Reactive species mechanisms of cellular hypoxia-reoxygenation injury

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Li, Chuanyu, and Robert M. Jackson. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol 282: C227–C241, 2002; 10.1152/ajpcell.00112.2001.—Exacerbation of hypoxic injury after restoration of oxygenation (reoxygenation) is an important mechanism of cellular injury in transplantation and in myocardial, hepatic, intestinal, cerebral, renal, and other ischemic syndromes. Cellular hypoxia and reoxygenation are two essential elements of ischemia-reperfusion injury. Activated neutrophils contribute to vascular reperfusion injury, yet posthypoxic cellular injury occurs in the absence of inflammatory cells through mechanisms involving reactive oxygen (ROS) or nitrogen species (RNS). Xanthine oxidase (XO) produces ROS in some reoxygenated cells, but other intracellular sources of ROS are abundant, and XO is not required for reoxygenation injury. Hypoxic or reoxygenated mitochondria may produce excess superoxide (O$_2^-$) and release H$_2$O$_2$, a diffusible long-lived oxidant that can activate signaling pathways or react vicinally with proteins and lipid membranes. This review focuses on the specific roles of ROS and RNS in the cellular response to hypoxia and subsequent cytolytic injury during reoxygenation.

anoxia; ischemia; reperfusion

BACKGROUND

Scope and Goals of the Review

This review focuses on reactive oxygen (ROS) and nitrogen species (RNS)-mediated mechanisms that lead to cellular injury during hypoxia and reoxygenation. Pathophysiology due to hypoxia per se is addressed when relevant to reoxygenation in the cellular context. Emphasis is on intracellular mechanisms that generate ROS and RNS and their roles in cellular signaling or as mediators of cell death.

Clinical Importance of Ischemia-Reperfusion Injury

Both hypoxia (lack of oxygen relative to metabolic needs) and reoxygenation (reintroduction of oxygen to hypoxic tissue) are important in human pathophysiology because they occur in a wide variety of important clinical conditions. Prominent examples of tissue hypoxia that predispose to injury during reoxygenation include circulatory shock, myocardial ischemia, stroke, and transplantation of organs (67, 76, 102). Because diseases due to ischemia (e.g., myocardial infarction and stroke) are exceedingly common causes of morbidity and mortality and because organ transplantation is increasingly frequent, understanding the role of ROS and RNS in reoxygenation injury has the potential to lead to therapies that could improve public health. Cellular models of hypoxia-reoxygenation have provided useful tools for the study of reactive species-mediated mechanisms of cellular dysfunction in ischemia-reperfusion injury (127).

Cellular Hypoxia and Reoxygenation Injury

Cellular necrosis inevitably follows extended periods of anoxia (i.e., oxygen absent) or severe hypoxia (i.e., oxygen supply decreased relative to metabolic demand). Hypoxic tolerance of various cell types differs, depending on the metabolic rate and intrinsic adaptive mechanisms of the tissue. Sublethal hypoxia, which
may be transient and have no apparent consequences, can be followed by enhanced resistance to reoxygenation injury (conditioning), recovery, or exacerbated cellular injury (reoxygenation injury). Posthypoxic injury is due to a combination of changes in cellular energy charge, oxidant generating systems, and antioxidant defenses (76).

Cellular hypoxia appears to be a key signal that activates transcriptional regulators, including hypoxia-inducible factor-1 (HIF-1) (109), nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and some mitogen-activated protein kinase (MAPK) pathways (46). A redox-sensitive human antioxidant response element induces gene expression in response to low oxygen concentrations in some malignant cells (124). Two overlapping manifestations of cell death, necrosis and apoptosis, can be initiated by cellular hypoxia-reoxygenation (105).

Cells undergo specific changes in enzyme activities, mitochondrial function, cytoskeletal structure, membrane transport, and antioxidant defenses in response to hypoxia, which then collectively predispose to reoxygenation injury (1). In contrast to the adaptive effects of sublethal hyperoxia on ion transport (74), hypoxia downregulates proteins that maintain alveolar ion transport, including the Na⁺-K⁺-ATPase (sodium pump) and the epithelial Na⁺ channel (ENaC) (23). Hypoxia causes time- and concentration-dependent decreases in α-, β-, and γ-subunits of ENaC mRNA and decreases both β₁- and α₁-subunits of the Na⁺-K⁺-ATPase. Hypoxia itself also impairs cation transport in both A549 lung epithelial cells and rat alveolar cells (73). Oxidative inhibition of membrane Na⁺-K⁺-ATPase activity by H₂O₂ produced as a result of hypoxia-reoxygenation may be an important mechanism leading to swelling and cytolysis (52). In lung hypoxia-reoxygenation injury (e.g., after lung transplantation), reduced transepithelial Na⁺ transport and fluid resorption by the alveolar epithelium would increase pulmonary edema formation and impair its clearance.

A number of mitochondrial enzymes, including cytochrome oxidase and manganese superoxide dismutase (Mn SOD), decrease in activity in hypoxia (81, 101, 113), with predicted effects on oxygen metabolism. Cellular hypoxia inhibits expression of the multienzyme mitochondrial oxidase (complex IV), the final intramitochondrial site of oxidative phosphorylation. Cytochrome oxidase activity of aerobic mouse lung macrophages decreases ~40% when incubated anaerobically for 96 h (113). Loss of cytochrome oxidase activity leads to cellular injury during reoxygenation, because absence of the final electron acceptor increases ROS production by more proximal complexes (33).

Cytoskeletal changes occurring in hypoxia would likely alter endothelial and epithelial permeability. ATP-depleted endothelial cells display shortening and disassembly of F-actin filaments (41), which lead to increased endothelial permeability (71). Hypoxia-reoxygenation has specific effects on the cytoskeleton of renal epithelial cells (88), which influence transmotional motion of membrane lipids. Cellular hypoxia may cause membrane protein aggregation, alterations in protein polarization (altered apical-basolateral orientation), protein degradation, and changes in molecular chaperones or growth factors. Renal epithelial cells develop increased membrane permeability, because of changes in transmembrane adhesion molecules, in a coordinated response characterizing the ischemic phenotype (12).

McCord (76) proposed the seminal model to explain how reoxygenation worsens ischemic (i.e., hypoxic) injury through increased ROS production. Superoxide radical (O₂⁻), generated by xanthine oxidase (XO), was postulated initially to be the ROS responsible, but now the entire spectrum of ROS and RNS [including peroxynitrite (ONOO⁻)] has been implicated in hypoxic cellular injury. ROS generated by hypoxia or reoxygenation are now recognized as interacting with physiological signal transducers (19, 46, 104, 110) rather than behaving as simple reactants that peroxidize membrane lipids, oxidize DNA, or denature enzyme proteins.

Free radicals (defined chemically as molecules containing an odd number of electrons) (33), such as O₂⁻, the hydroxyl radical (·OH), and other ROS and RNS species (especially ONOO⁻) are detected during, and likely account for, some manifestations of postischemic injury (Table 1). Perhaps of more physiological importance, both ROS and RNS can affect signal transduction in posthypoxic cells, and ROS are able to initiate cell death programs in the form of apoptosis or necrosis. Diverse sources of ROS (e.g., enzymes, mitochondria) exist normally within cells, some of which produce excess reactive species during hypoxia-reoxygenation. Excess ROS from endogenous sources can account for autocrine and paracrine cellular injury during reoxygenation.

