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REVIEW

The Mitochondrion as a Key Regulator of Ischaemic Tolerance and Injury

Denis N. Silachev, PhD [a], Egor Y. Plotnikov, PhD [a], Irina B. Pevzner, PhD [b], Ljubava D. Zorova, PhD [c], Valentina A. Babenko, MD [b,d], Savva D. Zorov, PhD [b], Vasily A. Popkov [b], Stanislovas S. Jankauskas, MD [b], Valery P. Zinchenko, PhD [e], Gennady T. Sukhikh, PhD [d], Dmitry B. Zorov, PhD [a*]

[a]A. N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russian Federation
[b]Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Moscow, Russian Federation
[c]International Laser Center, Lomonosov Moscow State University, Moscow, Russian Federation
[d]Research Center of Obstetrics, Gynecology and Perinatology, Moscow, Russian Federation
[e]Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Russian Federation

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Vascular pathologies pose a significant health problem because of their wide prevalence and high impact on the rate of mortality. Blockade of blood flow in major blood vessels leads to ischaemia associated with oxidative stress, where mitochondria act as a major source of reactive oxygen species (ROS). While low levels of ROS perform a necessary function in normal cellular signalling and metabolism, elevated levels under pathological conditions are detrimental both at the cell and organ level. While cellular oxygenation is necessary to maintain tissue viability, a key pathological occurrence when restoring blood flow to ischaemic tissues is the subsequent burst of ROS generation following reoxygenation, resulting in a cascade of ROS-induced ROS release. This oxygen 'paradox' is a constraint in clinical practice, that is, the need for rapid and maximal restoration of blood flow while at the same time minimising the harmful impact of reperfusion injury on damaged tissues. Mitochondria play a central role in many signalling pathways, including cardioprotection against ischaemic injury and ROS signalling, thus the main target of any anti-ischaemic protective or post-injury therapeutic strategy should include mitochondria. At present, one of the most effective strategies that provide mitochondrial tolerance to ischaemia is ischaemic preconditioning. In addition, pharmacological preconditioning which mimics intrinsic natural protective mechanisms has proven effective at priming biological mechanisms to confront ischaemic damage. This review will discuss the role of mitochondria in contributing to acute ischaemia-reperfusion (IR) injury, and mechanisms of cardioprotection in respect to mitochondrial signalling pathways.

Keywords Reperfusion • Preconditioning • Antioxidants • Kidney • Brain • Heart

Introduction

Pathologies associated with acute circulatory disorders in organs and their consequent complications occupy a leading position as a cause of worldwide mortality. It is estimated more than 17 million people died worldwide from cardiovascular diseases (CVD) in 2008, with three million of these deaths occurring before the age of 60, many of which could have been prevented. The percentage of premature deaths from CVD ranges from 4% in high-income countries to 42% in low-income countries, leading to growing inequalities in the occurrence and outcome of CVD between countries

*Corresponding author at: A. N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992, Leninskye gory, house 1, building 40, Moscow, Russian Federation, Tel.: +7 495 9395944; fax: +7 495 9390338., Email: zorov@genebee.msu.su

and populations (Fig. 1) [1]. Among the causes of death, stroke takes second place after cardiovascular pathologies, with the consequences of stroke being the leading cause of primary disability. Population studies have shown that less than 15% of patients who have undergone stroke returned back to work or full implementation of their previous domestic responsibilities, with the remaining 85% requiring life-long medical and social support due to their disabilities [2]. In addition, 40-50% of survivors suffer permanent deterioration of cognitive functions such as loss of memory and attention span [3]. Other common pathologies associated with ischaemia are prerenal and renal aetiologies of kidney dysfunction. The mortality rate from acute kidney failure is currently around 22-25% [4]. Despite the development of treatment methods, the number of patients with ischaemic acute renal failure has not reduced [5].

Previous work on ischaemia-reperfusion injury mechanisms indicates that the main damaging effects involve the pathological consequences following restoration of blood flow to the tissue, rather than ischaemia itself. Reperfusion following cessation of blood flow leads to the development of oxidative stress, which is a profound and largely irreversible destructive process leading to the death of reoxygenated cells and may eventually lead to organ failure [6].

