Chapter 3

The Hypoxia-Reoxygenation Injury Model

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Additional information is available at the end of the chapter

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Abstract

Hypoxia-reoxygenation injury is a commonly used *in vitro* model of ischemia, which is useful to study the recovery processes following the hypoxic period. Hypoxia can be rapidly induced *in vitro* by replacing the culture atmosphere with hypoxic or anoxic gas mixture. Cellular injury mostly occurs as a result of energetic failure in this model: the lack of oxygen blocks the mitochondrial respiration and anaerobic metabolism becomes the major source of high-energy molecules in the cells. In the absence of glucose, glycolysis and pentose phosphate pathway fail to suffice the cellular energy prerequisite and longer periods of oxygen-glucose deprivation (OGD) can completely deplete the cellular NAD^+ and ATP pools. The lack of NAD^+ results in severe metabolic suppression and predisposes the cells to other injury types. This includes oxidant-induced damage, since oxidative stress activates poly(ADP-ribose) polymerase (PARP) that further depletes the cellular NAD^+ pool and leads to excessive cell death. The impaired mitochondrial respiration also leads to an increase in the mitochondrial membrane potential and augments the mitochondrial superoxide generation leading to oxidative stress. The above processes ultimately lead to necrotic cell death, but in certain cell types, mitochondrial damage can also trigger apoptosis.

Keywords: hypoxia-reoxygenation injury, poly(ADP-ribose) polymerase, energetic failure, mitochondrial dysfunction, oxidative stress

1. Introduction

This chapter gives an overview of the hypoxia-reoxygenation model, provides guidance to perform hypoxia-reoxygenation or oxygen-glucose deprivation (OGD) experiments and discusses the mechanism of cellular damage in this model.

In vivo ischemia-reperfusion models are technically simple and reproduce many aspects of ischemic diseases, but *in vitro* models are equally important, because they allow detailed study of the mechanism of cellular damage and make it possible to test large chemical libraries or sets of human small interfering RNAs (siRNAs) that are essential for early phase drug discovery [1–5]. Chemical hypoxia models that use mitochondrial uncoupling agents or respiration

blockers reproduce the rapid onset of ischemia but there is no way to study the recovery processes that occur during reperfusion [6–9]. The significance of true hypoxia-reoxygenation models is in the capacity for recovery from the hypoxic phase, which makes these models especially useful for ischemia-related experiments. Oxygen-glucose deprivation is a variation of the hypoxia model to mimic the shortage of nutrients in ischemia.

2. Hypoxia-reoxygenation induction

The hypoxia/OGD models are simple experimental models that do not require expensive laboratory instruments. Regular cell culture plasticware can be placed in a gas-tight chamber and the culture atmosphere replaced with oxygen-free gas mixture using an inexpensive flow meter. In addition, OGD can be induced by replacement of the culture medium with glucose-free medium. The reoxygenation period is initiated by glucose supplementation and by returning the culture vessels to regular atmosphere. The severity of the injury can be adjusted to specific needs by varying the length of the hypoxic/OGD period. Therapeutic interventions may be delivered prior to hypoxia induction or immediately following the reoxygenation modelling preventive or reperfusion therapies.

In most hypoxia experiments, above the hypoxic and OGD groups it is essential to use normoxia controls with normal glucose concentration or to expose normoxic controls to glucose deprivation (GD). Since the normoxic and hypoxic cells must be physically separated during the hypoxic period, identical cell plates must be prepared for the hypoxia and simultaneous normoxia exposures. Culture medium is replaced with fresh medium either containing glucose or without glucose prior to the induction of hypoxia. Serum deprivation may be necessary for complete removal of glucose in OGD injury. To induce hypoxia, the culture plates are placed in gas-tight incubation chambers (Billups-Rothenberg Inc., Del Mar, CA) and the chamber is flushed with oxygen-free gas mixture at 25–30 L/min flow rate for 5–10 min to completely remove oxygen [1–5, 7, 10]. Hypoxia is maintained by clamping and incubating the chambers at 37°C for the requested period. The composition of the gas mixture may vary depending on the bicarbonate content of the culture medium and the required level of acidity change (pH level), since hypercapnia can mimic the rapid development of acidic pH of ischemic tissues [11]. The CO_2 content is typically between 5 and 20% with 80–95% N_2. This procedure removes oxygen from the atmosphere but dissolved oxygen remains in all fluids in the chamber including the culture medium and additionally in water used for humidification, thus complete anoxia is reached with a delay, following depletion of the remaining oxygen. Following the hypoxic exposure, restoration of the normal culture conditions is achieved by supplementing the culture medium with glucose and foetal bovine serum (FBS) and by reoxygenating the culture vessels in regular culture atmosphere. In most cells, the cellular ATP level is recovered during a recovery period of 16–24 hours that might be the period of interest in most experiments.

Drug treatments may be administered before the hypoxia induction to test preventive effects or following the hypoxic period to test the therapeutic potential in ischemic diseases [1–3]. For gene silencing small interfering RNAs may be added 48 hours prior to the hypoxia exposure to

effectively reduce RNA and protein levels of the gene of interest at the time of the hypoxia experiment [4, 5]. Unfortunately, gene silencing cannot be selectively used to study the hypoxic or the reoxygenation phase. Pharmacological treatments using small compounds allow specific post-hypoxic treatments that permit the specific study of the recovery phase.

3. Mechanism of cellular damage in hypoxia-reoxygenation injury

3.1. Cellular energy depletion

Hypoxia and glucose deprivation cause energy depletion in the cells and may be directly responsible for the viability reduction caused by the injury. Since the lack of oxygen blocks aerobic metabolism, which is responsible for the larger part of ATP production in the cells, the cells need to use other pathways to produce sufficient ATP for survival. Most cells can adapt to low oxygen conditions in cell culture, producing ATP solely by anaerobic metabolism if adequate glucose supply is present. However, the anaerobic pathways, glycolysis and pentose phosphate pathway need to use high amounts of glucose to produce comparable output. Glycolysis produces only two ATP molecules, but oxidative phosphorylation is capable to produce ~30 ATPs per glucose molecule oxidized [12]. The typical mitochondrial ATP production is lower than the theoretical maximum, since up to 20% of the basal metabolic rate may be used to drive the proton leak [13], but it is still more than 10 times higher than the anaerobic ATP production. The compensatory increase in anaerobic metabolism would be stopped by the limited availability of NAD^+, since protons are transferred to NAD^+ by glyceraldehyde phosphate dehydrogenase to produce NADH during glycolysis, if lactate dehydrogenase (LDH) did not recycle NAD^+. This step helps maintain the higher anaerobic metabolic rate, but at the expense of metabolic acidosis (lactic acidosis).

However, in the absence of glucose, the ATP production will drop rapidly as the cellular energy storage is depleted and cell death will be induced. Most cells can survive in culture if the cellular ATP concentration will be reduced by less than 75–80% the normal ATP level [1–3, 5]. Following an OGD injury that does not reduce the cellular ATP concentration below 20% of the initial baseline value full recovery is expectable if optimal culture conditions are provided. Since the cells try to maintain normal ATP level and use all resources that can be utilized for energy production during the OGD phase, the recovery process is time-consuming: all precursor molecules need to be resynthesized in the cells. A more robust injury that decreases the cellular ATP concentration below 20% will initiate severe viability loss in the cell population [2] (**Figure 1**).