**ROS Mediate Reoxygenation Injury**

General evidence for involvement of ROS in hypoxia-reoxygenation injury includes detection of lipid peroxidation and protein nitration products in reperfused brains (117), protection of various reperfused organs by antioxidant enzymes including SOD (76), and inhibition of postsischemic injury by allopurinol, an XO inhibitor structurally related to purines (91). ROS (including ·OH radical) have been confirmed by electron paramagnetic resonance and spin trapping to occur in reoxygenated endothelial cells (135).

Neutrophils are important sources of ROS, but activated neutrophils are not required for reoxygenation injury. Injury to cultured endothelial cells, cardiac myocytes, hepatocytes, and other cell types occurs after anoxia-reoxygenation in vitro, even in the absence of neutrophils. Endothelial cells themselves subjected to anoxia-reoxygenation release superoxide anions (O₂⁻) into the extracellular medium, as demonstrated by SOD-inhibitable, extracellular cytochrome c reduction and other assays (108). The quantity of O₂⁻ produced by reoxygenated cells depends on the duration of both anoxia and reoxygenation (99).
In vitro reoxygenation decreased the viability of rat hepatocytes as a function of time in hypoxia (26). Extracellular SOD and catalase completely prevented reoxygenation injury, confirming involvement of at least extracellular ROS. Electron transport chain inhibitors cyanide and antimycin A increased the severity of cellular injury in this model, suggesting that mitochondria may be a source of ROS (27). Reoxygenated hepatocytes released increased quantities of O$_2$ and catalase completely prevented increased cellular injury. Hypoxia-reoxygenation also stimulates vascular endothelial cells to release extracellular O$_2$ (118). The anion channel blocker DIDS, which prevents extracellular release of O$_2$ through anion channels, protected from reoxygenation injury as well as extracellular SOD did. Injury to reoxygenated endothelial cells appears to involve generation of OH$^-$ by surface-associated iron that reacts with H$_2$O$_2$, because iron chelation decreased cellular injury.

Hypoxia Apparently Increases ROS Production

Oxidants are produced in excess during reoxygenation, but ROS production also may increase in the reduced state that characterizes cellular hypoxia. Pulmonary artery smooth muscle cells, cardiomyocytes, and several other cell types produce increased ROS in hypoxia that are usually detected as oxidation of the fluorescent probe DCF (54, 133). Hypoxic pulmonary artery smooth muscle cells significantly increased DCF fluorescence fivefold above that of normoxic cells (55), and simulated ischemia (hypoxia and low glucose) of embryonic ventricular myocytes increased DCF fluorescence threefold over that of normoxic cells (133).

Table 1. Important mechanisms and sequelae of cellular reoxygenation injury

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ROS, reactive oxygen species; XD, xanthine dehydrogenase; XO, xanthine oxidase; GSH, glutathione; NF-κB, nuclear factor-κB; PGI$_2$, prostacyclin; NOS, nitric oxide synthase; RNS, reactive nitrogen species; PKC, protein kinase C; HSP, heat shock protein.

In vitro reoxygenation decreased the viability of rat hepatocytes as a function of time in hypoxia (26). Extracellular SOD and catalase completely prevented reoxygenation injury, confirming involvement of at least extracellular ROS. Electron transport chain inhibitors cyanide and antimycin A increased the severity of cellular injury in this model, suggesting that mitochondria may be a source of ROS (27). Reoxygenated hepatocytes released increased quantities of O$_2$ and catalase completely prevented increased cellular injury. Hypoxia-reoxygenation also stimulates vascular endothelial cells to release extracellular O$_2$ (118). The anion channel blocker DIDS, which prevents extracellular release of O$_2$ through anion channels, protected from reoxygenation injury as well as extracellular SOD did. Injury to reoxygenated endothelial cells appears to involve generation of OH$^-$ by surface-associated iron that reacts with H$_2$O$_2$, because iron chelation decreased cellular injury.

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Simulated in vitro ischemia of cardiomyocytes also increased dihydroethidine (DHE) oxidation due to ROS, so these observations are not simply an artifact related to the use of oxidizable DCF. Both myxothiazole and rotenone inhibited the DHE oxidation, suggesting that mitochondrial complexes III and I produced increased ROS. SOD inhibited the DHE oxidation, providing indirect evidence that the mitochondrial electron transport chain generated O$_2^-$ during in vitro ischemia, probably from the ubisemiquinone site (6). Hypoxia-induced tyrosine hydroxylase gene expression also is paralleled by increased ROS production in PC-12 cells (42), as shown by increased oxidation of dihydrorhodamine. Human lung epithelial cells likewise have been observed to increase ROS production significantly after 24-h incubation in hypoxia (<1% O$_2$) (69). Examples of typical data from such experiments are shown in Fig. 1, which demonstrates increased DCF fluorescence in hypoxia-preexposed lung epithelial cells by both fluorescent microscopy and flow cytometry. The sulfhydryl compound N-acetylcysteine and the antioxidants ebselen (a peroxide scavenger) and Tempol (a superoxide scavenger) inhibited DCF fluorescence. Ebselen was
the most effective at inhibiting the DCF signal from reoxygenated cells, suggesting that peroxides (probably H$_2$O$_2$) accounted for the most of the increased DCF fluorescence.

Hypoxia preexposure appears to increase cellular ROS production, probably from mitochondrial electron transport complexes. Excess ROS and RNS production has been well documented during reoxygenation. In vitro experiments have demonstrated that hypoxia-reoxygenation is sufficient to cause injury to various cell types, even in the absence of activated neutrophils. Both inhibitor (antioxidant) studies and detection of lipid and protein oxidation have confirmed a role for endogenously generated reactive species in cellular reoxygenation injury.

**INTRACELLULAR SOURCES OF ROS DURING HYPOXIA-REOXYGENATION**

**Xanthine Dehydrogenase-Oxidase**

Two closely related enzymes potentially capable of ROS generation, aldehyde oxidase and XO, exist in most animals. Activities are concentrated mainly in the liver and intestine (63). XO is the more important source of both O$_2$ and H$_2$O$_2$, but XO varies widely in abundance among cell types, organs, and species. XO normally occurs in vivo as an NAD$^+$-dependent dehydrogenase [xanthine dehydrogenase (XD)] incapable of ROS production. XD activity converts by sulfhydryl oxidation or limited proteolysis (conditions that exist during reoxygination) to an oxidase that produces both O$_2$ and H$_2$O$_2$ (91). XO is phosphorylated in hypoxic microvascular endothelial cells through a mechanism involving p38 MAPK and casein kinase II, and phosphorylation appears to be necessary for hypoxia-induced enzyme activation (54). However, reoxygenation injury also unquestionably occurs in cells in which XO activity is exceedingly low or absent. For example, human and rabbit hearts produce only minute quantities of urate because of their extremely low XO activities, yet both species develop myocardial infarctions and ischemia-reperfusion injury (82).

Rat tissues contain relatively high levels of XD-XO activity. Rat pulmonary endothelial cells are protected equally from reperfusion injury by antioxidant enzymes or allopurinol, confirming that XO is an important source of ROS during reoxygination (1, 99). XO also appears to be responsible for reoxygenation-induced O$_2$ formation and injury of rat kidney cells. Rat proximal tubule epithelial cells subjected to 60-min hypoxia and 30-min reoxygenation released increased quantities of LDH, whereas XO inhibitors allopurinol and dietary tungsten, which depletes XO and dietary tungsten, which depletes XO activity, attenuated both injury and O$_2$ production (37).