Under physiological conditions reactive oxygen species (ROS) formation may vary widely depending on the type of tissue, their functions and conditions (See [7],[8]). In actively proliferating cells and in those which are most often subjected to attacks by pathogens (for example, lung or blood cells) endogenous levels of ROS are high and very much determined by the metabolism and the degree of pathogenic burden, while in poorly metabolising cells with small proliferative potential these levels are much lower. Thus, in actively metabolising tissues such as the brain, heart and kidneys, the problem of homeostasis of ROS is of paramount importance. While ROS play an essential function as intracellular signalling elements, transition to pathological

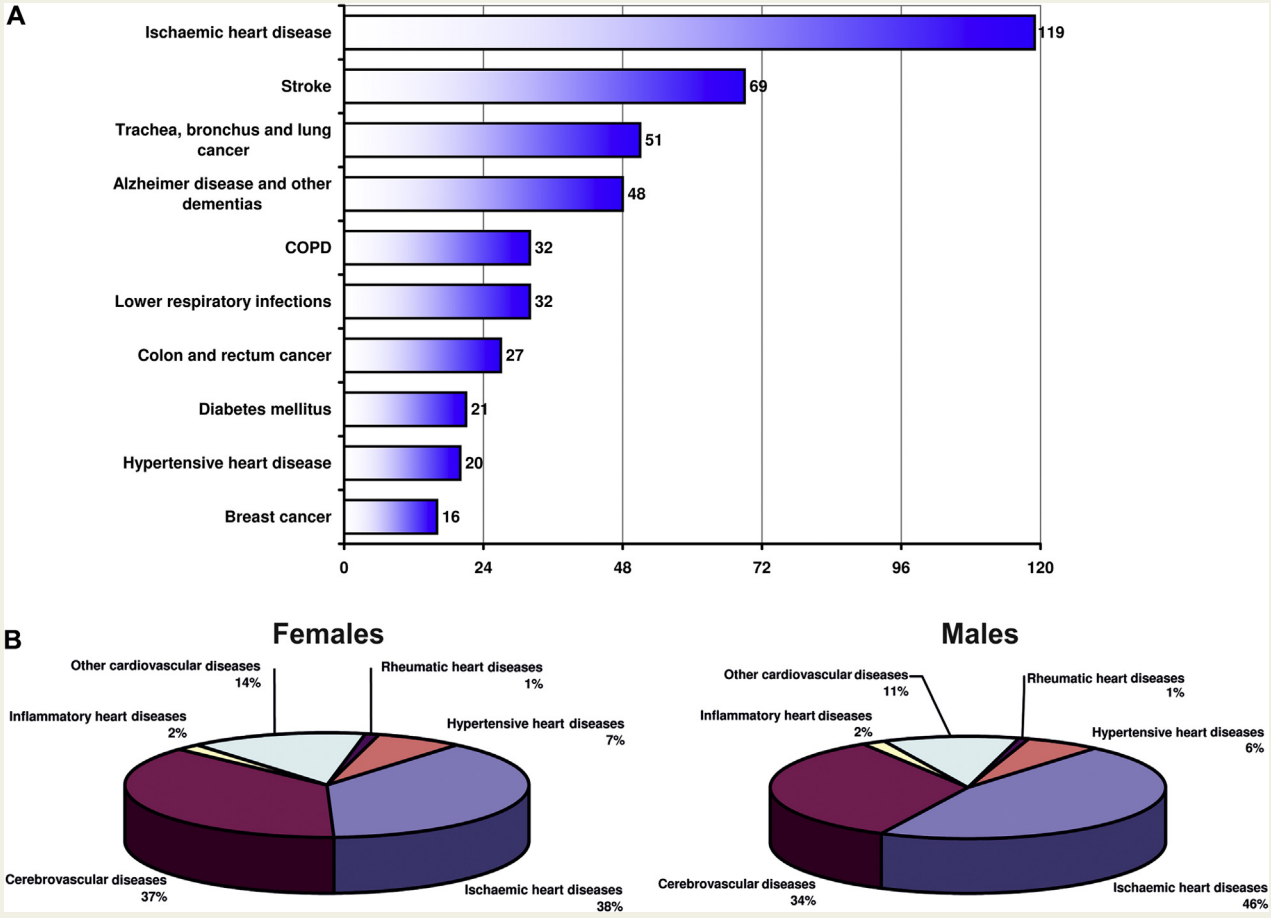

Figure 1 The epidemiology of cardiovascular diseases. (A) The 10 leading causes of death in high income countries (2011), adapted from the public domain article by the World Health Organization (http://www.who.int/mediacentre/factsheets/fs310/en/index1.html). (B) Distribution of deaths from cardiovascular diseases including heart attacks, strokes and other types of cardiovascular diseases. Reproduced with publisher's permission, from the World Health Report: Global Atlas On Cardiovascular Disease Prevention And Control. Geneva, World Health Organization, 2011. (http://whqlibdoc.who.int/publications/2011/9789241564373_eng.pdf).

