suggests a relationship between autophagy induction and liver viability although further work is needed to fully establish this relationship.

As discussed above targeting of autophagy needs to be considered in terms of the timing of intervention, the cell type targeted and the part of the autophagy pathway targeted. During NMP-L there are two forms of intervention that could modulate autophagy in endothelial cells; mechanical or pharmacological.

Livers exposed to NMP-L are associated with increased cellular ATP levels in comparison to cold static stored livers. For instance in a porcine model of donation after cardiac death (DCD) livers exposed to 1 h warm ischaemia, followed by 2 h of cold ischaemia and then 4 h of NMP-L increased cellular ATP levels by 80% in comparison with livers maintained in cold static storage for 2 h[59]. Higher ATP levels within liver grafts prior to liver transplantation are associated with better patient outcomes in some series[60,61]. Although it must be remembered that these studies have assessed global liver ATP levels and not specifically LSEC ATP levels. Additionally, NMP-L is associated with preservation of liver architecture and integrity of the mitochondria[59]. The regulation of mitochondrial function and number is regulated in large part by the specific autophagy process termed mitophagy[62]. The regulation of mitophagy during NMP-L and liver IRI is now being actively investigated as a potential method to reduce liver injury following transplantation[62]. The manipulation of mitophagy in LSEC is emerging as a method to reduce liver injury. However despite this it remains to be established whether LSEC autophagy contributes to increased ATP levels during NMP-L and whether autophagy, through promoting survival of LSEC, improves the survival of neighbouring liver parenchymal cells.

NMP-L may also regulate autophagy activation through calcium signalling. During NMP-L the perfusion fluid is supplemented with calcium to maintain physiological extracellular levels of the ion ensuring that the electrochemical gradient of calcium is maintained across cell membranes[58]. Low intracellular levels of calcium are reported to induce CaMMk-β activation followed by mTOR inhibition and therefore ULK1/autophagy activation[34]. Physiological calcium levels during NMP-L potentially promote normal autophagy activity within LSEC and other liver cells ensuring homeostasis is maintained.

The mechanical manipulation of autophagy during NMP-L is dependent upon shear stress. NMP-L has the advantage of providing an adjustable vascular/laminar flow rate to livers. In turn these flow rate provides

a near physiological shear stress known to promote autophagy induction[43]. Activated autophagy in endothelial cells is fundamental for eNOS transcription, NO production and maintenance of vascular tone maintenance[47]. Increased NO may also reduce platelet aggregation and leukocyte adhesion in endothelial cells thus reducing IRI and hepatic microcirculatory disturbance[63].

Pharmacological modulation of autophagy during NMP-L may also allow the targeting of endothelial cells dysfunction in donor liver grafts prior to transplantation. Extended criteria liver donors (*e.g.,* DCD grafts and steatotic grafts) tend to be associated with lower cellular ATP content and increased ROS production increasing their susceptibility to IRI[57,59]. Pharmacological agents promoting activation of autophagy during NMP-L may promote elimination of damaged organelles and toxins prior to graft implantation. In particular aiding mitophagy function may be crucial to maintaining LSEC function during NMP-L and thus aiding the survival of other liver cells. As described above, many drugs are already known to regulate autophagy activation and the use of these drugs in experimental NMP-L perfusion offers an important method to evaluate these interventions. This manipulation of autophagy in LSEC using NMP-L is an exciting area of interest for research groups working on liver IRI particularly as this technology will become more widespread in addition to the development of new autophagy modulating drugs.

CONCLUSION

In summary, autophagy is a complex metabolic process that is essential for the cell survival; it promotes clearance of harmful substances and provides energy to the cell during cellular stress. The role of autophagy in endothelial cells and its manipulation using NMP-L offers significant promise in reducing liver IRI thereby improving the quality of donor liver organs used for transplantation.

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