**Ferrylhemoglobin**

Hemoglobin and myoglobin, which are released into plasma after trauma, can mediate endothelial cell oxidative stress, and a ferrylhemoglobin intermediate has been detected in cellular hypoxia-reoxygenation models. Incubation of hemoglobin (Hb) or myoglobin during hypoxia-reoxygenation with endothelial cells causes transient oxidation of Hb to the reactive ferryl species (Fe$^{4+}$). Lipid peroxidation, which results from exposure of endothelial cells to ferrylhemoglobin, increased after reoxygination. Incubation of endothelial cells with Hb also caused a dose-dependent decrease in intracellular glutathione (GSH), confirming an oxidative stress (78). Nitric oxide (NO) acts as an antioxidant to inhibit lipid peroxidation and injury due to ferrylhemoglobin, because 400 µM arginine inhibited both lipid peroxidation and formation of ferryl intermediate (3). Hemoproteins oxidized to their ferryl forms could account for heme-mediated injury of endo-
Endothelial cells express a membrane NADPH oxidase, similar to the membrane enzyme complex that produces the respiratory burst in granulocytes. The membrane oxidase complex is an important source of ROS in ischemic mouse lungs (4) and probably other reoxygenated tissues. ROS produced by lungs exposed to normoxic ischemia or high-K⁺ buffer (24 mM) eminate from the membrane-associated NADPH oxidase complex in the vascular endothelial cells. p47\(^{phox}\), a subunit of the NAPDH oxidase, is detected by Western blotting of endothelial proteins. Endothelial cells adapted to flow in vitro for 2–7 days showed a nearly twofold increase in ROS production during simulated no-flow ischemia, compared with continuously perfused cells (129). Absence of flow causes flow-adapted cells to activate both NF-κB and AP-1. Transcription factor activation appears to be dependent on transduction of mechanical signals rather than altered oxygenation, showing that ischemia rather than hypoxia is the more important determinant in flow-adapted cells. In contrast, in isolated perfused lungs, anoxia-reoxygenation rather than ischemia-reperfusion causes XO to produce ROS (4).

The GTPase binding protein Rac1 regulates the membrane NADPH oxidase that produces ROS during mouse liver ischemia-reperfusion. A dominant negative Rac1 construct completely inhibited ischemia-reperfusion induced ROS production, NF-κB activation, and liver necrosis (86). The dominant negative gene product (N17rac1) inhibits the intracellular ROS burst after reoxygenation. N17rac1 expression protects smooth muscle cells, fibroblasts, endothelial cells, and ventricular myocytes from hypoxia-reoxygenation-induced death (58), indicating an important role for Rac1. A constitutively active Rac1 mutant does not increase intracellular ROS. Rac1 GTPase is, therefore, a necessary but not sufficient component of the pathway leading to ROS production during reoxygenation (87).

Multiple sources of ROS exist with cells and may account for increased reactive species production during reoxygenation. Although XD was identified early as a source of ROS during reoxygenation, other intracellular sources including redox cycling of iron and NAD(P)H oxidases have been demonstrated to produce ROS and contribute to oxidant stress during reoxygenation.

**ANTIOXIDANT DEFENSE MECHANISMS**

**Antioxidants Inhibit Cellular Reoxygenation Injury**

Endogenous antioxidant systems are critically important in limiting reoxygenation-induced cellular damage. The preponderance of data shows that exogenous antioxidant enzymes and low-molecular-weight ROS scavengers inhibit reoxygenation injury in cellular models. However, studies employing scavengers do not by themselves identify specific ROS or RNS responsible for reoxygenation injury, and they do not exclude other mechanisms. The level and duration of hypoxia are themselves important in regulating levels of both the antioxidant enzymes and low-molecular-weight ROS scavengers. including glutathione, which ultimately might determine the extent of reoxygenation injury (60, 81, 112).

Reoxygenation (after 12-min hypoxia) of hippocampal slices in vitro increased LDH release and lipid peroxidation significantly. Exogenous SOD and catalase inhibited injury, demonstrating involvement of ROS (43), which appeared to arise from prostaglandin synthesis, XO, and mitochondria, on the basis of the inhibitor profile. O₂⁻ is generated at complex III during reoxygenation (13), and antimycin A (a mitochondrial complex III inhibitor) typically increases its production. Antimycin A increased reoxygenation injury, as would be predicted if increased mitochondrial O₂⁻ production were responsible. Antimycin A is a quinone analog that binds to cytochrome b₅₅₉ and blocks transfer of electrons to the ubiquinone. It thus increases the complex III semiquinone concentration, which donates single electrons to oxygen.

Further indirect evidence from inhibitor studies for the involvement of ROS is that sodium salicylate, a nonspecific radical scavenger, prevents reoxygenation injury of rat livers perfused with hypoxic buffer (24). Livers released increased LDH and developed increased protein carbonyl and malondialdehyde content during reoxygenation. Salicylate (2 mM) inhibited development of markers of oxidant stress in reperfused livers, including LDH release, carbonyl formation, and malondialdehyde production.

Endogenous low-molecular-weight antioxidants are important in protection from reoxygenation injury. Thioredoxin (TRX; human T cell leukemia-derived factor) proteins act as disulfide oxidoreductases and electron donors for thioredoxin peroxidases. Thioredoxins function as disulfide bond reductants, similar in mechanism to N-acetylcysteine. Oxidized thioredoxin is reduced by thioredoxin reductases requiring NADPH.

Thioredoxin protects lung cells from reoxygenation injury. Viability of murine endothelial cells cultured in thiol-free medium decreased significantly after hypoxia-reoxygenation, but injury was diminished by exogenous TRX (100 μM). Because TRX does not decrease cellular ROS production or inhibit loss of GSH, it rather appears to act directly as a scavenger in reoxygenation models (47).

**Effects of Hypoxia on Antioxidant Enzymes**

Alveolar (AM) or peritoneal macrophages (PM) exist in high (P\(_{O₂}\) > 100 mmHg) or low (P\(_{O₂}\) < 20 mmHg) oxygen environments, respectively. Activities of the enzymes of oxidative phosphorylation and glycolysis differ significantly, with oxidative phosphorylation being higher in aerobic AM and glycolysis higher in hypoxic PM. Hypoxia decreases apparent expression of cytochrome oxidase in AM, a change in the electron transport chain that is consistent with elevated oxygen demands in hypoxic environments.
transport system that would predispose to O₂ formation (101). The antioxidant enzymes SOD, glutathione peroxidase (GP), and catalase are all two- to threefold higher in aerobic AM than in hypoxic PM (113), apparently reflecting the different levels of oxygenation. Similar changes in antioxidant enzyme phenotype occur when normally aerobic cells are cultured in hypoxia (48, 59, 101).

Mitochondrial DNA content, reflecting the abundance of mitochondria, does not change significantly in rat skeletal muscle cells cultured in hypoxia (81). However, a number of mitochondrial enzymes in these cells and in lung macrophages decrease in a coordinated fashion in hypoxia. Citrate synthase, NAD-isocitrate dehydrogenase, malate dehydrogenase, and cytochrome oxidase all decreased significantly after incubation of cells in hypoxia. (Mn SOD activity was not measured.) These data suggest a loss of mitochondrial enzyme activities due to hypoxia without a change in the number of mitochondrial. Brain capillary endothelial cells similarly respond to hypoxia preexposure by decreasing activities of GP, glutathione reductase (GR), catalase, and SOD, as well as cellular total GSH content (94). Such changes, which represent possibly adaptive downregulation of antioxidant defenses by unspecified mechanisms in hypoxia, would predispose to increased ROS production by reoxygenated mitochondria.