functions of ROS through non-selective oxidative modification of proteins, lipids, nucleic acids and other components of cells and tissues, confer varying degrees of dysfunction. Such a transition as noted earlier involves oxidative stress exceeding the threshold that can be managed by intrinsic mechanisms. However, complete blockade of ROS may also be detrimental, particularly if vital survival or regulatory cell signalling roles of ROS are not served and pathological effects occur causing unnecessary reduction of cellular components (reductive stress, Fig. 2) [9].

ROS and Oxidative Stress - Some Basic Principles Considered

The main regulator of ROS levels in the cell and tissue is oxygen content, which is mostly reliant on active transport in the blood by haemoglobin molecules, in the muscle tissue by myoglobin [10], and in other tissues by physical diffusion of oxygen from the capillaries into the tissue [11], reviewed in Zorov et al. [8]. When oxygen supply to the tissue ceases, tissues with high metabolic requirements very quickly become hypoxic. In turn, cessation of blood flow leading to both the loss of oxygen supply and lack of nutrient delivery leads to the state of ischaemia. In normal tissue, there is a certain reserve of nutrient substrates (free glucose, amino acids, glycogen). The availability in the tissues of proteolytic and lipolytic enzymes provide some, albeit limited flux of oxidative substrates, which the tissue utilises until they reach the limitation of available oxygen. The time that tissue can tolerate hypoxia before organ failure occurs is very tissue-specific. Cardiac or renal tissue may tolerate this state for minutes or even hours. In the brain after a few minutes of hypoxia irreversible changes occur associated with the

