BIOCHEMICAL CHANGES IN MITOCHONDRIA AND ITS ROLE IN CELL DEATH DURING MYOCARDIAL ISCHEMIA-REPERFUSION INJURY.

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Summary

Myocardial ischemia-reperfusion injury is a major contributor to the mortality associated with coronary artery disease. Although mitochondria maintain normal heart function by providing virtually all of the heart´s ATP, is also implicated in the development of ischemic damage. While ischemic injury has long been considered to result in necrotic tissue damage, studies over the past decade have focused attention on apoptosis as a significant component of cell loss following myocardial infarction. Also a substantial body of evidence implicates reactive oxygen species and oxidative stress in the cellular injury induced by ischemia-reperfusion in the heart. Cell damage can occur through mechanism involving lipid peroxidation, covalent modification of protein and mitochondrial DNA oxidation. Reactive oxygen species contributes to irreversible damage of mitochondrial function and consequent, impaired recovery of physiological function and cell death.

Key Words: mitochondria, ischemia-reperfusion, oxidative stress, cardiomyocyte

Introduction

Myocardial ischemia-reperfusion (IR) injury is a major contributor to the mortality associated with coronary artery disease (1). The level of IR-induced myocardial injury can range from a small insult resulted in limited myocardial damage to a large injury culminating in myocyte death. Importantly, major IR injury to the heart can result in permanent disability or death (2).

Heart tissue is remarkably sensitive to oxygen deprivation. Although heart cells, like those of most tissues, rapidly adapt to anoxic conditions, relatively short periods of ischemia and subsequent reperfusion lead to extensive tissue death during cardiac infarction. Heart tissue is not readily regenerated, and permanent heart damage is the result (3). Paradoxically, however, the major damage to ischemic cells comes on the re-introduction of oxygen (reperfusion). During reperfusion, the cells typically undergo further contractions and membrane damage, followed by cell death (4,5).

Although mitochondria maintain normal heart function by providing virtually all of the heart´s ATP, is also implicated in the development of ischemic damage. While mitochondria do provide some mechanisms that protect against ischemic damage (such as an endogenous inhibitor of the F_1F_0-ATP_{ase} and antioxidant enzymes), it also possesses a range of elements that exacerbate it, including reactive oxygen species (ROS) generators, the mitochondrial permeability transition pore ($_m$PTP), and their ability to release apoptotic factors (3).

While ischemic injury has long been considered to result in necrotic tissue damage, studies over the past decade have focused attention on apoptosis as a significant component of cell loss following myocardial infarction. Intense investigation in recent years has revealed the critical importance of mitochondria in the apoptotic cascade. Mitochondria plays a key role in cellular homeostasis, but also in responding to pathologic stresses, including extreme calcium influxes, oxidative stress, protein kinases, activation of pro-apoptotic Bcl-2 family members. Signaling between mitochondria serves to coordinate and integrate the overall cellular response (6).

Mitochondria as an important organelle within cell

Mitochondria are located in the cytoplasm of all eukaryotic cells and are involved in many processes essentials for cell survival and function, including energy production, redox control, calcium homeostasis and certain metabolic and biosynthetic pathways (7). Mitochondria contain two compartments bounded by inner and outer membranes. The outer membrane is permeable to small metabolites, whereas the permeability of the inner membrane is controlled to maintain the high electrochemical gradient created by mitochondrial respiratory chain that is necessary for energy conservation and ATP synthesis (8).

Amidst the many functions described for the mitochondria, the most significant is oxidative phosphorylation for the generation of cellular energy. Oxidative phosphorylation involves the coupling of electron transport, through the electron transfer chain (ETC), to the active pumping of protons across the inner mitochondrial membrane and ATP formation by the F_1F_0-ATP synthase (9). The mitochondrial electron transport chain is made up of > 80 component proteins that constitute five complexes designed for cellular energy production: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinone cytochrome c oxidoreductase), complex IV (cytochrome oxidase) and complex V (F_1F_0-ATP synthase) (8). The latter one is a complex enzyme, comprising a transmembrane H^+ channel (F_0) and a nucleotide-binding extrinsic sector (F_1) (10,11).

Oxidation of reduced nutrient molecules, such as carbohydrates, lipids and proteins trough cellular metabolism yields electrons in the form of the reduced hydrogen carriers NADH and $FADH_2$, which are referred to as reduced cofactors. These reduced cofactors donate electrons to the ETC. The movement of electrons between the components of the ETC is driven by a redox potential that is present across the chain. Complexes I, III and IV pump protons across the inner membrane as electrons pass down the respiratory chain. This produces an electrochemical potential difference across the mitochondrial inner membrane known as the proton motive force (Δp), consisting mostly of an electrical gradient

(membrane potential) and a small chemical gradient (pH difference). The energy that is conserved in the proton gradient across the mitochondrial inner membrane is used by complex IV to synthesize ATP from ADP and inorganic phosphate (P_i) while protons are transported back from the intermembrane space into the mitochondrial matrix. The ultimate destination for the electrons is molecular oxygen, which is reduced to water by complex IV in the last step of the ETC (8).

