

Intracellular Signaling in Ischemia/Reperfusion Injury (IRI): From Mechanistic Insights to Therapeutic Options

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Abstract

Transplantation of solid organs is invariably linked to a disruption of oxygen and nutrient supply. Damage initiated in the ischemic period is greatly enhanced during reperfusion. In particular the excessive production of reactive oxygen species (ROS) plays a key role in the development of ischemia/reperfusion injury (IRI), which in the clinical setting is difficult to control through the use of antioxidants. Ischemia/reperfusion (IR) is also marked by the activation of intracellular signaling pathways, which may have protective but also damaging effects. Modulating intracellular signaling thus may hold the promise to prevent or minimize IRI. Most intriguingly, some of these pathways have been shown recently to control mitochondrial events, including the production of ROS. Understanding this cytoplasmic/mitochondrial crosstalk will be the basis for the development of novel approaches for the prevention of IRI.

Introduction

Organ transplantation is essential to assure prolonged survival beyond the step of individual organ failure. Besides the challenges inherent in the surgical procedures, the prevention of rejection was the main obstacle in the past on the way to clinical implementation. A major problem, which still persists, is directly associated with the need to procure an organ from a donor and to implant it in a recipient. This causes disruption of blood and oxygen supply (ischemia) and their subsequent restoration (reperfusion). Prolonged ischemia by itself is a condition of cellular stress eventually resulting in cell death. Reperfusion is vital for organ recovery and continued function. However, it has been observed that resumption of the metabolic activity is linked to the collapse of mitochondrial and cellular homeostasis. Lack of ATP production, inability to maintain ion gradients across membranes, excessive production of reactive oxygen species (ROS) and perturbation of Ca^{2+} trafficking, leading to mitochondrial Ca^{2+} overload and cell death occur during this time period. Cells, which are key to organ function are usually metabolically highly active and thus will be affected most prominently. As a consequence malfunction or death of a relatively low number of cells will have major consequences. Collectively these changes leading to temporal or permanent functional impairment of an organ are referred to as ischemia-reperfusion injury (IRI).

Intracellular signaling as a mode of communication and regulation in physiological and non-physiological processes is well documented. Most obvious is this in settings where the function of signaling proteins is affected by mutations resulting in the gain or loss of function. Understanding aberrant signaling in disease and pathological conditions holds the promise for novel therapeutic approaches. Reactive oxygen species (ROS) which are abundantly produced early during reperfusion may have direct toxic effects on biomolecules (nucleic acids, proteins, lipids) but also function as signaling molecules. However, canonical signaling pathways are also activated, both during ischemia and upon reperfusion. This review will attempt to emphasize the concept that the crosstalk between these two modes of signaling is important for shaping the outcome of IR. Understanding its mechanisms thus may provide novel therapeutic approaches. We do not intend to cover the whole field of signaling in ischemia/reperfusion with its often conflicting data, but restrict ourselves to the discussion of general processes and regulatory mechanisms, which are at work during IR in a largely organ-independent fashion.

Signaling at the Mitochondria: ROS, Ca^{2+} , - Big Tasks for Small Molecules

Mitochondria are essential for cell survival, both because of their roles as energy producers and as regulators of programmed cell death [1]. Our current understanding of IRI sees perturbation of mitochondrial homeostasis as a main initiating step. Such deviations from the physiological state of mitochondria result among others in abnormally high mitochondrial Ca^{2+} levels and increased oxidative stress [2]. Mitochondrial dysfunction thus is a major feature of IRI, in its extremist leading up to necrotic or apoptotic cell death. During ischemia the lack of oxygen inhibits electron flow through the electron transport chain resulting in a shortage of ATP. The arising lack in ATP is partially resolved by a switch to anaerobic glycolysis leading to intracellular acidification. In the attempt to restore the intracellular pH the Na^+/H^+ exchanger (NHE) is activated increasing cellular Na^+ levels. This leads to the activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCE) raising cellular Ca^{2+} levels and causing mitochondrial Ca^{2+} overload and depolarization. During reperfusion repolarization of the mitochondrial transmembrane potential coupled with an increased cytosolic Ca^{2+} leads to a further increase in mitochondrial Ca^{2+} via the calcium uniporter (CaU). With the recovery of the pH, high Pi, excessive ROS and Ca^{2+} overload upon reperfusion, opening of the mitochondrial permeability transition pore (mPTP) is favored [3]. The mPTP is a multiprotein complex forming non-selective pores in the inner mitochondrial membrane (IMM). Long-lasting mPTP opening can lead to excessive water entry into the matrix, matrix swelling and outer mitochondrial membrane (OMM) rupture. This causes the release of pro-apoptotic molecules from the intermembrane space (IMS) leading