The cellular energy production remains impaired following an OGD injury: the cellular ATP production is slow even if the energy sources are provided in liberal amounts. The loss of all high-energy molecules is responsible for the diminished ATP synthesis following OGD. Not only ATP, but also adenosine diphosphate (ADP) and NAD^+ are greatly reduced in the cells to minimize the ATP loss that will sustain the metabolic suppression [5]. ATP is the central coenzyme in the cells that functions as universal energy currency to transfer chemical energy. ATP molecules are generated in large quantities by constant recycling of ADP to ATP; the daily

estimated ATP synthesis is around 1000 g/kg bodyweight [14]. Organic compounds are catabolized via a series of redox reactions in the cells and ultimately generate carbon dioxide and water. During these reactions, energy is collected via transferring electrons from organic donors to the acceptor molecule NAD^+ and reducing it to NADH. Energy is retrieved from NADH in the mitochondria as the electrons are gradually transferred to oxygen through the electron transport chain and ATP is produced in the coupled oxidative phosphorylation reaction. Thus, the energy stored by NAD^+ molecules is interconvertible to ATP molecules and the lack of NAD^+ can severely limit the ATP generation.

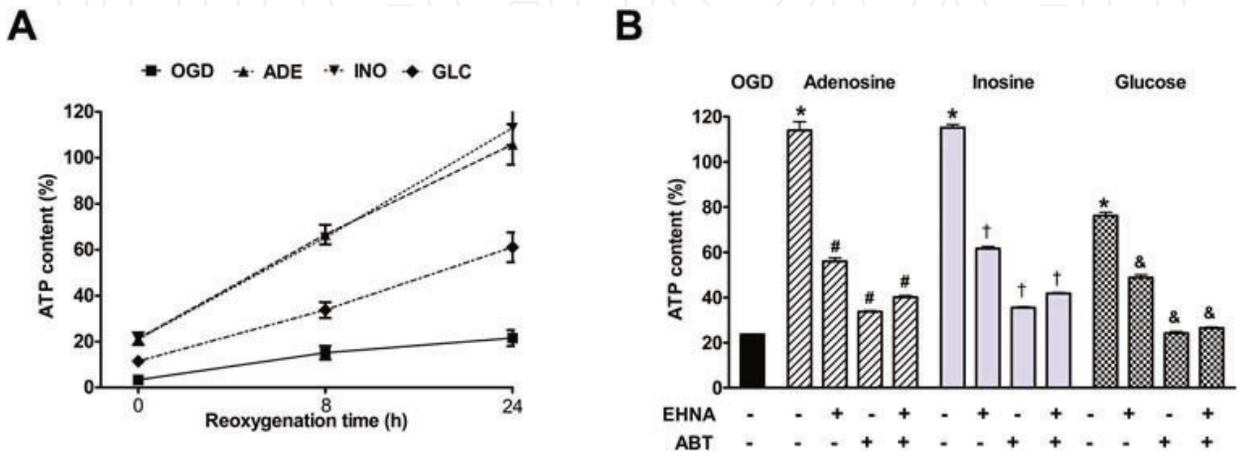

Figure 1. Post-hypoxic recovery of the cellular ATP content. (A and B) LLC-PK1 cells were subjected to hypoxia in the absence (OGD) or presence of 300 μM adenosine (ADE), inosine (INO) or glucose (GLC) to reduce the cellular ATP content to 5, 10 or 20% of normoxic controls, and ATP concentration was measured during the 24-hour-long recovery period. (A) ATP content gradually increased proportional to the hypoxic ATP depletion. (B) ATP resynthesis requires both adenosine deaminase (ADA) and adenosine kinase (AK) activity in the cells. Blockage of ADA by EHNA (10 μM) and/or AK by ABT 702 (ABT, 30μM) blocks the recovery of the cellular ATP content. (Data are shown as mean ± SD values. *$p < 0.05$ compared to OGD, #$p < 0.05$ compared to adenosine, †$p < 0.05$ compared to inosine, &$p < 0.05$ compared to glucose.) From Ref. [2].

NAD^+ biosynthesis occurs either via the *de novo* (kynurenine) pathway from tryptophan or via the *salvage* pathway using nicotinamide as substrate [15–17]. NAD^+ is not only used in cellular energy production reactions catalyzed by dehydrogenases, but it is also utilized by poly(ADP-ribose) polymerases (PARPs) in ADP-ribosylation reactions and by sirtuins in deacetylation reactions that produce nicotinamide [18, 19]. Nicotinamide can be reused for NAD^+ synthesis via the *salvage* pathway: an energy-requiring (endothermic) two-step process that uses ATP. The *salvage* pathway is considered as the main NAD^+ biosynthesis pathway in humans and the major substrate is nicotinamide, since nicotinamide deamidase, the enzyme that catalyzes the conversion of nicotinamide to nicotinic acid, is missing in humans [20]. In the first step, nicotinamide is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NamPRT) using phosphoribosyl pyrophosphate (PRPP) as cosubstrate and one ATP molecule (**Figure 2**). The second step is the conversion of NMN to NAD^+ by nicotinamide mononucleotide adenylyl transferases (NMNATs) that also requires one ATP molecule. This step is catalyzed by NMNAT-1 in the nucleus, NMNAT-2 in the Golgi and NMNAT-3 in the mitochondria [21, 22]. Since the conversion of ribose 5-pyrophosphate (coming from the degradation of ADP-ribose polymers) to PRPP requires a third ATP molecule, altogether three ATP molecules are necessary

for the resynthesis of one NAD^+ molecule [15, 16, 20]. NamPRT is recognized as the rate-limiting enzyme in NAD^+ *salvage*, partly because this step requires more energy, if PRPP synthesis is also considered, and because it relies on a single enzyme, while the cells contain multiple NMNAT isoenzymes. NAD^+ biosynthesis is an energy-requiring process, and it is further complicated by sequestered localization of NAD^+ in the cells: there are separate mitochondrial, cytoplasmic and nuclear NAD^+ pools and they are not completely exchangeable [16]. NAD^+ biosynthesis is estimated to occur at 5g/kg tissue/day [16] suggesting that nicotinamide may be recycled several times a day. Still, the recovery occurs at a slower rate following a severe OGD injury, because the lack of ATP limits the NAD^+ turnover and the low NAD^+ availability blocks the ATP generation from metabolic sources.