However, not all of the energy available in the electrochemical gradient is coupled to ATP synthesis. Some is consumed by "proton leak" reactions, whereby protons pumped out of the matrix are able to pass back into the mitochondria through proton conductance pathways in the inner membrane which bypass the ATP-synthase (9,12-14).

So the energy derived from the oxidation of metabolic fuels is dissipated and released as heat. These non-productive proton leak pathways are physiologically important and account for 20-25 % of basal metabolic rate. Several different functions have been suggested for proton leak. These include thermogenesis, regulation of energy metabolism or carbon fluxes, control of body mass, and attenuation of ROS production (15).

Normal cardiomyocyte metabolism

Fatty acids provide the main energy source for the healthy heart, supplying 60-80 % of its energy requirements (3). Over 90 % of heart metabolism is aerobic, and the heart is a highly oxidative tissue, with an oxygen utilization rate of 60-150 mmol/min in humans (16). Because of this high oxidative metabolism, heart cells have a high oxidative capacity. It has been estimated that 25-35 % of total cardiomyocyte volume is occupied by mitochondria (17). Two forms of mitochondria have been recognized in heart cells on the basis of position, and ease of extraction, and these are termed interfibrillar and subsarcolemmal (18). It is thought that interfibrillar mitochondria provide most of the ATP for the contractile apparatus, although it is unclear whether there is a significant functional difference between these classes of mitochondria (3).

Approximately two-thirds of the ATP hydrolyzed in the cardiomyocytes is used by the contractile apparatus while the remaining third is used by pumps which maintain ion balance, in particular by the sarcoplasmic reticulum Ca^{2+}-ATP_{ase} and the sarcolemmal Na^+/K^+-ATP_{ase} (19). The ATP used in these processes is regenerated by the mitochondrial F_1F_0-ATP synthase using the energy released by the respiratory chain (3).

ATP in cardiac cells is in rapid equilibrium with creatine-phosphate, which acts a temporary store of "high-energy" phosphate bonds. The enzyme involved is creatine kinase which is present in the mitochondrial intermembrane space and the sarcoplasm. There have been some claims that, aside from store of energy, this system may also promote the apparent diffusion rate of ADP to the mitochondria and thus this organization is important for the efficient functioning of oxidative phosphorylation (20).

In the short-term, the contractile mechanism of the cell is controlled by the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_c$). Beats are characterized by $[Ca^{2+}]_c$ transients, with $[Ca^{2+}]_c$ rising from approximately 100 to 500 nM, due to release of Ca^{2+} from the sarcoplasmic reticulum and uptake from outside the cell via the slow sarcolemmal Ca^{2+} channels (both

uniports). The low level of $[Ca^{2+}]_c$ is then restored owing to the action of the sarcoplasmic Ca^{2+}-ATP_{ase} and the sarcolemmal Na^+/Ca^{2+} exchanger. These transients repeat whit a period of approximately 1second, depending on the organism (21).

The concentration of free intramitochondrial Ca^{2+} ($[Ca^{2+}]_m$) responds to $[Ca^{2+}]_c$ via the inner membrane Ca^{2+} uniport (22), which mediates Ca^{2+} entry, and the mitochondrial Ca^{2+}/Na^+ exchanger, which mediates Ca^{2+} exit. However, these act slowly relative to the above-mentioned transients, and the rapids changes in $[Ca^{2+}]_c$ are damped, leaving a $[Ca^{2+}]_m$ that reflects the time-averaged level of $[Ca^{2+}]_c$. These changes in $[Ca^{2+}]_m$ are known to regulate dehydrogenases of the tricarboxylic acids cycle, and certainly play a role in regulating heart metabolism. However, the large Ca^{2+} capacity of the mitochondria in heart cells has permit to the suggestion that, in addition cardiac mitochondria might themselves be involved in controlling $[Ca^{2+}]_c$ (23).

Myocardial ischemia-reperfusion injury

Ischemic heart disease (IHD) is associated with high morbidity and mortality and its prevalence is continuously increasing worldwide (24). The most common cause of IHD is atherosclerosis of coronary artery along with artery thrombosis, spasm and coronary embolus (25,26). Myocardial ischemia is a condition in which heart tissue is slowly or suddenly starved of oxygen and other nutrients leading to death of an affected cardiac muscle (27,28).