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to cell death via caspase-dependent or -independent mechanisms [1]. Recent evidence suggests that mitochondrial permeability transition in ischemia reperfusion injury is not triggered by the same proapoptotic members of the Bcl-2 family [4] normally involved in this process but that mitochondrial ROS causes mPTP opening, mitochondrial depolarization and cell death [5]. Mitochondria also respond to cellular stress with changes in their morphology by undergoing fission resulting in fragmented mitochondria. Inhibiting the collapse of the mitochondrial network was shown to be protective in a model of simulated IR [6].

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide or hydroxyl radical are products of normal oxygen metabolism in living cells. They are highly reactive small molecules potentially harmful for cellular components like proteins, lipids or nucleic acids [7]. However, ROS, especially hydrogen peroxide, can be beneficial for cells and tissues mainly through their function in normal cellular signaling [8]. Therefore, levels of ROS in a cell must be tightly regulated. Cells possess several sources for ROS production including mitochondria, peroxisomes, NAD(P)H oxidases, nitric oxide synthase and xanthine oxidase, and complex anti-oxidant defense machineries for their elimination that includes enzymatic (e.g. catalase, superoxide dismutase, glutathione peroxidase) as well as non-enzymatic systems (e.g. glutathione and vitamins A, C and E) [9]. At the physiological level ROS control the function of signaling proteins through redox modification [10,11]. Different stimuli like growth factors and cytokines induce ROS formation [8] and transcription factors such as AP-1 and NF κ B have been shown to be activated by ROS resulting in the expression of genes associated with inflammatory and immune responses [12,13]. Excessive production of ROS, has been implicated in many pathologies, including cancer, hypertension, type II diabetes, atherosclerosis, chronic inflammatory processes, various neurodegenerative diseases and IRI [14]. Their essential role in IRI is supported by the studies showing that pretreatment with antioxidants or overexpression of antioxidant enzymes protect cells during IR [15].

Calcium ions are universal second messengers involved in many different intracellular processes including enzyme activation, gene expression, secretion, cell proliferation, cell differentiation and cell death [16]. The concentration of cytoplasmic calcium in resting cells is maintained at a low level, strictly controlled by Ca²⁺ uptake from extracellular space, release from intracellular calcium stores, in the endoplasmic reticulum (ER), the buffering capacity of mitochondria and by proteins capable of binding Ca²⁺ (e.g. calmodulin) [17]. During ischemia/reperfusion the loss of calcium homeostasis is observed, marked by increased cellular and subsequently mitochondrial Ca²⁺ levels resulting in massive ROS production and oxidative stress [18]. Oxidative stress again drives release of Ca²⁺ from ER and contributes to mitochondrial Ca²⁺ overload, which triggers the events leading up to cell death [19].

Signaling Changes in Oxygen and Nutrient Availability

Since inadequate oxygen supply profoundly affects cellular physiology, cells are equipped with the ability to sense and respond to changes in cellular oxygen levels. This involves the HIF-oxygen-sensing transcriptional pathway, which may compensate for hypoxia by regulating the transcription of an increasing number of genes through binding to hypoxia regulatory elements (HRE) [20]. HIF facilitates oxygen supply by advancing iron delivery, improving blood flow by e.g. promoting angiogenesis and reduces oxygen consumption by favoring the switch to the less efficient but lifesaving glycolytic pathway. HIF is a heterodimeric transcription factor consisting of a stable β and a