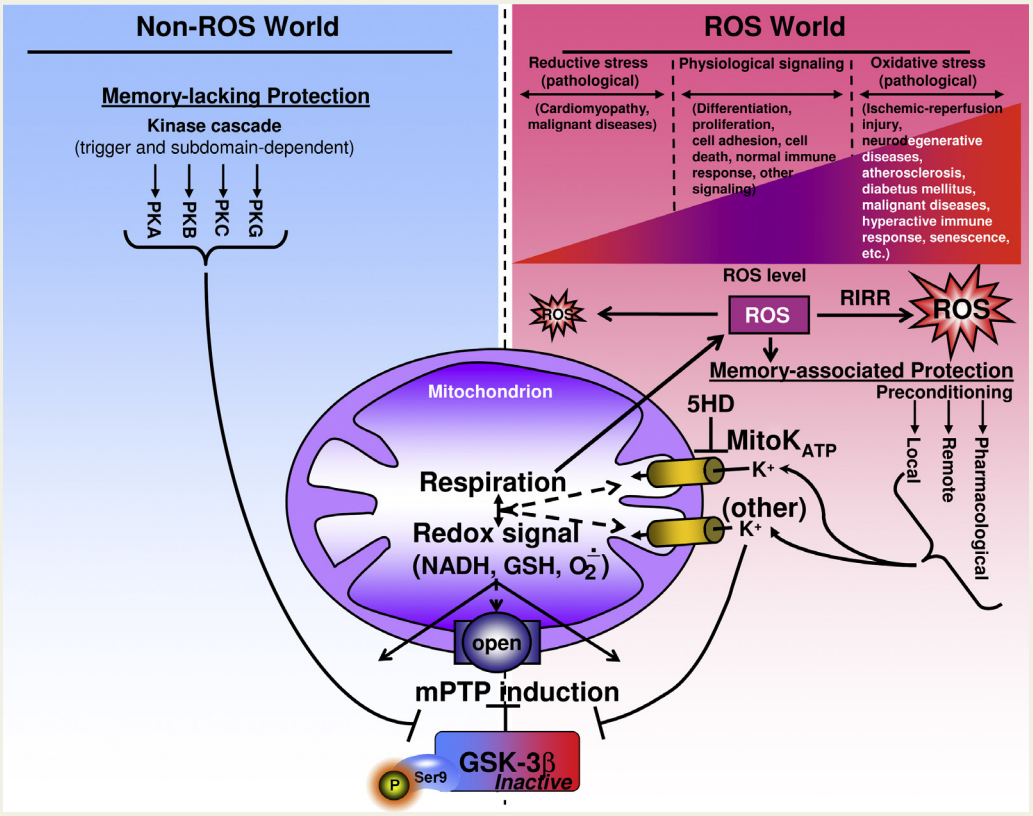

Figure 2 Two major signalling worlds: ROS and Non-ROS. Some of the key processes activated by very low amounts of ROS, moderate ROS levels and high ROS which respectively determine reductive stress, physiological homeostasis and oxidative stress (upper right part). Under normal physiological conditions, mitochondrion serves as a hub collecting signalling pathways to evoke protection of the cell. Under pathological conditions mitochondria generate pathological amounts of ROS (via ROS-induced ROS release, RIRR [8]) igniting oxidative stress. As mitochondria determine the redox state of the cell (producing and releasing reducing and oxidative equivalents shown as NADH, GSH and superoxide anion) in extremely reductive situations they cause reductive stress. Physiological signalling amounts of ROS are involved in a cell protection which is preserved for some time after removal of the stimulus (memory associated protection [38,48]). The same levels of protection can be afforded without ROS (shown in Non-ROS world) but it lacks 'memory' and vanishes after removal of the stimulus. The end-effector of all protective signalling is the mitochondrial permeability transition pore (mPTP) and its upstream effector GSK-3β which in phosphorylated form prevents mPTP activation.

release from the brain cells of toxic doses of glutamate [12], which until then, but being released in small doses, carried out a signalling function, however in these toxic doses promotes apoptotic death via neuronal mitochondria [13]. Thus the extent of ischaemic tolerance also depends on the extent of toxic cellular metabolic waste accumulation and the capacity for recovery of function is dependent on the extent of cell injury.

This critical vital time window that tissue can survive without any consequences for the organism is very important not only for understanding the pathogenesis, but for a strategy of action in the face of such pathology. Currently, the underlying effects of prolonged hypoxia on tissue function remain unclear (except, perhaps, as we indicated, the brain), but the seeming simplicity of the solution associated with the desire in clinics by any ways to restore blood flow, can be fatal, because in hypoxic tissue, depending on the time spent in hypoxic phase irreversible phenomena can occur, in which the restoration of access of oxygen can be not only therapeutically ineffective but also destructive [14–16].

As a result, tissue reperfusion after ischaemia, necessarily required for the survival of cells is a ''double-edged sword'' because it is associated with a further damage, known as reperfusion injury [17]. Mechanisms of reperfusion damage of organs require a general understanding, since they reflect not only the physiological function of oxygen as the main player in essential oxidative metabolism, but also as a possible participant in unwanted oxidative processes in the organism, which are a source of a number of pathologies.

Under conditions of oxygen deficiency, oxygen molecules in the mitochondrial respiratory chain can accept only one electron, instead of four under normal conditions, producing the superoxide anion radical, one of the elements of ROS, of which a distinctive feature is high oxidative capacity. Notably, in normal circumstances a few percent of utilised oxygen is processed to ROS (superoxide anions), which are a vital component of intra- and intercellular signalling (Fig. 2) [17]. ROS homeostasis is mandatory to normal downstream physiological function, whereas excessive or insufficient ROS leads to oxidative or reductive stress, respectively [7,9,18]. In vivo there is constant interconversion of ROS according to redox potential and oxidising capacity, differing ROS life times and varying capacity to enter cell and intracellular compartment membranes. In addition, ROS homeostasis is also supported by endogenous redox buffers, such as glutathione or major non-catalytical proteins, such as albumin.