Global ischemia of isolated rabbit hearts decreases the rate of oxidative phosphorylation but does not eliminate H₂O₂ production. Hypoxia-reoxygenation significantly decreased activities of SOD and GP by ~40% in mitochondria isolated from rabbit hearts (112). Glucose-free hypoxia, used to model ischemia in vitro, also resulted in significantly depressed SOD and GP activities in perfused rat hearts. Rat hearts hypotrophied due to chronic pressure overload had increased SOD and GP activities (on a per mg protein basis), which appeared biologically important during recovery. Yet, during hypoxia, SOD activity decreased significantly in both control and hypertrophied hearts (60). Similarly, hypoxic exposure of cardiac myocytes (30 min) in vitro also caused a decrease in Mn SOD and GP activities but caused no change in catalase activity (59).

The response of alveolar type II (ATII) cells in primary culture to hypoxia exemplifies the effects of hypoxia on antioxidant defenses. Exposure of isolated rabbit ATII cells to hypoxia in vitro (<1% O₂ for 24 h) caused a significant decrease in Mn SOD activity and protein content (103). Mn SOD and Cu,Zn SOD mRNA expression also decreased significantly in ATII cells cultured in hypoxia for 24 h (48). The decrease in ATII cell Mn SOD mRNA expression (~69% compared with air controls by semiquantitative PCR) was greater than the decrease in Cu,Zn SOD mRNA expression. The decrease in Mn SOD mRNA content was due, in part, to decreased Mn SOD mRNA stability in the hypoxic ATII cells. Mn SOD enzyme specific activity did not change.

Hypoxia-induced decreases in cellular antioxidant enzymes, especially Mn SOD, have potentially important biological consequences because they could lead indirectly to cellular reoxygenation injury by exacerbating oxidant stress (69). LDH release from lung epithelial cells maintained in hypoxia (<1% O₂) for 24 h before treatment with antimycin A plus carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), an in vitro oxidant stress that increases mitochondrial O₂ production, was significantly greater than LDH release from vehicle-treated cells. The sulfhydryl antioxidant N-acetylcysteine (1 mM) significantly inhibited the increase in LDH release due to antimycin A plus FCCP.

The general response to hypoxia, therefore, appears to be decreased activity or expression of antioxidant defenses. Hypoxia-induced downregulation of antioxidant enzymes leads to reoxygenation injury, but the mechanism by which antioxidant enzyme expression is regulated by hypoxia is not known with certainty.

**RNS MEDIATE HYPOXIA-REOXYGENATION INJURY**

-NO Itself Can Cause Cellular Injury

Both ROS and RNS mediate some aspects of reoxygenation injury, but ·NO can produce either damaging or protective effects (49). In many cell types, inducible nitric oxide synthase (iNOS) is induced by hypoxia. iNOS induction during hypoxia is controlled by HIF-1 (51), a basic helix-loop-helix-PAS heterodimer regulated by oxygen tension (125). ·NO reacts rapidly with O₂ when both are produced simultaneously. The reaction product, peroxynitrite anion (ONOO⁻), is a potent oxidant that, when protonated (pKₐ 7.49; ONOOH), has ·OH radical-like reactivity (7). ONOO⁻ nitrates protein tyrosine residues to form 3-nitrotyrosine (3-NT) in reoxygenated tissues, but alternate pathways of tyrosine nitration utilizing myeloperoxidase and H₂O₂ exist and probably predominate (122). Thus finding 3-NT in reoxygenated cells does not unequivocally identify ONOO⁻ as the nitrating species, because nitrite (NO₃⁻) can react with peroxidase and H₂O₂ to form nitrogen dioxide (NO₂), which also contributes strongly to tyrosine nitration. Diverse cellular proteins may be nitrated during reoxygenation, but the specific functional consequences of most nitration reactions are unknown.

Human umbilical vein endothelial cells exposed to anoxia-reoxygenation release decreased quantities of prostacyclin (PGI₂) in response to thrombin, calcium ionophore A-23187, or arachidonic acid (40). Endothelial cells decrease prostacyclin production during short (15 min) hypoxia exposures (126). Decreased cyclooxygenase activity is due, in part, to ROS, because SOD and catalase inhibit the decrease in PGI₂ production. In addition, nitration of PGI₂ synthase correlates with decreased PGI₂ formation after reoxygenation. ONOO⁻ apparently nitrates and inactivates PGI₂ synthase, leaving unmetabolized prostaglandin H₂ (PGH₂), which can cause vasospasm, platelet aggregation, and thrombus formation by stimulating the thromboxane A₂ (TXA₂)/PGH₂ receptor (134).
NO itself may be cytotoxic, especially when present at supraphysiological concentrations (57). NO can react reversibly with enzyme 4Fe-4S (iron-sulfur) centers, yielding inactive 4Fe-4S-NO derivatives. NO thus inactivates mitochondrial aconitase, NAD-ubiquinone oxidoreductase (complex I), and succinate-ubiquinone reductase (complex II), resulting in inhibited mitochondrial respiration (32). NO inhibits respiration directly by binding cytochrome oxidase. NO-derived reactants, especially ONOO−, also potentiate inflammation by inactivating Mn SOD protein through nitration of its tyrosine residues (72), as well as by depleting low-molecular-weight antioxidants like GSH and ascorbate.

-NO Protects Cells From Hypoxia-Reoxygenation Injury

In contrast to its cytotoxic effects, NO more often acts as an antioxidant and attenuates reoxygenation injury. NO is capable of inhibiting lipid peroxidation chain reactions, and NO has clear protective effects in many cellular hypoxia-reoxygenation models. Hypoxia preexposure increases iNOS mRNA in myocardial cells and protects against subsequent damage from prolonged hypoxia (98). NO from hippocampal constitutive NOS (cNOS) may be involved in neuroprotection afforded by hypoxic preconditioning, because NOS inhibition blocks the development of preconditioning after anoxia exposures (17).

Activation of cGMP-dependent pathways by NO appears to be protective. For example, L-arginine, an NOS substrate, limits myocardial cell death due to hypoxia-reoxygenation through a cGMP-dependent mechanism (2). L-Arginine increases cGMP in cardiac myocytes and inhibits LDH release due to reoxygenation (2). Cardiomyocytes preconditioned with 90 min of simulated ischemia (i.e., near anoxia and glucose-free medium) followed by 30-min reoxygenation were protected from subsequent ischemia (98). The NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) blocked preconditioning, and the NO donor S-nitroso-N-acetyl-L-penicillamine (SNAP) protected cells from reoxygenation. Preconditioning required synthesis of NO and was cGMP-dependent, but protection due to NO appeared independent of protein kinase C (PKC) activation or ATP-sensitive K+ (KATP) channels. Similarly, decreased cNOS expression due to reoxygenation appears to be related to development of apoptosis of coronary artery endothelial cells. Anoxia-reoxygenation of the cells significantly decreased cNOS and Bcl-2, whereas it increased Fas expression (70).