Myocardial reperfusion is the restoration of blood flows to an ischemic heart. Early reperfusion minimizes the extent of damage to heart muscle and preserves the pumping function of the heart. However, reperfusion after a prolonged period of ischemia produces a marked damage in myocardium rather than restoration of normal cardiac function. Thus, IR injury could be defined as the damage to heart when blood supply is restored after a prolonged period of ischemia resulting in oxidative damage, inflammation and cardiac dysfunction (29).

Depending upon the duration of ischemia, three levels of IR-induced cardiac injury have been described. The first detectable level of injury is the generation of reperfusion-induced cardiac arrhythmias. In general reperfusion after 1-5 min of ischemia can result in ventricular tachycardia or fibrillation without cell death, or a deficit in ventricular contractile performance. After an ischemic period of 5-20 min, reperfusion results in the second level of myocardial injury, known as “myocardial stunning” (30). Myocardial stunning is characterized by a deficit in myocardial contractility that occurs without myocardial cell death. Typically, IR-induced myocardial stunning results in ventricular contractile deficits lasting 24-72 h after the IR event. The third and highest level of IR injury occurs when ischemia is extended beyond 20 min. In these circumstances, cardiac myocytes become irreversibly damaged, resulting in cell death (i.e., myocardial infarction) (2).

Despite the complexity in the mechanisms responsible for the IR-induced myocardial damage, essential factors leading to IR-induced cellular injury have been delineated. It is now clear that IR-induced cardiac myocyte death occurs due to both apoptosis and necrosis,

and that mitochondrial injury plays a major role in both forms of cell death (31,32). Evidences indicate that several interrelated factors, including a decrease in cellular ATP levels, productions of ROS, accumulation of hydrogen ions, calcium overload, calpain activation, and leukocyte activation, contribute to IR injury (33,34).

Myocardial ischemia leads to various cellular events like altered membrane potential, altered ion distribution, cellular swelling and cytoskeleton disorganization (29). Ischemia is also associated with proteolytic conversion of xanthine dehydrogenase to xanthine oxidase (XO), which at the time of reperfusion metabolizes hypoxanthine and xanthine to uric acid resulting in large amount of generation of ROS such as: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (35). These ROS by causing membrane injury and activating intracellular signaling pathways ultimately lead to myocardial cell injury and death (29)**(Figure 1)**. It has been well documented that increased ROS generation occurs in ischemic myocardium exposed to reoxygenation upon reperfusion and the maximal release or ROS occurs during the first 15 min of reperfusion (36).