As mitochondrial ROS are both important regulatory elements and major contributors to ischaemic tissue damage, a paradox arises. In terms of chemistry, the generation of ROS (as 'relatives' of oxygen) is expected to be directly proportional to the concentration of molecular oxygen (generation of ROS from oxygen is the reaction of first order). That is, the lower the concentration of molecular oxygen it follows that there must be lower generation of ROS. Although this does occur at certain intervals of partial oxygen pressure, below the threshold of a tissue-dependent hypoxic minimum, further decreases of partial oxygen pressure actually causes an increase in ROS formation rather than a decrease [14–16]. The phenomenon of enhanced generation of ROS under hypoxic and ischaemic conditions was coined the 'oxygen paradox' [14], particularly as the restoration of blood flow by reperfusion actually caused greater post-ischaemic ROS-related tissue injury. On the one hand, this paradox may be explained by a mechanism of generation of ROS in mitochondria, when oxygen levels in the vicinity of mitochondria are less than the affinity of mitochondrial cytochrome oxidase to oxygen, the super reduction of mitochondrial carriers normally responsible for the ROS production occurs, resulting in a phenomenon whereby all utilised oxygen forms ROS. In addition, the activation of ROS-induced ROS release may also be stimulated to occur at exacerbated pathological levels [19,20], (reviewed in Zorov et al. [8]), to drive 'bursts' of injurious ROS.

In summary, while hypoxia itself can be recognised as conditionally a cause of injury, it is the transition from hypoxia to normoxia (hypoxia-reoxygenation or as ischaemia-reperfusion) [21–23] that causes the most overt cell damage and tissue failure, known as reperfusion injury. Presently, although the molecular pathogenesis of ROS-induced ROS release in post-ischaemic tissue damage has not been fully resolved, it is important to be factored in the algorithm of clinical interventions and procedures (timing and rate of reperfusion in angioplasty, surgery, transplantation).

Mitochondria-directed Therapeutic Approaches

Linear logic for solving this problem has prompted the broad use of antioxidants as pharmaceutical agents for therapy of ischaemic disorders. However such active compounds vary considerably by their mechanisms of actions and targets ultimately affecting the processes of free radical oxidation of cellular structures and biomolecules, including peroxidation of membrane phospholipids. Attempts to achieve therapeutic effect in ischaemic pathologies with the use of antioxidants (vitamins C, E, beta-carotene, some synthetic compounds) have revealed no positive clinical outcome [24,25] and sometimes have been implicated in adverse effects associated with the loss of ROS-dependent physiological function (Fig. 2). The only promising direction of antioxidant development has been in the targeted design of antioxidants directed exclusively to the mitochondria [26]. In recent years a new range of targeted agents have been reported to be protective against reperfusion injury of numerous tissue types beyond heart [27–37]. However the basic premise targeted by such agents is that pro-apoptotic mitochondrial permeability transition is largely prevented allowing cellular protection to occur at a very early stage of signalling [38–41].

Although the protein structures and all the elements involved in governing mitochondrial permeability transition are far from fully determined [41–45], one element, an enzyme, responsible for activation/deactivation of this complex, has been clarified. The name of this enzyme (glycogen synthase kinase-3β, GSK-3β) does not represent the variety of

its functions. GSK-3β phosphorylates not only glycogen synthase, but also a number of other proteins, in particular, those belonging to the complex, responsible for the induction of nonspecific mitochondrial permeability. By definition, activation of this kinase (dephosphorylation of serine in the 9 position of a polypeptide chain) promotes activation of the nonspecific pore, while the inhibition of its activity (after phosphorylation of serine-9) prevents the generation of mitochondrial membrane permeability pores (Fig. 2) [39,46]. Thus, cellular protection requires that mitochondrial GSK-3β should be maintained in a phosphorylated state. Surprisingly lithium and drugs with lithium ion-based salts have been shown to be effective inhibitors of GSK-3β [47].