The protective effects of NO depend critically on the Po2 and balance of NO and O2− production (45). When perfused rabbit lungs were ventilated with N2 and subjected to ischemia-reperfusion, NOS inhibition decreased, whereas an NOS substrate increased, the degree of lung injury. In contrast, the NOS inhibitor L-NAME significantly worsened injury of lungs ventilated with air. Protective effects of N2 ventilation may be due to a lower rate of NO production or altered peroxynitrite production.

Consistent with apparent antioxidant effects of NO noted above, we observed that inhibition of endogenous NOS activity markedly worsens lung epithelial cell reoxygenation injury in vitro. Cultured, human lung epithelial cells were incubated with aminoguanidine (50 μM), an inhibitor of iNOS, during reoxygenation. iNOS inhibition markedly increased LDH release, suggesting that NO inhibits reoxygenation injury [similar results have been found with N(G)-monomethyl-L-arginine (L-NMMA)]. An example of such data is shown in Fig. 2. Similarly, L-NMMA significantly increased cytolysis of Mn SOD-deficient mouse lung fibroblasts (−/−) during reoxygenation (Jackson and Li, unpublished data), again suggesting that NO, probably derived from iNOS, modulates oxidative stress in reoxygenated lung cells.

Effects of NO on Mitochondrial Function During Hypoxia-Reoxygenation

NO has important effects on cellular respiration and mitochondrial ROS production, which could either contribute to or protect from hypoxia-reoxygenation injury. Cellular respiration is regulated by ADP availability to F2-ATPase, O2 availability to cytochrome oxidase, and the concentration of NO (10). NO participates in redox reactions in the mitochondrial matrix, which regulate both intramitochondrial NO concentration itself and production of other reactive species (O2− and ONOO−). Exogenously produced NO markedly

Fig. 2. Nitric oxide synthase (NOS) inhibitors increase lung epithelial cell death. To test the hypothesis that nitric oxide (NO) might attenuate lung epithelial cell reoxygenation injury, H441 lung epithelial cells were incubated with the inducible NOS (iNOS) inhibitor aminoguanidine (50 μM) (4 bars at right) during reoxygenation. In the absence of aminoguanidine (4 bars at left), antinmycin A and FCCP (Anti A + FCCP) caused a moderate increase in lactate dehydrogenase (LDH) release from hypoxia-preexposed cells (solid bars) compared with vehicle (veh)-treated, hypoxia-preexposed controls. Inhibition of iNOS significantly increased LDH release from both air controls (shaded bars) and hypoxia-preexposed cells (solid bars) exposed to the oxidant stress, suggesting that NO inhibits reoxygenation injury (similar results obtained with N(G)-monomethyl-L-arginine). These data are consistent with others in the literature showing a protective and apparently antioxidant effect of NO in cellular hypoxia-reoxygenation models. *P < 0.05 compared with the respective vehicle by ANOVA.
decreased O$_2$ uptake of isolated rat heart mitochondria (50% inhibition at 4 μM -NO) (96). -NO inhibits cytochrome oxidase between cytochromes b and c. Binding at this site accounts for its inhibitory effects on O$_2$ uptake. -NO also regulates O$_2$ production, detected as H$_2$O$_2$. Mitochondrial -NO is metabolized by pathways leading to ONOO$^-$ formation and ubiquinol oxidation and only secondarily by reversible binding to cytochrome oxidase (95). Whereas low concentrations of -NO inhibit cytochrome oxidase, higher concentrations of -NO inhibit other respiratory chain complexes, possibly by nitrosylation, oxidation of protein thiols, or disruption of Fe-S centers (11).

In contrast, ONOO$^-$ irreversibly inhibits mitochondrial complexes I, II, IV, and V, as well as aconitase. Decreased maximal uptake rate and increased affinity constant for O$_2$ of the enzyme characterize ONOO$^-$ inhibition of cytochrome oxidase (111). Intramitochondrial ONOO$^-$ reacts with NADH, changing the mitochondrial redox state. ONOO$^-$ treatment of submitochondrial particles leads to ubiquisemiquinone autoxidation increasing O$_2$ production.

Mitochondrial proteins such as Mn SOD may be nitrated chemically by high concentrations of ONOO$^-$ (200 μM in vitro). However, the intramitochondrial steady-state concentration of ONOO$^-$ remains around 2 nM (121), indicating that this mechanism may be less relevant in vivo. Mitochondrial creatine kinase (CK) exists predominantly as an octamer, and ischemia decreases the ratio of octamers to dimeric forms and so inhibits CK activity. An identical decrease in CK activity and change in structure occurs after ONOO$^-$ exposure that inhibits transport of high-energy phosphate out of mitochondria, resulting in impaired cardiac performance (114).

Although -NO can injure cells directly or after reaction with O$_2$ to from the oxidant ONOO$^-$, much evidence indicates that -NO is protective against cellular hypoxia-reoxygenation injury. Probable protective mechanisms include innate antioxidant properties of -NO: increasing cellular cGMP, and partial inhibition of cellular respiration.

**ROLE OF MITOCHONDRIA IN HYPOXIA-REOXYGENATION INJURY**

**Mitochondrial ROS Production**

ROS and possibly RNS generated by mitochondria may damage the organelles themselves and other cellular constituents during reoxygenation. Protein carbonyls, a marker of oxidant stress, are detected in mitochondrial proteins after 10-min hypoxia and 5-min reoxygenation (100). The mitochondrial electron transport chain generates superoxide anions radicals (O$_2^-$) during reoxygenation that dismutate rapidly either enzymatically or spontaneously to form H$_2$O$_2$, which reacts with iron to form hydroxyl radicals (-OH) (30). Mitochondrial oxidative phosphorylation, uncoupled respiration, and ADP-to-oxygen ratios are all decreased by excess O$_2$ during reoxygenation. The mitochondrial outer membrane also contains monoamine oxidase, which catalyzes oxidative deamination of biogenic amines and is itself a source of H$_2$O$_2$ (13).

**Effects of Hypoxia on Mitochondrial ROS Production**

The respiratory electron transport chain becomes reduced (i.e., the complexes harbor electrons) during anoxia, and the reduced state potentiates O$_2$ production (20). Mitochondria generate excess partially reduced oxygen species (primarily O$_2$) and its dismutation product, H$_2$O$_2$ during reoxygennation, which react with respiratory chain proteins (especially those containing Fe-S centers) (120) or diffuse as H$_2$O$_2$ out of mitochondria. H$_2$O$_2$ produced by mitochondria also might function as a signal transducer, allowing mitochondria to act as oxygen sensors (84) that regulate extramitochondrial signaling pathways.

**Effects of Reoxygenation on Mitochondrial Function**

Decreased Mn SOD activity, protein, or gene expression caused by hypoxia could influence the steady-state concentration of mitochondrial O$_2$ production. Antimycin A-sensitive generation of O$_2^-$ originates from the ubiquinone-cytochrome c reductase (complex III), specifically from the Q$_i$ semiubiquinone. When Mn SOD-depleted submitochondrial particles are inhibited with myxothiazole or stigmatellin, O$_2^-$ is detected by coelen terazine oxidation. Mn SOD eliminates ROS generated at the Q$_i$ site, but in the absence of Mn SOD, O$_2^-$ from that site is free to react (97). This leads to increased O$_2$ production, a situation that might occur if Mn SOD synthesis were inhibited (e.g., by hypoxia) or if Mn SOD were inactivated (e.g., by ONOO$^-$).