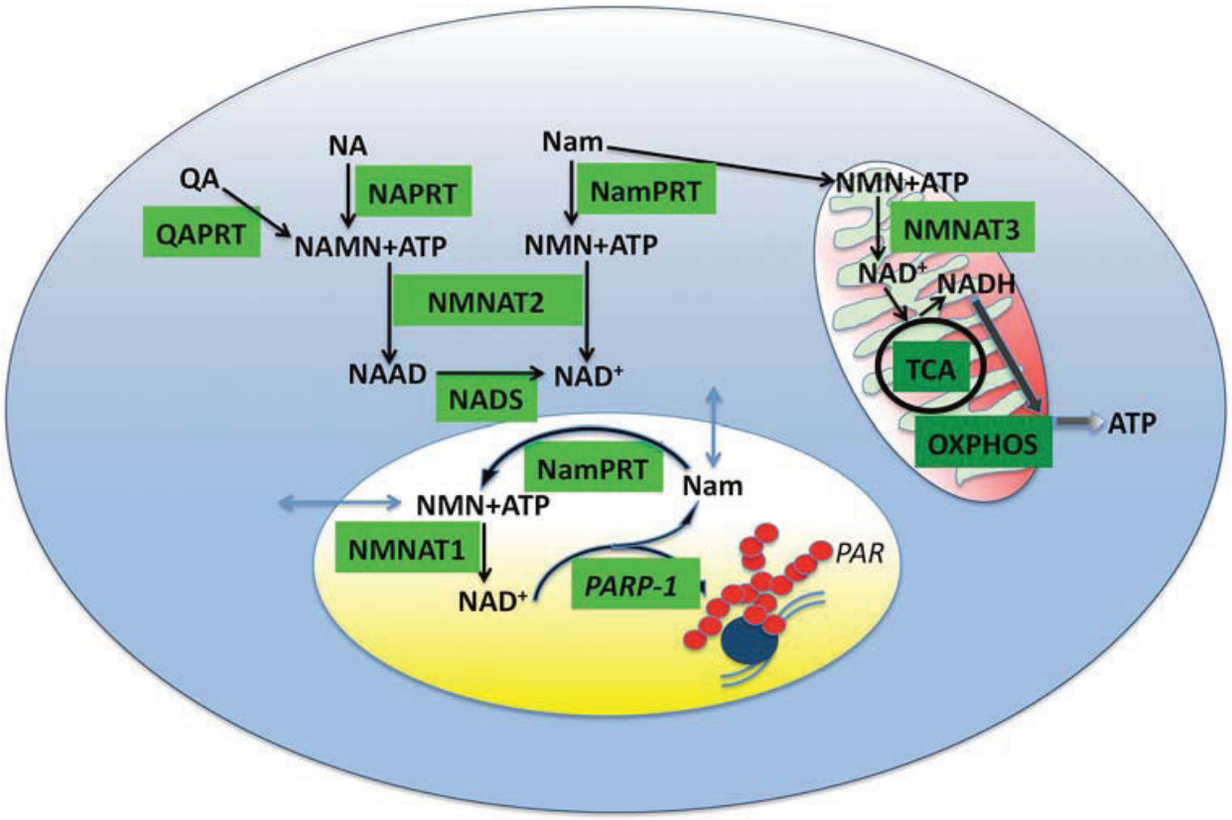

Figure 2. Compartmentalization of NAD^+ biosynthesis. The '*de novo*' synthesis of NAD^+ starts from tryptophan and produces the precursor quinolinic acid (QA), while the '*salvage*' pathway utilizes the NAD^+ break-down products nicotinamide (Nam) and nicotinic acid (NA). QA, NA and Nam are converted to nicotinic acid mononucleotide (NAMN) and nicotinamide mononucleotide (NMN) by the respective phosphoribosyltransferases (QAPRT: quinolinic acid phosphoribosyltransferase, NAPRT: nicotinic acid phosphoribosyltransferase, NamPRT: nicotinamide phosphoribosyltransferase). NAMN and NMN are converted to nicotinic acid adenine dinucleotide (NAAD) and nicotinamide adenine dinucleotide (NAD^+) by transferring the adenylate moiety of ATP to the mononucleotides by compartment-specific NMN adenylyl transferase (NMNAT) enzymes. NAAD is amidated by NAD synthetase (NADS) using glutamine as an ammonium donor. There are three NMNAT isoforms: NMNAT1 is ubiquitous and is localized to the nucleus, NMNAT2 is cytoplasmic and is predominantly expressed in the brain and NMNAT3 is present in the mitochondria. PARP-1 utilizes NAD^+ as a substrate to produce ADP-ribose polymers and nicotinamide.

The lack of NAD^+ affects both mitochondrial respiration and anaerobic metabolism following the OGD injury [5]. Severe metabolic suppression is detectable following the OGD injury if the

resynthesis of NAD^+ is prevented by NamPRT inhibition: the mitochondrial oxygen consumption of the cells is severely reduced in the cells (**Figure 3**). The respiratory capacity of the cells is suppressed following OGD and while normal cells typically use no more than ~50–60% of their