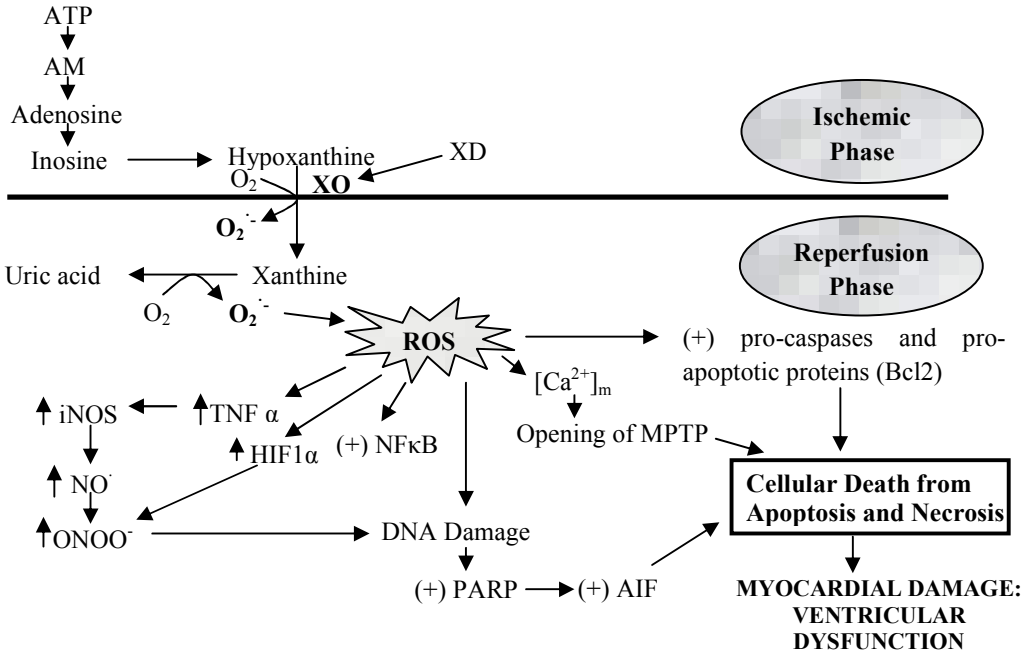

Figure1. Role of ROS in mechanisms of cellular signaling that take place during episodes of myocardial IR. XD: xanthine dehydrogenase, XO: xanthine oxidase, $O_2^{\cdot-}$: radical anion superoxide, iNOS: nitric oxide sintase inducible, TNFα: tumoral necrosis factor α, HIF1: Hypoxic inducible factor 1, NFκB: nuclear transcription factor κ B, MPTP: mitochondrial permeability transition pore, $NO^{\cdot}$: nitric oxide, $ONOO^-$: peroxynitrite, PARP: poli (ADP-ribose) polimerase, AIF: apoptosis inductor factor, (+): activation.

IR cascade include various events such as micro-vascular damage, myocardial hibernation and no-reflow phenomenon (37). Myocardial hibernation is a reversible reduction in contractile function due to reduced coronary perfusion (38). Hibernation is an endogenous adaptative event in which the myocardium reduces its own contractility and metabolic needs in order to survive with minimal requirements due to diminished oxygen supply. However, on chronic hibernation, the normalization and recovery of myocardial function

could be delayed even the coronary blood flow gets normal (39). After re-opening of an occluded artery, reperfusion of the previously ischemic tissue depends on microvascular integrity. The no-reflow phenomenon may be defined as an incomplete and non-uniform reperfusion at the microvascular level despite the adequate re-opening of the proximal artery after a period of transient ischemia (40).

In addition the signaling pathways such as caspase-3, caspase-8 (41), interleukin-6 (IL-6) (42), and tumor necrosis factor-alpha (TNF-α) (43,44) have been implicated in IR-induced myocardial injury. Also, p38 α mitogen-activated protein kinase (p38α MAPK) (45), poly (ADP-rybose) polymerase (PARP) (46,47), janus kinase/signal transducer and activator of transcription (JAK/STAT) (48), mitogen-activated protein kinase (MEK 1/2) (49), c-jun-N-terminal kinase (JNK1/2) (50), poly (ADP-rybose) glycohydrolase (PARG), second mitochondrial-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pi (Smac/DIABLO) (51), and Rho kinase (52,53) have been shown to be implicated in the pathogenesis of myocardial IR injury and cardiac dysfunction.

ROS production contributes with ischemia-reperfusion injury

A substantial body of evidence implicates ROS and oxidative stress in the cellular injury induced by IR in the heart (54-57). Cell damage can occur through mechanism involving lipid peroxidation, covalent modification of protein (particularly on -SH groups) and mitochondrial DNA oxidation (3).

ROS production in the heart during both ischemia and reperfusion has been confirmed using electron paramagnetic resonance (58), and the quantity of ROS production in reoxygenated cells depends upon the duration of both anoxia and reoxygenation (2).

Experimental evidence for the involvement of ROS in myocardial IR injury includes detection of lipid peroxides, protein oxidation, and protein nitration products in reperfused heart (2). The importance of ROS-mediated damage to the heart after an IR insult has been confirmed by studies indicating that antioxidants can provide myocardial protection against IR-induced injury. Identifying the primary sources of ROS during both ischemia and reperfusion continues to be an active area of research. Existing evidence indicates that ROS are produced in both ischemic and reperfused cardiomyocytes from a variety of sources, including NADPH oxidase, XO, and mitochondria (58,59). Also, neutrophil infiltration during reperfusion can contributes to ROS production in the heart, but activated neutrophils are not required for reoxygenation injury (2). More specifically, it is likely that mitochondrial production of ROS plays a dominant role in IR-mediated injury (58,59).

Redox components of the ETC, particularly impaired complexes I and III in their reduced state, have been shown to produce ROS **(Figure 2)**. Complex I, in particular, is impaired during IR, and may be considered as the source of damaging radicals, although experiments using the electron transfer inhibitors amytal and myxathiazole, suggest complex III as a major site of ROS production during ischemia (3). Other studies indicate that complex II may also produces ROS (60), particularly if certain mutations are present in regions of the CoQ (ubiquinone) or the FAD-binding sites (61).