Mitochondrial complex I dysfunction occurs after reoxygenation of hypoxic mitochondria (62). Complex I defects increase cellular production of ROS, which may influence Mn SOD expression (93). Reoxygenated kidney tubule cells develop energy deficits due to complex I dysfunction that occur before onset of the mitochondrial permeability transition (MPT) or loss of cytochrome c. Supplementation with citric acid cycle metabolites that anaerobically generate ATP may prevent energy deficits (125). Anaerobic metabolism of α-ketoglutarate and aspartate generate sufficient ATP to maintain mitochondrial membrane potential. Proximal tubules are protected from hypoxia-reoxygenation-induced mitochondrial injury by anaerobic metabolism of citric acid cycle intermediates and of succinate (130). Whereas complex I dysfunction occurs as a result of ROS produced during reoxygennation, substrates that bypass complex I provide sufficient energy to maintain viability of reoxygenated cells (131).

Transient, but large, cellular and mitochondrial Ca$^{2+}$ fluxes occur during hypoxia-reoxygenation. Elevated intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), causes mitochondrial depolarization. Anoxia depolarizes guinea pig ventricular myocyte mitochondria, and recovery of normal mitochondrial membrane potential is essential to avoid hypercontracture. Reoxygenation caused significant elevation in intramitochondrial [Ca$^{2+}$], the amplitude of which correlated with the extent of hy-
percontracture (28). Intramitochondrial [Ca\(^{2+}\)] also increases markedly ([Ca\(^{2+}\)] \(_{\text{in}}\) \(>350\) nM) during rat cardiomyocyte hypoxia-reoxygenation, whereas cytosolic [Ca\(^{2+}\)] falls. Myocyte mitochondrial Ca\(^{2+}\) uptake during hypoxia occurs largely through the Na\(^{+}/Ca\(^{2+}\) exchanger, rather than the Ca\(^{2+}\)-uniporter (38). Extracellular catalase inhibited the increase in myocyte [Ca\(^{2+}\)] and [Na\(^{+}\)] content and LDH release due to hypoxia-reoxygenation, indicating that extracellular H\(_2\)O\(_2\) contributed to the ion fluxes.

Release of Ca\(^{2+}\) from storage sites stimulates Ca\(^{2+}\)-dependent proteases, nucleases, and phospholipases that trigger apoptosis. Ca\(^{2+}\) leaves mitochondria during reoxygenation through pore formation, reversal of unipor influx carrier, the Ca\(^{2+}/H^+\) antiport system, or channel-mediated Ca\(^{2+}\) pathways. Increased intracellular Ca\(^{2+}\) also appears to be related to oxidation of adenine nucleotides or by thiol oxidation, because over-expression of the antiapoptotic protein Bcl-2, which has antioxidant properties, in mitochondrial membranes inhibits Ca\(^{2+}\) efflux due to oxidants (31).

The long-lived oxidant H\(_2\)O\(_2\) stimulates human aortic endothelial cell [Ca\(^{2+}\)] oscillations. Reoxygenation similarly initiates [Ca\(^{2+}\)] oscillations, which depend on Ca\(^{2+}\) release from the intracellular pool and require extracellular Ca\(^{2+}\). The Ca\(^{2+}\) oscillations caused by NADPH oxidase-derived H\(_2\)O\(_2\) appear to play an important role in signal transduction (44). Removal of Ca\(^{2+}\) from the medium or Ca\(^{2+}\) chelation prevented killing of rat hepatocytes by rotenone and anoxia, but not by cyanide. Ca\(^{2+}\) depletion prevented the MPT in anoxic or rotenone-treated cells, and Ca\(^{2+}\) influx is required to kill cells after complex I inhibition (92). Although anoxia is associated with mitochondrial Ca\(^{2+}\) influx, hypocalcemic medium does not always prevent reoxygenation injury. Low extracellular Ca\(^{2+}\) at the time of reoxygenation significantly worsened cellular injury of rat hepatocytes (61). When the Na\(^{+}/H^+\) exchanger and Na\(^{+}/HCO_3^-\) symporter were both inhibited, intracellular pH recovery did not occur normally, although Ca\(^{2+}\) oscillations and hypercontracture were attenuated (107).

Preconditioning involves both the K\(_{\text{ATP}}\) channel and ROS production. K\(_{\text{ATP}}\) channel antagonists and antioxidants blocked both preconditioning and ROS production. Transfer and expression of genes encoding ATPase channel subunits render COS-7 monkey kidney cells resistant to hypoxia-reoxygenation injury (50).

Mitochondrial complex III is an important site of O\(_2\) production during hypoxia-reoxygenation. Reoxygenation impairs mitochondrial complex I function and inhibits energy production. Reoxygenation causes increased ROS production and a significant increase in mitochondrial calcium concentration. Mitochondrial Ca\(^{2+}\) fluxes may be associated with the activation of intracellular proteases that trigger apoptosis.

**REACTIVE SPECIES IN INTRACELLULAR SIGNALING**

ROS act as signaling molecules in various cell types (46, 119), participating in or modifying physiological events related to receptor-ligand binding and transcriptional activation (35, 39, 90). Intracellular signaling pathways are implicated in cell death following reoxygenation, although the specific pathway leading to apoptosis or necrosis might vary among cell types. Several protein kinase signaling pathways involved in cellular reoxygenation injury are shown in Fig. 3. ROS interact with a number of specific molecular targets in reoxygenated cells, including extracellular signal-regulated kinases (ERK) and MAPK that mediate proliferation, stress-activated protein kinases (SAPK) implicated in apoptosis, NF-\(\kappa\)B, and several caspases (132). The GTPass binding protein Rac1 regulates some kinases (e.g., SAPK, p38 MAPK, and c-Jun NH2-terminal kinases (JNK)). These kinases may be regulated further by ROS from NAD(P)H oxidase, which requires Rac1 for activity (46). Inhibition of Rac1 lessens both ROS production and reoxygenation injury (58).

ROS also regulate or participate in growth, apoptosis, and the adaptive response to injury or stress (35). Platelets exposed to anoxia-reoxygenation generate O\(_2\) and -OH, which activate arachidonic acid metabolism via phospholipases A\(_2\) and C (66). Platelet activation is associated with inositol 1,3,4-trisphosphate and TXA\(_2\) production, and platelet activation is inhibited by antioxidants.

**Stress Kinases, JNK, and AP-1**

ROS produced during reoxygenation appear to stimulate SAPK, including JNK and p38 MAPK (65). Anoxia followed by hyperoxia (reoxygenation) causes apop-

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**Fig. 3.** Protein kinase signaling pathways possibly involved in reoxygenation injury. The mitogen-activated protein kinases (MAPK), which phosphorylate target molecules at threonine-X-tyrosine domains, have been implicated in cellular hypoxia-reoxygenation models. GTPass binding proteins and kinases experimentally implicated in cellular reoxygenation injury models are shown in bold italics. Hypoxia or reoxygenation appears to activate the c-Jun NH2-terminal kinase (JNK) and p38 MAPK. During reoxygenation, protein kinase C-\(\zeta\) is activated by phosphoinositide 3-kinase, which induces MAPK activation through MEK1/2. As noted in the text, permeability changes in a cell culture model due to hypoxia and low glucose (simulated ischemia) could be blocked by PKC and PKG inhibition or by inhibition of p38 MAPK-1 (89). RNS, reactive nitrogen species.
tosis of mouse embryo fibroblast (NIH/3T3) cells. Fibroblast arrest in G1 during anoxia was reversed on reoxygenation. c-jun and c-fos expression increased during anoxia. AP-1 binding increased markedly during reoxygenation, as did poly(ADP-ribos) polymerase (PARP) cleavage and caspase-3 activation. Induction of c-jun/c-fos (AP-1) expression during hypoxia was followed by PARP activation and histone H1 ADP-ribosylation. Activation of the AP-1 pathway was essential to initiation of programmed cell death (21).