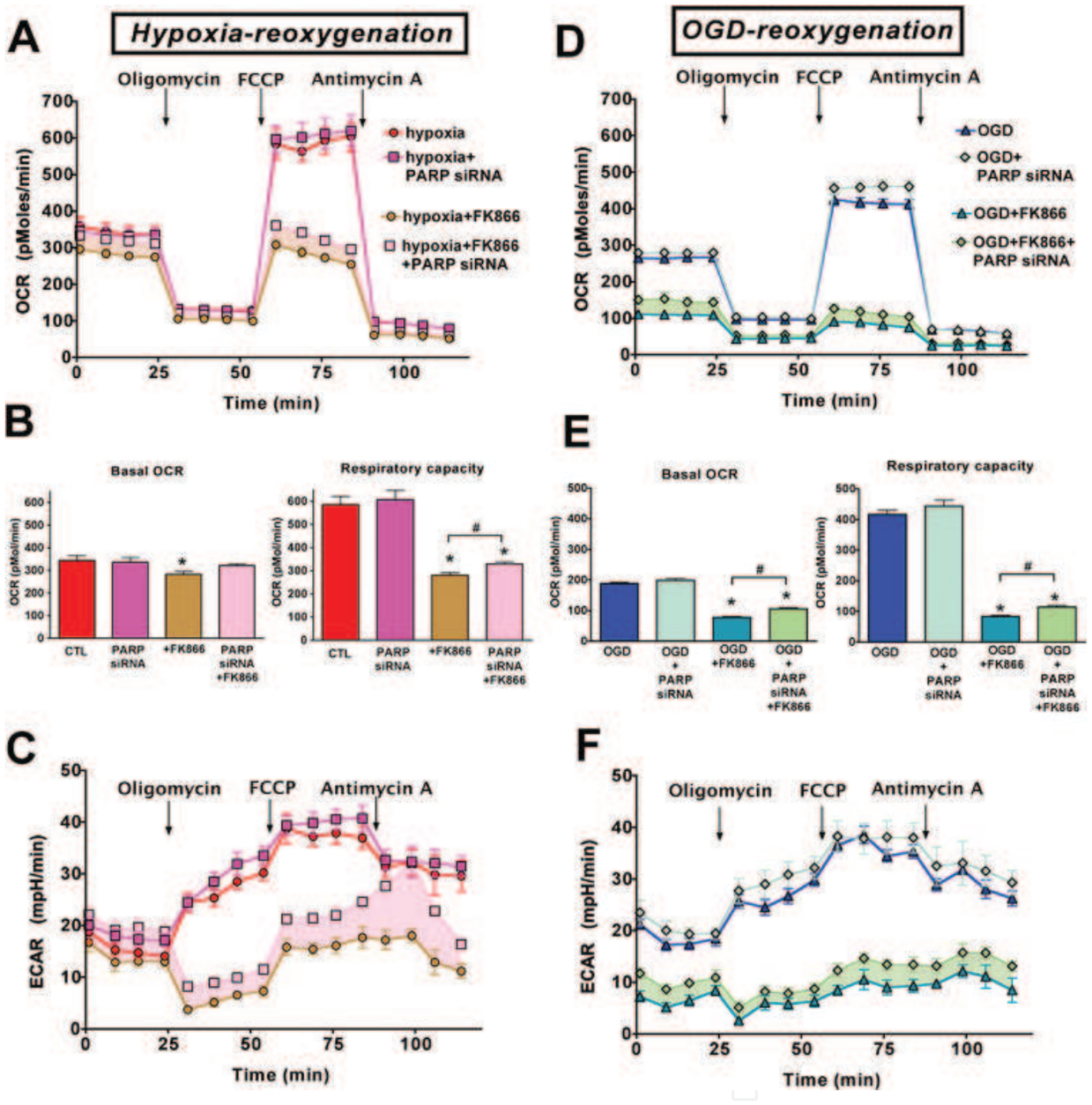

Figure 3. Suppressed cellular metabolism following oxygen-glucose deprivation (OGD). (A–F) H9c2 cells were transfected with PARP-1 (siPARP-1) or CTL siRNA and 48 hours later the cells were exposed to hypoxia or oxygen-glucose deprivation for 8 hours. Following the hypoxic phase, glucose and serum concentrations were normalized and the cells were treated with NamPRT inhibitor FK866 (10 μM) to block NAD^+ resynthesis (or vehicle) at normal oxygen tension for 16 hours. The metabolic profile of the cells was determined by extracellular flux analysis. (A and D) The oxygen consumption rate (OCR) and (C and F) the extracellular acidification rate (ECAR) were monitored using Oligomycin (1 μg/mL), FCCP (0.3 μM) and antimycin A (2 μg/mL) injections. (B and E) Basal oxygen consumption and total respiratory capacity were determined following the addition of FCCP. NamPRT inhibition severely blocks the recovery of the respiratory capacity and prevents the anaerobic metabolic compensation. PARP-1 silencing increases the respiratory capacity in cells with diminished NAD^+ content. ($n = 3$, $^*p < 0.05$ compared to CTL, $\#p < 0.05$ PARP-1 silenced cells compared to respective CTL siRNA treated cells.) From Ref. [5].

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respiratory capacity under baseline conditions, the cells use their full respiratory capacity following hypoxia of OGD injury. While the basal anaerobic metabolism is less affected by the lack of NAD^+ the anaerobic compensation is reduced by 70%, which makes the cells extremely sensitive to other injuries that require excess energy. At this stage, NAD^+ is functionally shared between the mitochondrial and cytoplasmic pools, as the blockage of mitochondrial NAD^+ recycling by inhibition of ATP synthase immediately draws a halt to anaerobic metabolism. This phenomenon can help explain the vulnerability of the cells: any injury that causes mitochondrial impairment can simultaneously block the anaerobic metabolism in the cells.

3.2. Oxidative stress during reoxygenation

Oxidative stress is an important contributor to cellular damage in hypoxia- or OGD-reoxygenation injury. While it is recognized as the major cause of cellular damage in ischemia-reperfusion injury *in vivo* [23–25], reoxygenation does not induce severe oxidative stress in the *in vitro* injury. Mitochondria are the major sources of oxidants *in vitro* following OGD or hypoxia. The mitochondrial respiratory chain is turned off by the lack of oxygen during hypoxia or OGD, but the electrons and protons are fed to the mitochondria as long as possible. As a result, the protons pumped from the matrix to the intermembrane space may increase the transmembrane gradient [5]. Mitochondrial uncoupling proteins are responsible for maintaining the physiological mitochondrial membrane potential [26]. They allow reverse transfer of protons from the intermembrane space to the matrix without coupled ATP synthesis. This proton leak may reduce the efficiency of ATP production, but it also helps against mitochondrial hyperpolarization [27–29].

Superoxide is produced by the mitochondrial electron transport chain itself, most importantly at complex III: a low percentage of electrons from quinone molecules are transferred to oxygen instead of complex III even in healthy mitochondria [30–34]. The amount of ROS generation is relatively low, approximately 0.2–2% of the oxygen consumed by the mitochondria is reduced to superoxide [28]. However, this process would leave behind excess protons in the intermembrane space and increase the mitochondrial membrane potential, if mitochondria did not possess a safety mechanism against it. Uncoupling proteins and especially UCP2 are responsible for protecting against hyperpolarization. The elevated mitochondrial membrane potential directly increases the mitochondrial superoxide generation [35, 36]. This action is reversible: if the mitochondrial membrane potential is normalized, the superoxide generation will decrease to normal levels [27, 34, 37]. However, the action of UCP2 and UCP3 is regulated by reactive oxygen species (ROS) generation as their activity is affected by glutathionylation: increase in ROS production prompts the deglutathionylation and activation of proton conductivity via UCP2 and UCP3, while at low ROS levels the uncoupling proteins are glutathionylated that effectively deactivates the proton conductance process [28, 38]. During hypoxia or OGD, the absence of oxygen completely deactivates UCPs in the cell and it excludes the compensation for the hyperpolarization in the beginning of the reoxygenation phase. While an increase is detectable in the mitochondrial membrane potential, the amount of superoxide generation hardly exceeds the normal levels immediately following hypoxia or

OGD due to the suppressed mitochondrial activity [5], but increased ROS production can be detected in the cells even after full recovery of the cellular ATP and NAD^+ contents [5] (**Figure 4**).