labile α subunit, which is regulated by hydroxylation of specific proline residues targeting the molecule for rapid degradation via the ubiquitin-proteasome pathway [21]. The stability of the α -subunit and thus signaling via HIF is tightly regulated in an oxygen-dependent manner. Under normoxic conditions HIF α is modified by prolyl-(PHD) and asparagyl hydroxylases (FIH) [22]. Both enzymes are capable of incorporating oxygen into specific amino acid residues of HIF α . The modification of prolyl side chains generates a binding site for proteins of the ubiquitination machinery (von Hippel-Lindau (VHL) complex) targeting the HIF α subunit for protein degradation. Besides protein stability HIF's ability to activate gene transcription is also regulated by intracellular oxygen levels. Hydroxylation of an asparagyl residue in the transactivation domain inhibits interaction with the cofactor p300, circumventing transcription of HRE regulated genes [23]. Oxygen is rate limiting in this type of regulation thus HIF heterodimerization can be accomplished under hypoxic conditions leading to the transcription of target genes.

Equally important is the ability to sense the energy status of the cells. While mammalian target of rapamycin (mTOR) is a central cell growth regulator stimulating energy consuming processes under nutrient rich conditions, AMP activated protein kinase (AMPK) dampens these processes under nutrient poor conditions and gets activated when energy levels are low which is reflected by a high AMP/ATP ratio [24,25]. Two distinct complexes of mTOR can be distinguished with only mTORC1 being sensitive to rapamycin and regulated by nutrients and AMPK. Besides energy stress, growth factors play a major role in mTORC1 regulation. Activation of PI3K pathway and its downstream effector AKT/PKB leads to the phosphorylation and inactivation of the upstream inhibitor of mTORC1 [26,27]. Similar effects have been ascribed to the mitogen-activated protein kinase (MAPK) ERK [28]. Upon ischemia, when growth factors are withdrawn, energy levels are low and oxygen is limited, signaling via the PI3K- and MAPK pathways is dampened while AMPK is activated, thereby alleviating mTORC1 signaling.

Signaling Under Ischemia/Reperfusion

The presence of cellular signaling events during IR is well documented but their regulatory roles are far from completely understood. Evidence comes from the direct study of signaling activities in tissue lysates and the large scale analyses of transcriptional events and post-translational modifications. Also genetically modified mice have been extensively studied to decipher the contribution of individual signaling proteins to the development of IRI. Overall, a complex picture emerges and frequently we lack insight, how signaling activities relate to the development or progression of IRI [29,30]. Mitogen-activated protein kinases (MAPKs) comprise a family of related kinases, which function downstream of similarly evolutionary conserved upstream kinases [31]. They participate in cellular responses to mitogens (ERKs), inflammatory cytokines or unphysiological stimuli (JNKs, p38) [32]. MAPKs are activated during ischemia and/or reperfusion and under these conditions ERK can be cytoprotective or neutral, p38 possesses pro- or anti- apoptotic effects, and also JNK has been discussed controversially [33-35]. Although ROS can lead to the activation of MAPK [36], these kinases may also be involved in modulating ROS levels [37,38]. Our own data showed a role for p38 in the regulation of mitochondrial ROS levels [38], while signaling through RAF-MEK-ERK protected against mitochondrial accumulation of ROS/Ca²⁺ and cell death [37]. Activation of NF κ B occurs in response to multiple stimuli and results in the transcription of an equally large number of target genes [39]. During IR NF κ B signaling may have both beneficial (e.g. anti-apoptotic) or adverse effects (e.g. induction of pro-inflammatory