Hypoxia-reoxygenation of cardiac myocytes caused a 10-fold increase in phosphorylation of the c-jun transcription factor. c-jun activation correlated with a decrease in GSH content, indicating that AP-1 activation occurred in association with oxidant stress. JNK activity was inhibited by a free radical spin trap (α-phenyl-N-tet-butylnitrone) and N-acetylcysteine, a sulfhydryl compound. Phosphorylation and activation of c-Jun protein are linked directly to intracellular redox status (65). SAPK activation also leads to H2O2-induced apoptosis and upregulation of intercellular adhesion molecule (ICAM)-1 (46).

Supporting evidence for activation of kinase pathways by ROS during reoxygenation includes inhibition of hypoxia-reoxygenation-induced apoptosis by an antisense oligonucleotide targeted to JNK-1. Human kidney cells in vitro undergo apoptosis during reoxygenation after 48-h hypoxia. Reoxygenation causes a time-dependent increase in activation of JNK-1, while p38 MAPK is activated by hypoxia. Activation of the JNK signaling cascade leads to apoptosis during hypoxia-reoxygenation (34).

Transient hypoxia causes apoptosis of developing rat brain neurons due to activation of AP-1-related transcription factors and JNK kinases. AP-1 expression increases in neurons during hypoxia. JNK-1 and JNK-3 are induced after 48-h reoxygenation. Hypoxia exposure increased c-Jun, Jun B, Jun D, c-Fos, and Fos-related protein expression. JNK-1 and JNK-3 both increased transiently 48 h after reoxygenation, when apoptosis occurred (22). JNK-1 activity translocated to the nucleus increases during reperfusion, at which time JNK-1 is serine-phosphorylated. An upstream kinase, SAPK/ERK kinase 1 (SEK1), activates JNK-1 in the nucleus. Another upstream kinase for the MAPK, MAPK/ERK kinase 1 (MEK1), in contrast, remains localized to the cytosol. Increased JNK-1 activity after ischemia depends on its translocation to the nucleus and activation of upstream protein kinases (probably SEK1) during reoxygenation (80).

**Protein Kinase C**

PKC-ζ acts upstream of the MAPK. PKC-ζ induces MAPK activity through MEK activation during reoxygenation. PKC-ζ is itself activated by phosphoinositide 3-kinase, which induces MAPK as a result of MEK1/2 activation in the nucleus of reoxygenated cardiomyocytes (79). Immunocytochemical staining of PKC-ζ in nuclei of cardiomyocytes increases when MAPK is phosphorylated. Phosphorylation and activation of MAPK during reoxygenation can be blocked both by PKC inhibition and phosphoinositide 3-kinase inhibitors. Activation of PKC also appears to be involved in the development of preconditioning or tolerance. Simulated ischemic preconditioning of endothelial cells prevented subsequent reoxygenation-induced expression of ICAM-1 associated with activation of PKC and production of NOS and ROS (5).

Chemical hypoxia (produced by the reducing agent thioglycolic acid) and lack of glucose increased permeability of cultured human dermal endothelial cells. Intracellular [Ca2+] increased as well under those conditions. Permeability changes due to hypoxia and low glucose could be blocked by chelating Ca2+, PKC, and protein kinase G (PKG) inhibition or by inhibition of p38 MAPK-1. Ca2+-induced dissociation of cadherin-actin and occludin-actin junctions might mediate increases in endothelial permeability (88), through pathways that involve PKC, PKG, and MAPK.

Ischemic preconditioning of hepatocytes decreases Na+ accumulation during hypoxia and decreases cellular death, as does PKC activation (16). PKC appears to activate H+ extrusion and maintain intracellular pH.

**Nuclear Factor-κB**

NF-κB activation is involved in hypoxia-reoxygenation injury, especially in the vascular endothelium, where NF-κB activation leads to neutrophil adhesion in vivo (56). NF-κB nuclear binding increased in microvascular endothelial cells during reoxygenation but not during hypoxia. NF-κB binding and ICAM-1 surface expression were induced, concurrent with ROS production. An -NO donor molecule, SNAP, blocked NF-κB activation and inhibited ICAM-1. Blockade of NOS by L-nitroarginine increased ICAM-1 expression after reperfusion. -NO thus appears to inhibit ICAM-1 activation by decreasing the level of oxidant stress responsible for NF-κB activation (64). NF-κB activation and nuclear translocation is required for development of tolerance to anoxia-reoxygenation (18). Inhibition of -NO synthase during a second anoxia-reoxygenation challenge prevents both inhibition of polymorphonuclear neutrophil adherence and NF-κB activation. Thus, while NF-κB activation is associated with the vascular inflammatory response in vivo, NF-κB activation is required for the development of tolerance to hypoxia-reoxygenation in vitro.

Reactive species may act as intracellular signals, or they may interact with specific molecules of intracellular signaling cascades. Stress-activated protein kinases, particularly JNK and p38 MAPK, have been most strongly associated with hypoxia-reoxygenation injury. PKC-ζ appears to be responsible for induction of MAPK activity during reoxygenation. NF-κB activation by hypoxia-reoxygenation can be blocked by -NO, and NF-κB appears to be required for development of tolerance to hypoxia-reoxygenation.

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DURING REOXYGENATION

Apoptosis

Both apoptosis and necrosis (and likely intermediate forms of cell death, as well) are linked to excess intracellular ROS production (70). ROS, for example, mediate PARP cleavage and lead to apoptosis (77). Apoptosis initiated by oxidant stress involves increased mitochondrial permeability and release of cytochrome c (14). Downstream signaling and enzymatic mechanisms, including activation of caspases, are all regulated by oxidant mechanisms (29). Apoptosis depends on the availability of cellular ATP, so reoxygenation could potentiate apoptosis by restoring cellular energy (77). Antioxidants inhibit DNA fragmentation and PARP cleavage, suggesting that ROS are responsible for these manifestations (79). Hypoxia-reoxygenation itself enhances expression of CD95L (APO-1/Fas), a transmembrane protein that induces apoptosis by ligand binding and subsequent activation of caspases (123).

Bcl-2 may exert some of its antiapoptotic effects through apparent antioxidant actions, because Bcl-2 inhibited H2O2 production due to ceramide or tumor necrosis factor-α. Bcl-2 overexpression allows cells to adapt to ROS overproduction (31). Apoptosis in an endothelial reperfusion model also required activation of NF-κB, because NF-κB decoy oligodeoxynucleotides decreased the number of TUNEL (TdT-mediated dUTP nick end labeling)-positive cells. NF-κB augments apoptosis by suppressing the antiapoptotic Bcl-2 expression in hypoxia (75).