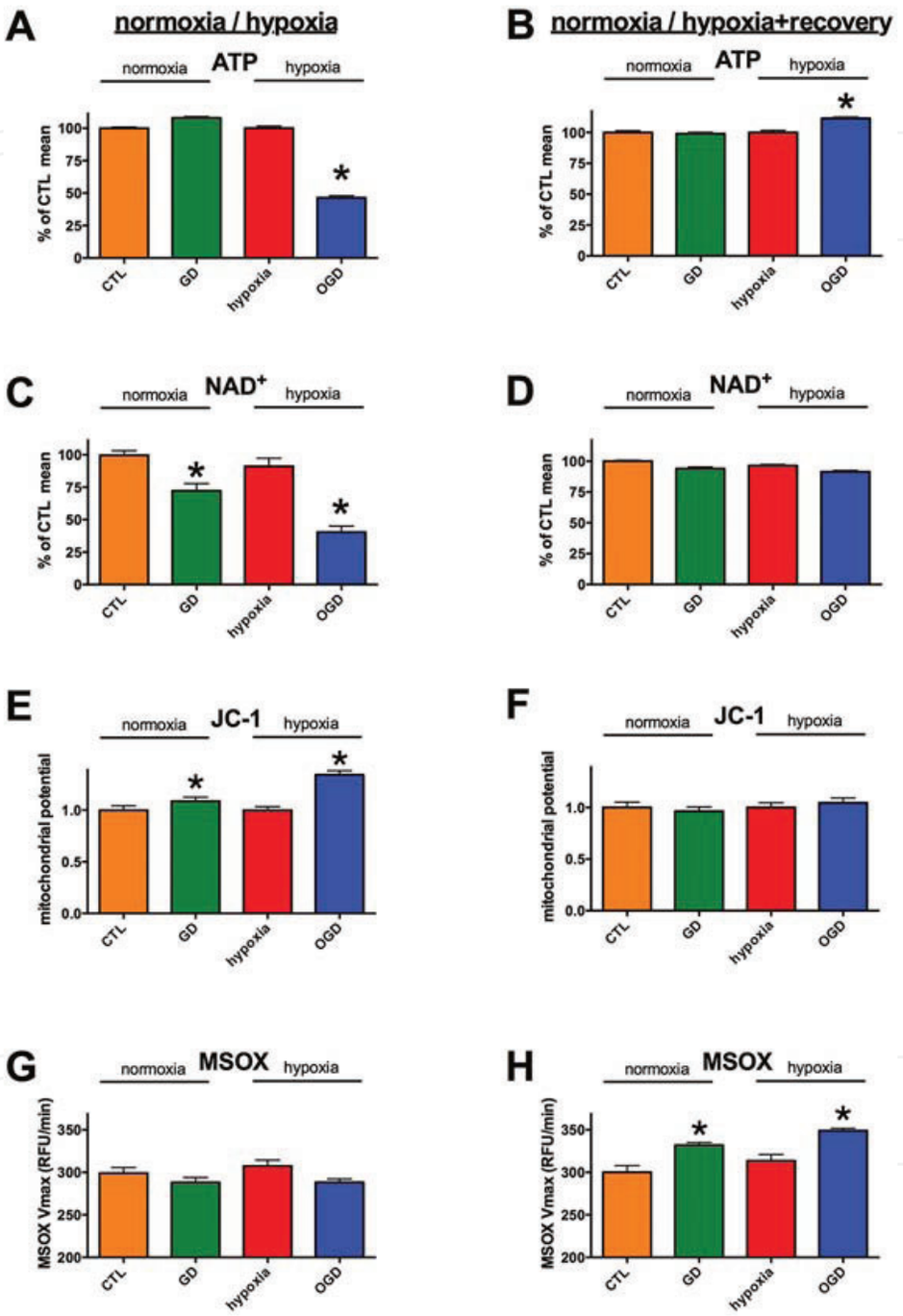

Figure 4. Mitochondrial oxidant production in hypoxia-reoxygenation injury. (A–F) H9c2 cardiomyocytes were exposed to hypoxia or oxygen-glucose deprivation (OGD) for 8 hours, followed by 16-hour-long recovery. Cells were simultaneously maintained at normoxia in glucose-containing culture medium as controls (CTL) or subjected to glucose deprivation (GD). (A and B) ATP and (C and D) NAD^+ contents were determined both at the end of the hypoxia (A and C) and following the recovery (B and D). (E and F) The mitochondrial potential and (G and H) superoxide production were measured by JC-1 and MSOX Red (MSOX) at the end of the hypoxia (E and G) and following the recovery (F and H). (n = 4, *$p < 0.05$ compared to CTL.) From Ref. [5].

Oxidative stress damages the DNA and RNA molecules causing modified bases and strand breaks and also induces oxidative protein damage. To minimize further dysfunction caused by impaired molecules, repair processes are promptly activated in the cells and PARP-1 is the key enzyme that orchestrates this process. The activation of PARP-1 is an easily detectable sign of oxidative stress in the cells and tissues [39–41].

3.3. The function of PARP-1 and its role in oxidative stress-induced cell death

PARP-1 is the major isoform of poly(ADP-ribose) polymerases in the cells that mainly resides in the nucleus. It detects DNA strand breaks and plays a role in base excision repair by adding multiple ADP-ribose units to the DNA associated histone proteins using NAD^+ as a substrate. It promotes DNA repair by recruiting components of the repair machinery and also by providing sequestered energy source for the repair in the form of ADP-ribose. Poly(ADP-ribose) (PAR) induces conformation changes in the DNA due to its negative charge, which may serve as a surface for interaction in DNA repair. The removal of PAR is catalyzed by poly(ADP-ribose) glycohydrolase (PARG), an enzyme that is mainly localized to the cytoplasm and needs to translocate to the nucleus to counteract PARP.

While the far-reaching activity of PARP-1 in DNA repair suggests that it is essential for DNA integrity and cell survival, PARP-1 knockout mice are viable and do not exhibit high susceptibility for spontaneous tumour development [42]. There is no human 'PARP-1 deficiency syndrome'. Single nucleotide polymorphisms of the PARP gene have been identified, but only few studies found association with functional changes and increased risk of cancer, nephritis or arthritis [43–46]. DNA repair processes possibly rely on redundant actions of many other components or PARP-1 is substituted by other PARP isoforms [47, 48]. On the other hand, the principal role of PARP-1 is indisputable in cell metabolism and oxidative stress-induced cell death.

In oxidative stress, the enzyme is capable of over-activation by creating huge branching PAR polymers within minutes, thereby depleting the available NAD^+ pool of the cells and causing energetic failure [40, 49, 50]. Inhibition of PARP activity prevents necrotic cell death in oxidative stress and promotes cell survival and apoptosis, a favourable cell death form. During apoptosis PARP is inactivated by caspase cleavage that dissociates the DNA binding and catalytic domains of PARP and prevents PARP activation by DNA strand breaks. Apart from caspases, various proteases (cathepsin, calpain, granzyme B) may inactivate PARP by proteolytic cleavage following OGD or hypoxia injury [2]. PARP also catalyzes its self-PARylation and this auto-modification reduces the catalytic activity of the enzyme, thus, it also serves as a control of its activity. It was suggested that other post-translational modifications of the enzyme (phosphorylation, acetylation) are also implicated in the regulation of PARP activity [49, 51].

PARP also regulates gene transcription via interacting with other transcription factors or by directly binding to promoter regions to control cellular metabolism [52, 53]. Among others, the PAR-degrading enzyme PARG, the nuclear NAD^+ synthesis enzyme NMNAT-1 and Nuclear Respiratory Factor 1, which activates the expression of metabolic genes regulating cellular

growth and mitochondrial respiration, were identified as PARP interactors [53–56]. The interplay between PARP-1 and the NAD^+ biosynthesis enzyme NMNAT-1 is particularly interesting because it suggests that under baseline conditions the nuclear NAD^+ utilization and recycling are fully coupled processes [54, 57].