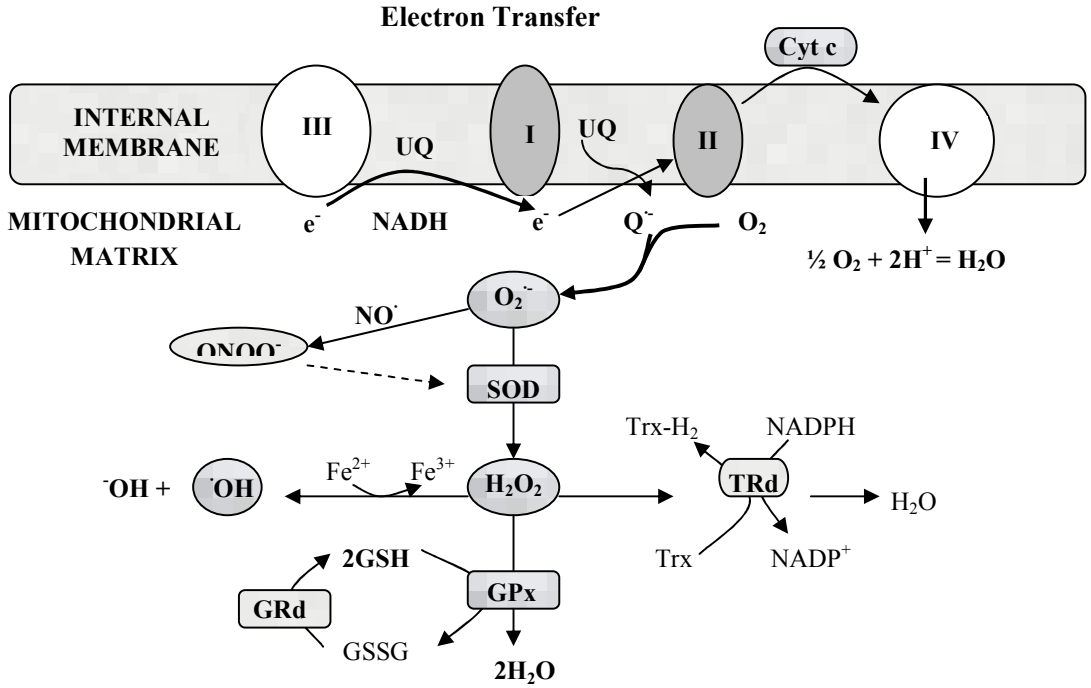

Figure 2. Generation of ROS during oxidative phosphorylation due to complexes I and III from the electron transfer chain. Complex I (NADH-ubiquinone oxide-reductase) catalyzes the first electronic transfer. After that, the ubiquinone Q10 (UQ) from complex III, catalyzes the conversion of molecular oxygen to superoxide anion ($O_2^{\cdot-}$). Other ROS can be generated from this radical, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). GPx: glutathione peroxidase, GRd: glutathione reductase, GSH: reduced glutathione, GSSG: oxidized glutathione, $NO^{\cdot}$: nitric oxide, $ONOO^-$: peroxynitrite, Cyt c: cytochrome c, TRd: thiorredoxin reductase, Trx: reduced thiorredoxin, Trx-H_2: oxidized thiorredoxin, NADPH: nicotinamide adenin dinucleotide phosphate (reduced form), $NADP^+$: nicotinamide adenin dinucleotide phosphate (oxidized form).

Several lines of evidence suggest that cross talk between ROS and calcium contributes to IR-induced cellular injury. First, increased cytosolic Ca^{2+} levels can contribute to cellular damage by activation of proteases, by promotion of mitochondrial damage, and by facilitating ROS production (2). Further, oxidative stress can contributes to Ca^{2+} overload in cells in several ways (62). For example, oxidant-generated formation of reactive aldehydes such as 4-hydroxyl-2,3-trans-nonenal has been reported to reduce plasma membrane Ca^{2+}-ATP_{ase} activity (63). It follows that an oxidative stress-induced decrease in membrane Ca^{2+}-ATP_{ase} activity would retard Ca^{2+} removal from the cell and therefore contributes to Ca^{2+}

accumulation. Accumulated Ca^{2+} is subsequently released from the mitochondria along with other matrix solutes, especially when Ca^{2+} sequestration is accompanied by oxidative stress and depletion of adenine nucleotides. This potentially deleterious effect of ROS production in mitochondria, known as Ca^{2+}- dependent mitochondrial permeability transition (MPT), which plays a key role in certain modes of cell death. This phenomenon is characterized by drastic changes in mitochondrial morphology and functional activity owing to the opening of a nonspecific pore in the mitochondrial inner membrane, commonly known as the MPT pore (MPTP) (64-66).

The basic unit of the MPTP is the VDAC-ANT-CyP-D (voltage dependent anion channel-adenine nucleotide translocase-cyclophilin D) complex located at contact sites between mitochondrial inner and outer membrane. Recent studies identified the ANT as one important target for ROS induced by anticancer drugs, such as doxorubicin and arsenic trioxide. The role of VDAC as target for ROS is less clear, although it has been demonstrated recently that $O_2^{\cdot -}$ modulates the opening of VDC reconstituted into liposomes (67).