Bax and p53 are induced in some cell types that undergo apoptosis after hypoxia. Proapoptotic Bax and the interleukin-converting enzyme subfamily of caspases increased after exposure of neurons to hypoxia followed by reoxygenation (8). Induction of Bax and p53 expression preceded neuronal apoptotic death. Apoptosis observed after in vitro reoxygenation of cardiomyocytes is associated with activation of caspases-3 and -9 and appearance of cytochrome c in the cytosol. Initiation of apoptosis depends on mitochondrial release of a caspase-activating factor (probably cytochrome c); Bcl-2 overexpression blocks both cytochrome c release and activation of caspases-3 and -9 from the reoxygenated cells (53). Hypoxia-reoxygenation decreases Bcl-2 expression and increases Fas protein, changes associated with cardiomyocyte apoptosis and necrosis. Caspase inhibition and Bcl-2 overexpression inhibit apoptosis during reoxygenation, but they do not inhibit death due to hypoxia.

Hypoxia causes accumulation of p53, as well as hypophosphorylated retinoblastoma protein (pRB), in S-phase melanoma cells. p53 protein was higher in S phase than in G1 or G2, while pRB was not cell-cycle dependent. However, hypoxic induction of p53 did not arrest cell growth (25). Hypoxia also induces p53 and enhances apoptosis of cultured trophoblasts. Apoptosis in hypoxic trophoblasts involves alterations in p53 and Bax expression, because both are increased in hypoxia, whereas Bcl-2 is decreased (68). In contrast to the proapoptotic effects of p53, epithelial growth factor and enhanced differentiation protect cells from hypoxia-induced apoptosis (68).

Hypoxia per se is insufficient to initiate apoptosis in some cells. Apoptosis of rapidly contracting hypoxic myocytes occurred only when extracellular pH decreased. Reoxygenation of the hypoxic acidotic myocytes induced apoptosis in 23–30% of cells, independently of changes in p53 expression. Therefore, exogenous lactic acid during reoxygenation can induce programmed cell death without an increase in cellular p53 (128).

Bax protein translocates to mitochondria during reoxygenation and triggers cell death. Maintenance of glucose during hypoxia prevented Bax translocation to mitochondria and reoxygenation injury, in addition to maintaining intracellular ATP levels. Likewise, Bcl-2 overexpression in kidney tubule cells minimized apoptosis. Bcl-2 overexpression preserved mitochondrial integrity and prevented both apoptosis (glucose present) and necrosis (glucose absent), suggesting a common pathway involving mitochondrial dysfunction in cell death (104).

Hypoxic preconditioning activates transcription of Bcl-2 and heat shock protein 70 (HSP70), which are apparently protective, in neurons. Preconditioning occurs, in part, by expression of antiapoptotic gene products including Bcl-2, HSP-70, and regulatory components of the cell cycle (9). Likewise, overexpression of HSP-70 attenuates hypoxic injury of coronary endothelial cells (115). HSP-72 appears to be an important part of the normal response to stress, protecting from hypoxia-reoxygenation. HSP-72 translocates to the cytoskeleton after of hypoxia-reoxygenation (106). Inhibition of HSP-72 using antisense oligonucleotides increases reoxygenation injury of cardiomyocytes (83).

Mitochondria are critical to initiation of apoptosis in reoxygenated cells. Increased ROS production, cytochrome c release from mitochondria, and cell death during reoxygenation are linked. Mitochondria from apoptotic cells produce increased quantities of O2•− because of a switch from normal four-electron reduction of O2 to one-electron reduction after cytochrome c release (14). Overexpression of the antiapoptotic protein Bcl-2 prevents increased O2•− production associated with apoptosis. Loss of cytochrome c from the mitochondrial matrix leads to activation of caspases, which participate in the apoptotic program. Caspases-3, -8, and -9 are activated by hypoxia, possibly through Ca2+ induced activation of other proteases. Serine protease inhibitors suppress cytochrome c-mediated caspase-9 activation and apoptosis during hypoxia-reoxygenation (29).

Necrosis

Necrosis is associated with tissue inflammation, but cytolysis occurs in cell culture reoxygenation models as well. Inhibition of apoptosis, because of lack of energy due to hypoxia-induced ATP depletion, may predispose...
to necrosis, and mechanisms of cell death typically overlap. DNA “laddering” into 180-bp fragments may occur in necrosis, although nucleosomal laddering is more characteristic of apoptosis. Multiple assays, including morphological examination, are required to define the actual characteristics (apoptotic, necrotic, or overlap) of cell death due to reoxygenation.

Human lung epithelial cells clearly undergo necrosis rather than apoptosis because of excess superoxide production in mitochondria after preexposure to hypoxia (1% O2 for 24 h). The Mn SOD activity of the cell decreases by ~50% after 24 h in hypoxia, as observed previously in ATII cells (103). The mechanism of decreased Mn SOD expression involves decreased stability of Mn SOD mRNA rather than direct inhibition of the Mn SOD promoter (85). ATP content did not change significantly, so cellular injury or lack of apoptosis could not be attributed to lack of energy per se. Antimycin A and FCCP generate excess mitochondrial O2* and cause necrosis in the hypoxia-preexposed cells. Neither hypoxia (24 h) nor reoxygenation (24-h air following hypoxia) by itself increased cellular LDH release significantly. Cell death rarely was associated with annexin V staining (1–3%), even at early time points during the oxidant stress. The epithelial cells did not display increased Hoechst nuclear staining (a morphological marker of apoptosis), PARP fragmentation, caspase-3 activation, or change in p53 protein levels on Western blots (69).

Cellular injury after reoxygenation was associated with increased ROS production. N-acetylcysteine (1 mM), a sulfhydryl compound, partially inhibited increased DCF fluorescence after hypoxia and protected the lung epithelial cells from cytolysis during incubation with antimycin A and FCCP. Inhibition of both ROS production and cytolytic damage by N-acetylcysteine supports the notion that oxidants produced by the epithelial cells are involved in reoxygenation injury. Thus lung epithelial cells may undergo necrosis directly without preliminary apoptosis, as a result of increased susceptibility to oxidant stress after hypoxia preexposure.

Thus both apoptosis and necrosis occur after hypoxiareoxygenation injury, and both appear dependent on increased reactive species production. Apoptosis after reoxygenation is associated with activation of caspases, and both caspase inhibition and Blec-2 overexpression inhibit apoptosis. Mitochondria are involved in posthypoxic apoptosis through increased ROS production and cytochrome c release. Cells are capable of undergoing necrosis directly after reoxygenation, and necrosis is also related to increased ROS production.

SUMMARY AND CONCLUSIONS

Adequate oxygenation is critical to cellular viability. The “paradox” of reoxygenation injury can be understood in terms of counteradaptive changes occurring in hypoxia that predispose to cellular dysfunction, apoptosis, and necrosis during reoxygenation. These changes include enhanced reduction of the mitochondrial respiratory chain, conversion of XD to XO, downregulation of antioxidant systems, increased reactive species formation, and activation of signaling pathways. Specific molecular mechanisms and pathways involving ROS and RNS that lead to necrosis or apoptosis during reoxygenation have been identified. Knowledge of these pathways has led to clinical attempts at minimizing reoxygenation injury in transplantation and myocardial reperfusion.

We thank M. Kennedy for transcribing the manuscript. This research was supported by The Veterans Affairs Research Service, National Institutes of Health Grants HL-57801 and DK-97010, Office of Naval Research Grant N00014-97-1-03-09, and an American Heart Association-Alabama Affiliate Grant-in-Aid.

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