PARP activation may cause mitochondrial dysfunction in cells exposed to oxidative stress that is best characterized by reduced mitochondrial reserve capacity [58]. The cellular NAD^+ pool is compartmentalized within the cells and since the NAD^+ pools are non-exchangeable between the nucleus and the mitochondria [22], the PARP-mediated nuclear NAD^+ depletion may develop mitochondrial failure via prior depletion of the cytoplasmic NAD^+ pool and inhibition of glycolysis. There seems to be a competition for substrate between PARP-1 and other NAD^+-consuming enzymes including the sirtuins [40, 59]. The sirtuin family members use NAD^+ for their deacetylation function and are mainly implicated in the regulation of glucose and lipid metabolism [59, 60]. The various sirtuins show distinct intracellular localization profile, Sirt1, Sirt6 and Sirt7 are predominantly nuclear proteins [59]. While PARP and sirtuins share their common substrate, the NAD^+ consumption by sirtuins is hardly comparable to that of PARP, thus competition for substrate has little impact on PARP activity. Still Sirt1 may affect the action of PARP-1 via direct interaction of the two proteins and by modulating PARP activity via deacetylation [61]. On the other hand, the nuclear sirtuins are possibly affected by PARP1-mediated NAD^+ consumption under oxidative stress, since the lack of PARP-1 increases Sirt-1 activity and stimulates the mitochondrial metabolism [62]. Thus, it suggests that sirtuins and especially Sirt-1 may play a role in PARP-mediated mitochondrial suppression, as PARP-mediated NAD^+ consumption decreases Sirt-1 activity and mitochondrial metabolism.

PARP-1 activation is generally associated with necrotic cell death, but PARP-1 may be involved in other cell death forms. The obligatory trigger of PARP activation is DNA single strand break, which can be induced by a variety of oxidants. In pathophysiological conditions, reactive species capable of inducing DNA strand breakage, and thereby PARP activation, include hydroxyl radical, nitroxyl radical, as well as peroxynitrite (a reactive oxidant produced from the reaction of nitric oxide and superoxide) [63–65]. In response to DNA damage, PARP becomes activated and, using NAD^+ as a substrate, catalyzes the building of homopolymers of adenosine diphosphate ribose units. Depending of the severity of DNA damage this process can be overwhelming and it may deplete the cellular NAD^+ and ATP pools and can eventually lead to cell death via the necrotic route [39]. Hypoxia- or OGD-reoxygenation injury predisposes the cells to PARP-1 mediated NAD^+ depletion: lower level of oxidative stress and PARP-1 activity can exhaust the cellular NAD^+ pool and lead to necrosis (**Figure 5**).

The activation of PARP-1 is a regulated process and the enzyme also plays an important role in programmed cell death forms [66, 67]. PARP-1 activity level depends on the severity of oxidative stress, and its high catalytic activity is necessary to promote immediate DNA repair. This protective mechanism helps maintain genome integrity: the ADP-ribose units provide energy source for base excision repair and the negatively charged polymer recruits other repair proteins to the site of the damage [68]. Low level of PARP activity is always detectable, and it is associated with normal gene expression and physiological maintenance of DNA integrity. Severe DNA damage that occurs under pathological conditions induces excessive activation

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of the enzyme that can rapidly deplete the cellular NAD^+ content. Less severe oxidative damage can induce moderate PARP activation to restore the DNA integrity and if the repair process is unsuccessful, apoptosis may be induced [39, 40, 66]. The apoptotic process follows the intrinsic or mitochondrial pathway in this case [69], and it requires a nuclear-to-mitochondrial signal for initiation. The signalling molecules have not been unequivocally identified, but PARP-1 and the PAR polymer might be directly involved in this process [70]. PARP-1 can generate large PAR polymers that may escape from the nucleus. The PAR polymer itself can induce membrane damage, mitochondrial depolarization and apoptosis-inducing factor (AIF) release [70]. AIF released from the mitochondria translocates to the nucleus and plays a role in cell death progression [71]. This PAR-mediated cell death program is occasionally discriminated from necrosis and apoptosis as parthanatos, a distinct cell death form [70]. Triggering of the mitochondrial apoptotic signal leads to caspase activation, which becomes detectable 1 hour following the start of reoxygenation and remains elevated for several hours in hypoxia-reoxygenation injury [2]. During apoptosis caspase cleavage inactivates PARP-1 by removing the catalytic region of the protein from the DNA binding region to avoid unnecessary NAD^+ consumption caused by the fragmented DNA [72].

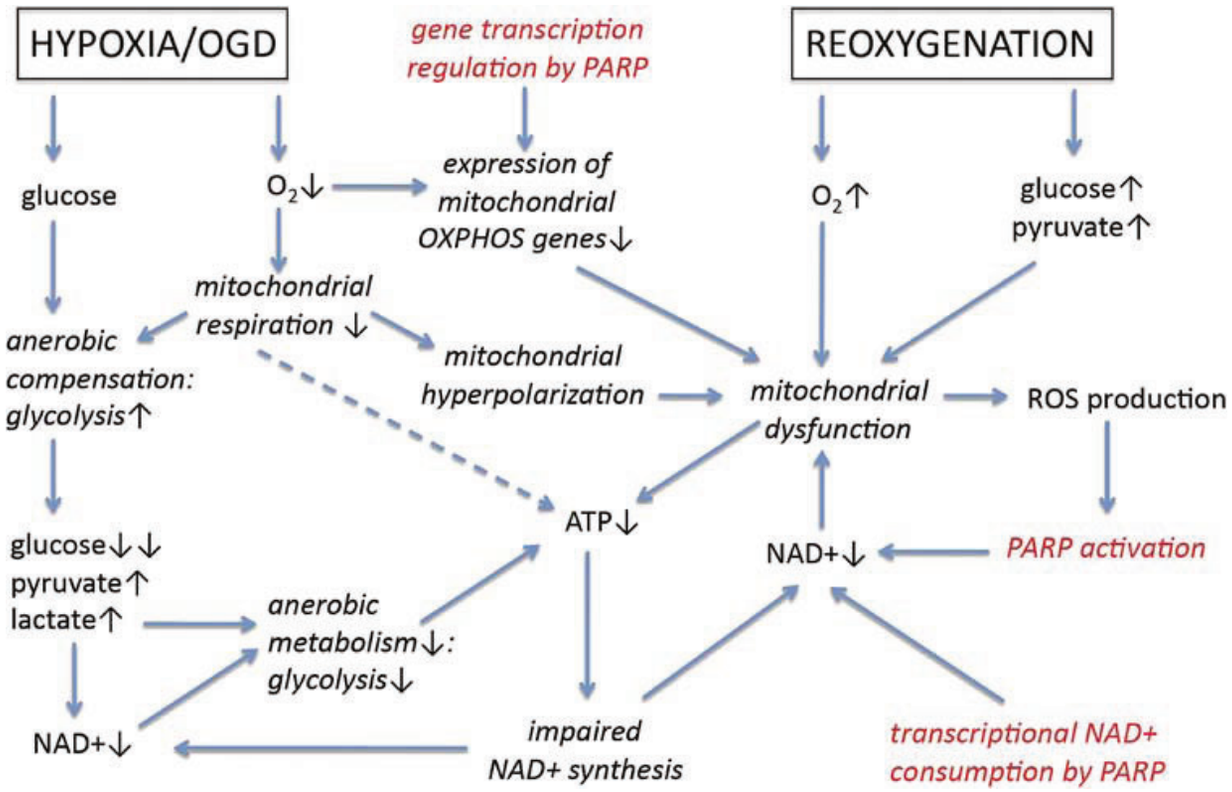

Figure 5. The mechanism of energetic failure in hypoxia-reoxygenation injury. The events of hypoxia/OGD-reoxygenation injury leading to ATP depletion with the contribution of PARP labelled in red.