Ischaemic Preconditioning and Pharmacological Activation of Common Protective Signalling

A major discovery has been the protocol of ischaemic (hypoxic) preconditioning of an organ, consisting of a series of intermittent episodes of ischaemia and reperfusion (usually 2-4 episodes by 5 min each) which ultimately signals for intrinsic mechanisms to adapt against a more sustained extended ischaemic episode that causes tissue infarction (cell death) [47–49]. Ischaemic preconditioning and post-ischaemic conditioning while potentially valuable to clinical exploitation has to-date been limited in practical universal clinical translation [49,50] and remains controversial despite intense study via numerous clinical trials.

Pharmacological activation of the intracellular protective signalling (in common with preconditioning) leading to mitochondrial tolerance against ischaemia-reperfusion injury may offer a useful alternative. It has been reported that pharmacological agents which provide protective 'preconditioning', are divided into two groups: those that activate mechanisms associated with mitochondrial swelling or those that are independent from them. All of these factors can be divided by the type of signal transduction which have either a high or low 'memory', (Fig. 2). In this context, the term 'memory' indicates sustained effects that do not require short-term additional stimulus for extended activation, from the start of the stimulus and which lasts for several hours, sometimes even days. Low 'memory' agents provide protection which dissipates within 10-15 minutes after stimulus withdrawal. The pharmacological agents which trigger sustained, 'memory'-associated protection have been reported to increase the inward flux of K^+ ions to mitochondria followed by their retention with subsequent swelling of the mitochondrial matrix. The result of this activity is the activation of mitochondrial respiration producing ROS signalling, which activates the nearest pool of protein kinase C with further signal transduction to the GSK-3β. In addition, the signal from protein kinase C provides the preservation of the mechanism of protection through the cascade loop of positive feedback, due to which the mitochondrial ATP-sensitive K^+ channel is retained in the open state [51]. This confirms the role of mitochondrial ATP-sensitive K^+ channels not only as activators, but also as the mediators of cell protection resulting in phosphorylated GSK-3β-dependent transmission of signal downstream to increase the sensitivity threshold of the non-specific mitochondrial pore to ROS that is necessary for the pore opening [39], (Fig. 2). In contrast, the mechanism of low memory protection is activated by agents such as insulin, insulin-like growth factor, Li^+, erythropoietin, and involves effects on phospho-inositol-3 kinase, protein kinases A, B, C, G, and their targets, including GSK-3β. This mechanism is not associated with mitochondrial swelling, but still ultimately increases the sensitivity threshold of mitochondrial transition pore to ROS.

Remote Ischaemic Preconditioning

When analysing the mechanisms of ischaemic tolerance and finding possible physical carriers of this phenomenon (low molecular weight molecules, peptides, hormones), remote ischaemic preconditioning is an intrinsic phenomenon where ischaemia-reperfusion of one organ protects remote organs due to release of protective signals into the circulation which are received and transformed by a targeted organ [52]. Cardiac remote preconditioning is often attributed to Przyklenk and colleagues (reviewed in Tapuria et al. [52]), who showed that ischaemic preconditioning of one vascular bed region (four brief episodes of circumflex branch occlusion) of the heart protected other 'virgin' regions of the heart subsequently exposed to sustained ischaemia-reperfusion (by left anterior descending coronary artery occlusion). However, preconditioning of the heart to tolerate sustained ischaemia by stressing a remote organ (kidney) was first reported by McClanahan et al. [53]. They found that short episodes of ischaemia-reperfusion of the kidney protected the myocardium from a long-term ischaemia-induced damage and reduced infarct size in the heart. Besides preconditioning of the kidney in order to salvage ischaemic myocardium brief episodes of ischaemia-reperfusion of the limb, gut or mesenteric artery have also been demonstrated to confer protection as measured by a reduced infarct size in myocardium or brain (reviewed in Tapuria et al. [52]). However, the most clinically feasible of all the methods of remote preconditioning is the preconditioning of the extremities, since this method does not require any surgical interventions and is performed by a simple clamping off limb circulation using a blood-pressure cuff. In various experiments on animals it has been shown that the ischaemic preconditioning of the limb reduces the functional disorders caused by ischaemia-reperfusion of the heart [54,55], brain [56] and lungs [57]. Similarly, it was shown that ischaemic preconditioning of the kidney reduces myocardial [58,59] and brain damage [32].