cytokines) [32,40-42]. Involvement in the control of IRI has also been suggested for JAK/STAT signaling [43,44]. Also activation of the PI-3 kinase (PI3K)/protein kinase B (PKB/AKT) may be involved in the protection of cardiac cells against hypoxia/reoxygenation-induced cell death [45,46]. The role of innate immune and inflammatory responses is well established in the progression of IRI, manifested by increased expression of proinflammatory and immunoregulatory cytokines during IR [47-50]. TLRs have been recently emerged as putative inducers of these innate immune and inflammatory responses and, more recently, of injury induced inflammation [51,52], making them central players in the development of IRI [53]. High-mobility box 1 (HMGB1) protein released during cellular damage can serve as ligand for TLRs [54]. In cultured hepatocytes HMGB1 release is an active process regulated by ROS [54]. TLRs predominantly activate NF κ B and stimulate the expression of immune and inflammatory responses [53]. Among TLRs, TLR4 and TLR2 have been extensively discussed for their role in IRI. Various studies using the TLR4-deficient mice, TLR4 antagonists, MyD88-deficient mice (MyD88 functions downstream of TLRs in signal propagation), dominant negative mutant of MyD88 have shown the deleterious role of TLR4 during myocardial IRI via NF κ B signaling mediated regulation of inflammatory cytokine production [55-58]. Parallel studies on the other organs such as brain, lung, liver, kidney and intestines that were subjected to IR also showed similar effects [53]. Reduced NF κ B binding activity and increased level of phosphorylated AKT were observed in the myocardium of TLR4-deficient mice subjected to IR. In addition, PI3K inhibition by pharmacological inhibitors completely abolished the cardioprotection in TLR4-deficient mice after myocardial IR injury, suggesting the presence of a crosstalk between the TLR4 and PI3K/AKT signaling pathways during myocardial IR [53,59,60]. The excessive production of ROS is a hallmark of IRI and has been recently shown to activate immune and inflammatory responses by activation of NF κ B through TLR4 dependent mechanism, suggesting that TLR4 mediated NF κ B activation is required for ROS activated intracellular signaling pathways (e.g. ASK1/p38, IKK- α / β and IRAK). Targeting of the TLR4-mediated NF κ B signaling could minimize ROS induced cellular damage [61,62]. There are controversial reports on the role of TLR2 in IRI, which may be due to the varying experimental conditions used and models employed in the studies.

Besides the pathways discussed above an increasing number of signaling molecules is being tested for a possible role in the development of IRI. Most recently two important developmental pathways were studied in this context: Wnt/ β -catenin signaling was activated by ROS and shown to protect against liver IRI through the activation of HIF1 α signaling [63]. NOTCH signaling affords protection of hepatocytes against IRI through suppression of ROS production [64].

Crosstalk between Cytoplasmic Signaling Cascades and the Mitochondria

Evidence for a link between intracellular signaling and the regulation of mitochondrial ROS production has been provided for p53 [65-67], PKA [68,69] and the survival proteins RAF, AKT and Bcl-2 [37]. STAT3 has been implicated in the regulation of mitochondrial energy production although effects on ROS production have not been studied [70-72]. A direct role in mitochondrial ROS production has been provided for p66SHC [73]. This protein represents the longest isoform of a family of proteins normally functioning as adaptor proteins in the activation of the small G protein RAS, downstream of protein tyrosine kinase receptors [74]. p66SHC is a redox enzyme that generates mitochondrial ROS through oxidation of cytochrome

c [73]. p66SHC^{-/-} mice show on average a 30% prolongation in life span, which correlates with increased resistance to oxidative stress, due to a decreased production of ROS, while scavenging systems are not affected [73]. Further work demonstrates that protein kinase C beta (PKC β) phosphorylated p66SHC on Ser36, which was required for mitochondrial accumulation of the protein [75]. Protection against IRI has been reported in p66SHC deficient mice [76].

Diagnostic Options: Gaining Insights through Real Time Live Confocal Microscopy of Tissue Biopsies