PARP-1 itself can exit the nucleus in oxidative stress and interact with cytoplasmic or mitochondrial proteins [4]. Thereby, PARP-1 can have direct access to the cytoplasmic or mitochondrial NAD^+ pools and can PARylate cytoplasmic and mitochondrial proteins [73]. In this process, the PAR-binding E3 ubiquitin ligase RNF146 (ring finger protein 146, dactylidin also

named Iduna) is involved [74–76], which can capture the PARP-1 protein and promote its ubiquitination and proteasomal degradation [4]. RNF146 was discovered as a neuroprotective gene product that when over-expressed exerted protection against NMDA excitotoxicity and MNNG-induced PARP-1 dependent cell death *in vitro* [77] and protects against oxidative stress-mediated neural injury in transgenic mice [78]. RNF146 is a 359 amino acid long, cytoplasmic protein that contains conserved Really Interesting New Gene (RING) and WWE domains. The special zinc finger domain (RING domain) between amino acids 38–75 is responsible for the E3 ubiquitin-protein ligase activity [79]. The WWE domain at 92–168 mediates specific interaction with ADP-ribosylated proteins (PAR-recognition sequence) and the carboxy-terminal half of the protein, which shows similarity to nucleoporin 155, a component of the nuclear pore complex, possibly plays a role in bidirectional trafficking of molecules between the nucleus and the cytoplasm [78, 79]. RNF146 can bind to the PAR polymer, thus it can recognize the auto-PARylated PARP-1 and other PARylated proteins, but their distinct subcellular localization (PARP-1 is present in the nucleus and RNF146 in the cytoplasm) prevents their direct association under physiological conditions. However, when the nuclear membrane integrity is disrupted, RNF146 can translocate to the nucleus, directly interact with PARP-1 and both proteins are rapidly degraded by the proteasome [4]. This interaction affects PARP-1 removal during cell division: PARP-1 is sequestered and degraded during the mitotic phase, and also results in rapid PARP-1 removal in oxidative stress. In the latter case, not only PARP-1 but also its targets, the PARylated proteins are affected, including the NAD^+ biosynthesis enzymes NamPRT and nicotinamide N-methyltransferase and various metabolic enzymes, e.g. lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate dehydrogenase and succinate dehydrogenase [80] that can slow down the recovery following OGD or hypoxic injury. A small fraction of PARP-1 is localized to the mitochondria and plays a role in mitochondrial DNA repair, but it may also be involved in degradation of interacting mitochondrial proteins [73, 81, 82]. Inhibition of PARP-1 activity renders protection against reoxygenation-induced oxidative damage and cell death in OGD injury but this effect is limited by the low concentration of cellular ATP [2, 5] and may not be comparable to the beneficial effects of PARP inhibitors observed in ischemia-reperfusion injury *in vivo* [83, 84].

3.4. Increased sensitivity to oxidative damage

Post-hypoxic cells show increased sensitivity to oxidant-induced cellular injury due to (1) diminished ATP and NAD^+ pools, (2) low mitochondrial metabolic output and (3) reduced antioxidant capacity. Hypoxia and glucose deprivation decrease the intracellular concentrations of ATP and NAD^+ that greatly reduce the tolerance to cytotoxic injuries since they are associated with enhanced energy consumption. Oxidant-induced cellular damage is further aggravated by the diminished NAD^+ and ATP synthesis due to mitochondrial dysfunction and restricted glycolytic capacity. The exposure to low oxygen atmosphere induces down-regulation of antioxidant genes that reduces the buffering capacity during the reoxygenation phase [85, 86]. Changes in oxygen supply are detected via reduced levels of oxidants and hypoxia-inducible factor-α (HIF-1α) is responsible for transcriptional regulation of the antioxidant enzymes [87, 88]. The diminished scavenging capability and the higher oxidant generation

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during the recovery period greatly reduce the tolerance to oxidants. Overall, these factors increase the vulnerability of the cells and oxidants can induce devastating damage during the reoxygenation period.

The cells may be treated with exogenous oxidants following the *in vitro* hypoxic or OGD injury to better mimic tissue reperfusion, since (1) the infiltration and ROS production of circulating leukocytes is missing and (2) the ratio of culture volume/packed cell volume is a couple of orders of magnitude higher than the ratio of extracellular/intracellular space, thus oxidants produced by the cells are instantaneously diluted in *in vitro* hypoxia-reoxygenation injury. Oxidants induce more severe cell damage in post-OGD cells than in normal cells, since the cellular NAD^+ content is much lower following OGD exposure (**Figure 6**). The cellular NAD^+