Hence, oxidative stress and impaired Ca^{2+} homeostasis both contribute to mitochondrially mediated cellular damage. MTP is a mechanism causing mitochondrial failure, which can lead to necrosis owing to ATP depletion or to caspase-dependent apoptosis if MTP induction occurs in a subpopulation of mitochondria and remaining organelles are still able to maintain the mitochondrial membrane potential and produce ATP (68).

Anyway mitochondria itself has become a sensitive target for the damaging effects of oxygen radicals. One of these targets is mitochondrial DNA ($_m$DNA), which encodes 13 polypeptides, 22 transfer RNA ($_t$RNA$_s$), and 2 ribosomal RNA$_s$ ($_r$RNA$_s$), all of which are essential for electron transport and ATP generation by oxidative phosphorylation (69). $_m$DNA, therefore, represents a critical cellular target for oxidative damage that could lead to lethal cell injury through the loss of electron transport, mitochondrial membrane potential, and ATP generation. $_m$DNA is especially susceptible to attack by ROS owing to its close proximity to the ETC, the major locus for free-radical production, and the lack of protective histones (68).

Notably, cardiac ischemia induces increased generation of ROS, and subsequent reperfusion can result in toxic ROS overproduction that possibly contributes to irreversible damage of mitochondrial function and consequent, impaired recovery of physiological function and cell death.

Role of nitric oxide during ischemic-reperfusion injury

Nitric oxide ($NO^{\cdot -}$) has been associated with protection against ischemic cell death in a large number of studies (70), although the mechanism and site of action are not known. $NO^{\cdot -}$ is required for the cytoprotective effect induced by ischemic preconditioning in the heart (71), but it is not clear whether $NO^{\cdot -}$ acts before the ischemia, during the ischemia or during reperfusion (72).

Moreover, other studies suggest that $NO^{\cdot -}$ may potentiate apoptosis in response to IR (73,74) and that it can induce cell death in a dose-dependent manner (75). The abundance of

potential targets and the dose dependence of the responses may explain the diversity of effects that have been observed under different experimental conditions (72).

Physiologically low levels of $NO^{\cdot}$ during ischemia, either through exogenous application or through the activity of nitric oxide synthase (NOS), confer significant protection against cell death during reperfusion. $NO^{\cdot}$ seems to protect cell by attenuating the oxidation stress that occurs during ischemia, and this effect further attenuates reperfusion oxidative stress after the $NO^{\cdot}$ is removed. $NO^{\cdot}$ during ischemia can protect cells by inhibiting an oxidase system that is initiated during ischemia and that becomes amplified during the reperfusion phase or by lessing oxidative injury by scavenging ROS (72).

On the other hand, although $NO^{\cdot}$ plays a prominent role in regulating many biological functions, extensive evidence exists which indicates that a high concentration it can be also be cytotoxic and mutagenic (76). Mitochondria are considered a prime target for this $NO^{\cdot}$-induced damage. $NO^{\cdot}$ or its derivate secondary oxidants can have at least three types of actions on mitochondria: (a) reversible inhibition of mitochondrial respiration at cytochrome oxidase by NO, and irreversible inhibition at multiple sites by other nitrogen species; (b) stimulation of mitochondrial production of $O_2^{\cdot-}$, H_2O_2 and peroxynitrite ($ONOO^-$) by $NO^{\cdot}$; and (c) induction of the MPT by its secondary oxidant agents (77).

It is becoming increasingly clear that $NO^{\cdot}$ under certain circumstances can be genotoxic. It can cause both DNA damage and mutations. Moreover, it has been shown that $NO^{\cdot}$ damages $_m$DNA to a greater extent that nuclear DNA (78). $NO^{\cdot}$ can be generated within mitochondria (79), although the identity of mitochondrial NOS still remains uncertain, $NO^{\cdot}$ produced by mitochondrial NOS has been suggested to play important roles in oxidative stress and apoptosis (80), regulation of mitochondrial respiration (81) and modulation of intracellular Ca^{2+} homeostasis (82).

Two basic signaling pathways leading to $NO^{\cdot}$ -induced apoptosis have been identified. One is mitochondria-dependent (intrinsic), initiated by the release of cytochrome c from mitochondria, and leads to caspase dependent and/or –independent apoptosis. The other (extrinsic) is death receptor/caspase 8 dependent, and involves the interaction of death receptors with receptor-associated death proteases with the subsequent activation of downstream effector caspases (83,84). $NO^{\cdot}$, depending upon the concentration, cell type, presence of glucose, and its donor, has been shown to either induce or protect from apoptosis through the mitochondrial pathways (85-87). Several mechanisms have been purposed to explain NO-mediated apoptosis including increased p53 levels, ROS production, and/or opening of the MPTP (88,89). Opening of the MPTP quickly leads to cytochrome c release, which activates several well-established downstream signaling events, including formation of the apoptosome and activation of caspases (90).