Modern “omics” techniques for large scale protein and RNA expression screens have been applied to the study of IRI. Normal and genetically modified cells and animals have been used to address cellular processes and important regulators. The complexity of the events occurring during and after IR makes it a challenging task to link signaling to functional outcomes. Their study in transplanted organs requires novel approaches. Every organ consists of various cell types, which differ in function, metabolic activity or the nature of neighboring cells. These factors have pronounced effects on survival under cellular stress and may cause heterogeneity in cellular responses to IR. These complex responses are hard to document with classical biochemical assays, which only give us a momentary picture obtained from the entirety of cells present in an organ. A method, which is suitable to document stress or death in cells, tissues and even organs *in vivo*, *ex vivo* as well as *in vitro* in non fixed cells is Real Time Live Confocal Microscopy [77,78]. To gain functional insights into cellular changes occurring under IR and their regulation by signaling cascades, we have adapted this method to the study of fine needle biopsies obtained from the organ of interest, e.g. kidney, followed by live cell imaging with a confocal microscope allowing live cell imaging [79]. This method allows us to monitor various physiological parameters in defined compartments of complex organs like kidney with the perfect maintenance of the structural integrity. A wealth of fluorescent dyes is available, which allows monitoring of many parameters, e.g. ROS or Ca²⁺. This also provides insights into compartmentalized responses, as we expect that different structures in an organ will respond differently. Living tissues used in these studies may be maintained for hours in culture allowing manipulations like testing of signaling inhibitors, antioxidants or the performance of hypoxia/reoxygenation assays. In the example provided here, we obtained biopsies from rat kidneys spanning the whole length from the outer capsule to the innermost hilus through the kidney cortex and medulla. The biopsies were immediately transferred to tissue culture medium and incubated under normal culture conditions and live cell staining was performed. Exemplary stains are shown for Syto 16, propidium iodide (PI), tetramethylrhodamine methyl ester perchlorate (TMRM) or FITC-coupled wheat germ agglutinin (WGA) to visualize all nuclei, nuclei of dead cells, active mitochondria and cell/tissue morphology (Figure 1).

Conclusions and Outlook

Analyses of intracellular signaling during IR have provided insights into the complexity of these events. Further progress will mainly depend on understanding precisely the contribution of individual pathways to the progression or prevention of damage as a basis for future therapeutic interference. Of particular importance will be a detailed resolution of the sequence of events leading up to the manifestation of IRI. Given the proposed importance of ROS, produced early during reperfusion, for setting the stage for all the events to follow, we have to understand a possible crosstalk between early cytoplasmic signaling and mitochondrial events. ROS may be central players during IRI, which connect early events to later ones like the activation of the

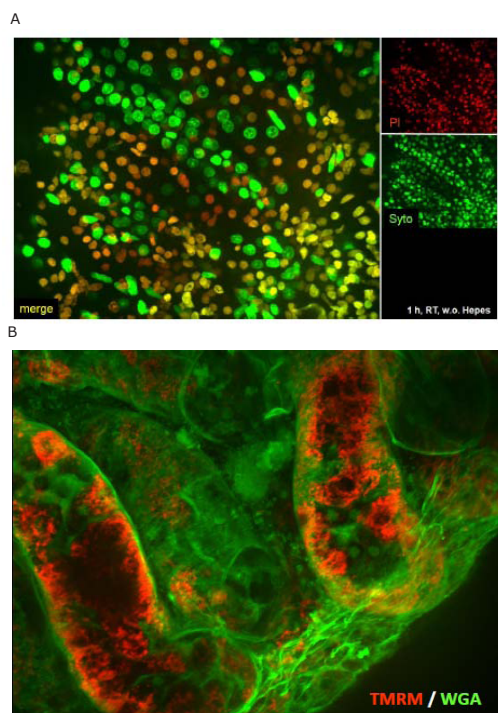


Figure 1: Application of live cell confocal imaging to the analysis of rat kidney biopsies.

(A) A combination of PI and Syto 16 was used to visualize the nuclei of dead (PI, red) and the nuclei of all cells (Syto 16, green) in the tubular area of the kidney. The insets at the top right side shows the single channels, the big image shows the merged image. The biopsy was incubated for one hour in standard culture medium at RT. Note the heterogeneity in cell death between neighboring tubules. (B) This image shows the result after staining such a kidney biopsy with TMRM (red) and WGA (green). Like in (A) the heterogeneity in cell survival is documented by TMRM fluorescence. Images were acquired using a 40x water immersion objective.

inflammasome or the regulation of autophagy. An increasing wealth of data supports the notion that mitochondrial function is regulated by intracellular signaling pathways, raising the hope for a therapeutic intervention before ROS are released, which are difficult to scavenge with existing antioxidants. Also; ROS are important modulators of classical signaling pathways and thereby affect cellular responses. Dissecting their contribution to the development of IRI may identify additional targets for therapeutic interference.

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