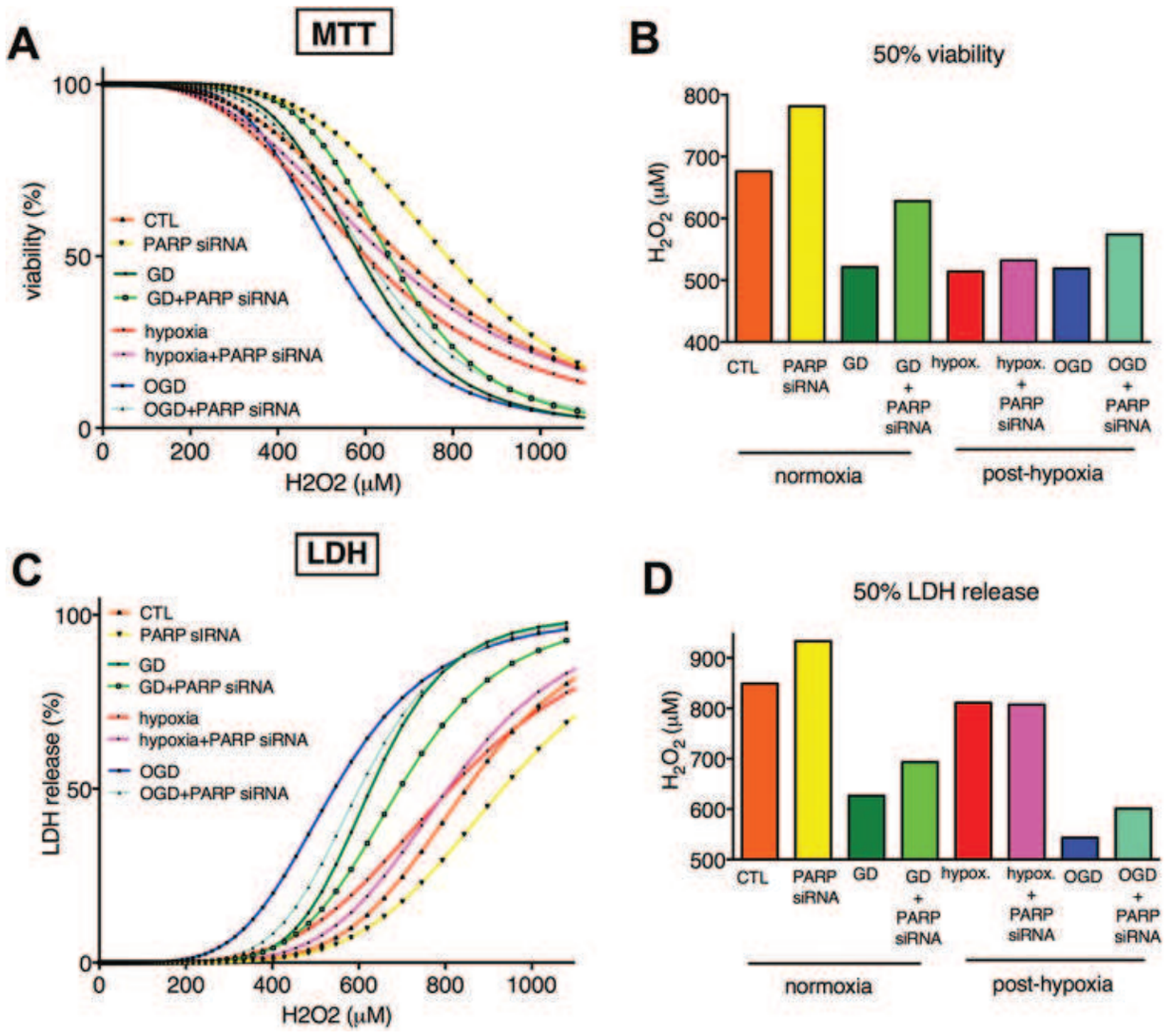

Figure 6. Hypoxia and OGD increases the sensitivity to exogenous oxidants. H9c2 cells were subjected to 8-hour-long hypoxia/OGD or GD, and then following the normalization of glucose concentration and oxygen tension the cells were exposed to various concentrations of H_2O_2 for 3 hours. (A and B) The viability of the cells was evaluated by the MTT assay. (C and D) LDH activity was measure in the supernatant. Non-linear curve-fitting was applied to the raw data (A and C) and the concentration of H2O2 that caused 50% reduction in the viability (B) or 50% increase in the LDH release (D) are shown. GD and OGD resulted in narrower range of H2O2 tolerance (steeper curves). From Ref. [5].

pools may be completely depleted by a moderate oxidative damage that hardly induces viability reduction in normal cells [5]. Cells undergoing OGD injury are less tolerant to oxidants and can survive oxidant-exposure in a narrow concentration range. PARP inhibition reduces the NAD^+ consumption and has protective effect against oxidant-induced cell damage in post-hypoxic or post-OGD cells that is in line with *in vivo* ischemia-reperfusion data [23, 67, 89–91].

4. Interventions to increase the recovery following hypoxia-reoxygenation or OGD-reoxygenation injury

In drug discovery, the ultimate goal of using *in vitro* models, like hypoxia-reoxygenation or the OGD-reoxygenation is to find novel drugs that show efficacy against ischemic diseases *in vivo*. The relative contribution of various pathways leading to cellular damage in hypoxia or OGD-injury has not been definitely established, thus it is unclear which pathways can serve as drug targets in this injury. Furthermore, there are notable differences between the hypoxia-reoxygenation model and ischemia-reperfusion injury that may result in discrepancy between the *in vitro* and *in vivo* drug efficacy [92, 93]. There are numerous factors that may account for this difference and their significance should be individually evaluated depending on the target disease for each organ or tissue type. While the organs consist of various cell types and the extracellular matrix, usually a single cell type is used in the *in vitro* model. This excludes the cells that build up the blood vessels and the circulating blood cells, and the secreted pro-inflammatory mediators and ROS produced by leukocytes are similarly absent. There are functional differences between tissues and cultured cells including muscle contraction, absorption of nutrients in the digestive system, kidney filtration, excretion and reabsorption and the detoxification function of the liver that all require lot of metabolic energy. Cultured cells may show slightly different expression profiles than their *in vivo* counterparts that may affect the expression level of drug targets and can change the observed responses. There are deficiencies of the *in vitro* model, which are associated with the differences in extracellular volume: the hypoxia induction is slower, the energy resources are more abundant and the dilutions of secreted cytotoxic or cytoprotective agents are greater than *in vivo*. On the other hand, there are no drug absorption, solubility and metabolism issues *in vitro* that may reduce the drug effects *in vivo*.

Interventions that reduce the cellular damage in hypoxia-reoxygenation injury and enhance recovery following hypoxia or OGD exposure may target (1) the metabolism and energy resources, (2) the oxidative stress pathways and antioxidant responses or (3) the proteasome and proteolytic activity. Apart from these universal cellular targets, some tissue-specific receptors were also found to have beneficial effects in some models. Energy replenishment using adenosine or inosine is effective in various cell types exposed to OGD injury since the pentose part of these nucleosides can be anaerobically metabolized through the pentose phosphate pathway [1–3]. Purine nucleosides are preferable to glucose in hypoxia since their metabolism can produce more ATP molecules than glycolysis and their utilization is more effective at low concentrations. Furthermore, they possess

anti-inflammatory and weak PARP inhibitor activity that supports their activity *in vivo* [94]. Various ROS scavengers and antioxidants also exert cytoprotective effect in hypoxia-reoxygenation models [95] and inhibition of the NAD^+ consumer PARP-1 that recognizes the oxidative DNA damage is also beneficial [96, 97]. Not only the necrosis-associated PARP-1 blockage is effective in OGD-reoxygenation injury, but also caspase inhibition has protective effect in select cell types, confirming that the cell death features both apoptotic and necrotic elements in this injury [2]. Proteasome inhibition that possibly prevents the degradation of key signalling proteins and metabolic enzymes is also beneficial in hypoxia-reoxygenation injury [98, 99].

5. Conclusion

The hypoxia-reoxygenation model is a valuable tool in hypoxia and ischemia research that may be combined with other injury models to fully reproduce features of inflammatory and vascular diseases. This low-cost model does not require advanced research skills and may be optimized within a short time in the laboratory. The cellular damage mostly occurs as a consequence of energetic failure and shows necrotic characteristics in this model. Both the hypoxic phase and the post-hypoxic recovery period involve massive changes in the cellular metabolism: a characteristic suppression of mitochondrial energy production is caused by the lack of oxygen and later by the shortage of NAD^+ supply. The recovery from this state is a delicate process that recreates the balance in cellular energetics.

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