Mutations in $_m$DNA could be partially the result of either increased damage to $_m$DNA or decreased repair of this damage, or combination of both factors. Because almost all of the $_m$DNA encodes for specific proteins of the ETC, any mutations or deletions in $_m$DNA could readily result in a loss of one or several protein essential for electron transport or mitochondrial ATP synthesis. This would in turn lead to decreased energy production, which is a hall mark of many mitochondrial disorders. Moreover, dysfunction of ETC could

cause additional production of ROS, and in this way enhance the cellular signals to initiate apoptosis, necrosis or a combination of both (77).

Metabolic changes in ischemia and reperfusion in cardiac muscle

Cardiac muscle obtains virtually all its energy from oxidative metabolism. Consequently, restriction of the blood supply to cardiac muscle has serious pathological consequences, leading to cell death in the oxygen-depleted region (infarct) (3). During hypoxia or ischemia, the supply of oxygen to the respiratory chain fails. Non-esterified fatty acids levels rise, although probably as a result of lipid breakdown rather than the concomitant cessation of fatty acid oxidation (91,92). The tricarboxylic acid cycle is blocked, and no energy is available from oxidative phosphorylation. This leads to an accumulation of cytoplasmic NADH, with the NADH/NAD^+ ratio increasing severalfold. In anoxia, ATP levels can still be maintained by glycolysis (93), but in ischemia this is accompanied by an accumulation of lactate and a decrease in cytoplasmic pH (5,5-6 after 30 min of ischemia) (94-96), and glycolysis is also inhibited (3).

On a simple model, it is expected that, during ischemia (as mitochondrial electron transfer is abolished) the electrochemical gradient of H^+ ($\Delta\mu_{H+}$) will fall rapidly. It should then rise during reperfusion, when electron transfer is restored. While this seems in principle to be the case, investigations reveal a greater complexity. First, in ischemia, a mitochondrial electrical potential is maintained for some minutes (97) due to the hydrolysis of cytoplasmic ATP by the F_1F_o-ATP synthase acting in reverse as an ATP driven pump (98,99). Conversely, during reperfusion, we should expect $\Delta\mu_{H+}$ to recover rapidly. A number of researchers have reported that, after reperfusion, mitochondrial electrical potential is depressed from its control value, and that mitochondria have an increased proton leak. This was originally demonstrated by analysis of flow force relationships in isolated heart mitochondria after ischemia (and/or reperfusion) (100-103).

It is possible, again, that this change represents either unavoidable mitochondrial damage during IR or the preferential use of oxidative energy to restore ion concentrations. However, a number of researchers have suggested that the depression of the electrical potential may play a protective role during early reperfusion, limiting the electrophoretic influx of Ca^{2+} and/or decreasing the production of ROS by facilitating electron transfer. Agents that promote the leakage of protons into mitochondria, such as uncouplers (104,105), or transfection with uncoupling proteins (UCPs) (106,107) have been shown to protect against IR injury.

Also during ischemia, creatine phosphate concentrations falls precipitately (to less than 10% after 10 min of ischemia), reflecting a sharp increase in free ADP levels. ATP levels fall rather more slowly, with 40-50 % of ATP levels remaining after 30 min of ischemia (108). During ischemia, the levels of total pyridine nucleotides seem to be roughly maintained, although there have been reports of significant loss (up to 30 %) of total nucleotides from the cell (109-111). This redox state, however, changes markedly, with [NADH] increasing sharply (112,113). The cytoplasmic [NADPH], in contrast, declines by approximately 30 %, resulting in a significant decrease in the NADPH/$NADP^+$ ratio. While at first this may appear surprising, the fall in [NADPH] could be due to the action of

glutathione reductase, which is particularly active under conditions of oxidative stress. In addition a contributory effect may come from the activation of aldose reductase, a member of the aldo-keto reductase family that utilize NADPH to reduce carbonyl compounds, including glucose, in the metabolism of the polyols (114).

Over long periods of ischemia, DNA and protein synthesis are suppressed (115), although some specific proteins e.g. HSP (heat-shock protein) 70, PKC (protein kinase C) and iNOS (inducible nitric oxide synthase) may be induced (116,117) or repressed, e.g. $ATP_{ase.}$(118) On reperfusion, electron transfer and ATP synthesis are restarted, and the internal cytoplasmic pH is restored to 7.0 (119,120). However, this leads in some way to a further deterioration of cell function. While ATP and creatine phosphate levels recover to some extent, the myocytes undergo further shortening (hypercontracture) and membrane damage, followed by cell death (5).