Cytoprotective mechanisms of remote ischaemic preconditioning also largely involve mitochondrial activity. As a result of preconditioning of a remote organ different humoral factors can be released which influence the targeted organ. In some studies it was shown that such substances can be adenosine [60], opioid peptides [61], erythropoietin [62], bradykinin [63] and some other compounds. Mechanisms of action of these agents, and hence of remote preconditioning in general, converge on mitochondria and have a protective effect on the

targeted organ by the mechanism which has been described above. The participation of mitochondria and GSK-3β in signalling underlying remote preconditioning has been confirmed in studies where the protective effect of remote preconditioning was abolished by blockers of mitochondrial ATP-sensitive K^+ channels [62,64].

Pharmacological Preconditioning

The approach to use pharmacological agents to activate G-protein-coupled receptors, downstream intermediates or targets that are activated by ischaemic preconditioning has been attractive for the potential to exert greater control over the process, in particular to regulate potency, timing, cell type-specific targeting and uniformity of action. A long search over recent decades has assembled numerous agents that mimic ischaemic preconditioning in experimental settings, such as adenosine, adenosine receptor agonists, opioids including morphine, bradykinin, endothelin, and antagonists of protein kinase C, drugs that opening the ATP-sensitive K^+ channels, nitric oxide donors and others [65]. However the introduction of such useful agents in clinical practice has been challenging due to many of these compounds exerting profound effects on haemodynamics, dose-toxicity or rapid metabolic clearance before action. For example, adenosine and openers of the mitochondrial ATP-sensitive K^+ channels simulate ischaemic preconditioning and reduce cell death in animal models, however they may cause significant hypotension in patients. Other unwanted side effects include arrhythmias (adenosine, ATP-sensitive K^+ channels openers) and possible carcinogenicity (activators of protein kinase C), thus excluding their application.

Despite this, some of the drugs that can mimic ischaemic preconditioning are already in the clinical use, although this use is not based on the concept of preconditioning. Nicorandil which opens ATP-sensitive K^+ channels is used clinically as an antianginal [66], and adenosine has been used in cardioplegic preparations. In the latter case, adenosine reduced the need for high doses of inotropic drugs prescribed after cardiac surgery [67]. Adenosine has also been tested as a supplement to reperfusion in patients with acute myocardial infarction [68] resulting in a reduction in anterior wall myocardial infarct size after reperfusion. In addition, inhalation anaesthetics reduces oxygen consumption of myocardium providing a beneficial effect on the oxygen balance during the perioperative myocardial ischaemia. However, as many of these agents are opiate-based their beneficial influence evoked endogenous signalling similar to ischaemic preconditioning [69]. Experimental studies have shown that many inhalation anaesthetics perform comparably to ischaemic preconditioning to afford protection for the brain, kidneys, lungs, liver, intestines [70,71].

Conclusion

Mitochondria are essential components of cells providing numerous bioenergetic, structural and regulatory signalling roles. Cellular and mitochondrial ROS are critical elements in signalling pathways, however under physiologically adverse conditions they may be destructive when they exceeded the normal threshold of ROS levels, as in ischaemia-reperfusion injury. Mitochondria work as a rheostat supporting cellular ROS homeostasis which is a requisite for a normal cell and organ function. Numerous intrinsic mitochondrial and cytoplasmic mechanisms manage ROS within physiological limits. However when this regulation is breached, high levels of ROS cause oxidative and low levels of ROS cause reductive stresses, and either when exacerbated contributes to disease onset. Under certain conditions when tissues are repeatedly exposed to small doses of ROS and within short time frames, greater tolerance (ischaemic preconditioning) to a larger and sustained exposure to ROS is conferred, thus limiting infarct size after a stroke or myocardial ischaemic injury. The adaptive mechanisms providing ischaemic tolerance involve ROS of mitochondrial origin. Protection against ischaemia-reperfusion caused by ischaemic preconditioning can be mimicked by pharmacological agents that activate common protective signalling pathways which all converge on mitochondria. However to-date there remains a major need to design clinically viable agents that overcome limitations in dose efficacy, target specificity and toxicity. Further research is required to target the mitochondrion and its metabolic management of ROS in the design of acute ischaemia-reperfusion treatment strategies.

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