Mitochondrial oxidation

It is widely accepted that during prolonged ischemia, the maximum capacity for respiratory chain oxidation is decreased, with the severity of damage depending on the length of exposure (121). In accordance with the decreased respiratory capacity, oxidative phosphorylation was also found to be depressed in experimental models of ischemia and reperfusion (122-124).

Ischemia/reperfusion does cause some damage to respiratory chain complexes, but as these complexes are normally present in excess (having a low flux control coefficient), this damage has little effect on normal respiratory rates. Indeed, respiration rates may be observed to rise owing to an increased proton permeability of the inner-mitochondrial membrane (decreased respiratory control). This rise, however, will be dependent on an ample supply of oxidizable substrate, which may, in some conditions, be restricted and itself limit the respiratory rate observed (3).

Many researchers have identified complex I as major site of damage to the respiratory chain in ischemia (99,100,124,125). They observed a reduction in oxidation rate for NADH-linked substrates by up to 60 % (126). The cause of complex I inhibition is unknown (3). Paradies et al. (127) associated the decreased in complex I activity under conditions of oxidative stress with the destruction of mitochondrial cardiolipin by ROS. Others researchers have suggested that NO may be the causative agent, possibly via peroxynitrite intermediates (128,129), since inhibitors of iNOS prevent the inactivation. This view is supported by Jekabsone et al. (89), who showed that complex I activity declines in isolated mitochondria exposed to NO, and that this decline is prevented by SOD. Thus either ROS or NO (or both) at the concentrations reachable during ischemia and reperfusion could inactivate the enzyme directly, or via an effect on cardiolipin.

Both complex II and III seem to be relatively resistant to ischemia and reperfusion. Rouslin (99) found that complex III activity did decline in ischemic dog heart, but more slowly than complex I activity. Its decrease paralleled that of succinate-supported oxygen uptake, implying that complex II was not affected by either ischemia or reperfusion.

The situation regarding complex IV is more complicated. Several reports indicate it to be virtually unchanged by ischemia (99,125,130-132). However, there did appear to be reduced electron flow through complex IV both in permeabilized ischemic tissue and mitochondria isolated from ischemic heart (133,134). This may be attributable to the loss of cytochrome c, which mediates electron transfer between complexes III and IV, from the intermembrana space in subsarcolemmal mitochondria during ischemia (3). Some authors claimed that cytochrome c oxidase activity decreased by nearly 30 % during ischemia (from 0.156 to 0.111 nmol/mg of protein) and that full respiration rates are restored by external addition of cytochrome c. These authors also showed that cytochrome c is found in the perfusate of hearts, following ischemia /reperfusion, suggesting it can indeed be lost from mitochondria in pathological situations (133). In contrast some other researchers claim that cytochrome c content of mitochondria is not significantly changed after ischemia (125). The effect of inhibition of cytochrome oxidase, either by direct damage or by loss of cytochrome c, might not be expected to have a major effect on respiratory rates *in vivo* (135,136).

The tendency of the F_1F_0-ATP synthase (also called complex V), to reverse during ischemia thus appears to be an inescapable consequence of the thermodynamics of the system, and in the absence of energy-yielding reactions of electron transfer, ATP synthesis is unfavorable and hydrolysis is favored (137-140).

Inhibition of wasteful ATP hydrolysis during ischemia might be assumed to have a beneficial effect on the heart. During reperfusion, when the ATP synthase should be working to synthesize ATP, however, inhibition of this enzyme would presumably delay recovery. This is explicable in terms of the properties of IF_1 (natural inhibitor protein of the mitochondrial F_1F_0-ATP_{ase}), which is known to bind to F_1F_0 at low pH and low electrical potential (such as occur in ischemia), but to be released at high pH and high electrical potential (such as occur in during electron transfer) (141), acting, effectively, as one way valve.

Despite of different viewpoints regarding the effect of ischemia and reperfusion on the respiratory chain, researchers have reached a consensus indicating that exposing hearts to 15-60 min of ischemia leads to a modestly impaired ETC, with rates reduced by approximately 30 % relative to the control value. Complex I appears to be damaged, and there is some restriction of electron flow through complex IV, possibly because of loss of cytochrome c. During reperfusion, further damage to electron transfer components occurs, in particular to complex IV, possibly due to the action of ROS (as described below), and a "vicious cycle" in which further inhibition leads to further ROS production, and so on, is a possible cause of irreversible damage to mitochondria (3).

Anyway, it is very important to take into account all the elements given, and to focus in new investigations regarding specially, the ROS production and its modulation in the ischemic environment. This fact should be handled carefully, because of the effect of the oxidative damage to mitochondria and its implication in many other degenerative disorders.

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