Oxidative and nitrosative stress during pulmonary ischemia-reperfusion injury: from the lab to the OR

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Abstract: Oxidative and nitrosative stress are an umbrella term for pathophysiological processes that involve free radical generation during inflammation. In this review, the involvement of reactive oxygen and nitrogen species is evaluated during lung ischemia-reperfusion injury (LIRI) from a surgical point of view. The main biochemical and cellular mechanisms behind free radical generation are discussed, together with surgical procedures that may cause reperfusion injury. Finally, different therapeutic strategies are further explored. A literature search was performed, searching for "lung ischemia reperfusion injury", "reperfusion injury", "large animal model" and different search terms for each section: "surgery", "treatment", "cellular mechanism", or "enzyme". Although reperfusion injury is not an uncommon entity and there is a lot of evidence concerning myocardial ischemia-reperfusion injury, in the lung this phenomenon is less extensively described and studies in large animals are not easy to come by. With increasing number of patients on waiting lists for lung transplant, awareness for this entity should all but rise.

Keywords: Ischemia reperfusion injury; oxidative stress; nitrosative stress; reactive oxygen species (ROS); reactive nitrogen species (RNS)

Introduction

Definition of pulmonary ischemia-reperfusion injury

Pulmonary ischemia is defined as a temporary arrest of circulation and/or ventilation in at least one lung. Resupplying lung tissue with blood and air, i.e., reperfusion, causes an inflammatory reaction called acute lung injury (ALI) or lung ischemia-reperfusion injury (LIRI). In the setting of a lung transplantation, this process is defined as primary graft dysfunction (1). This might lead, in its most extreme form, to the possibly lethal adult respiratory distress syndrome (ARDS). Differential diagnosis between ALI and ARDS is made by evaluation of radiological criteria and oxygenation status of the patient in absence of any cardiac or chronic lung disease (*Table 1*) (2). Whereas ARDS

is an acute pathology, LIRI can also compromise graft survival in the long run. There is now evidence that LIRI is a risk factor for development of the bronchiolitis obliterans syndrome, the most prevalent cause of long-term morbidity and mortality following lung transplantation (3).

Pulmonary IRI can be caused by either anoxic ischemia, in which the complete pulmonary hilum is occluded, or by ventilated ischemia, where the pulmonary artery (PA) is occluded selectively.

Anoxic ischemia typically occurs during cold ischemia storage of donor lungs, acute trauma surgery and in complete cardio-pulmonary bypass. It implies that PA, pulmonary vein, bronchus and bronchial artery are all temporarily occluded. The bronchial artery derives directly from the aorta and supplies the bronchial epithelium and

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Table 1 Criteria to differentiate between ALI and ARDS

	ALI	ARDS
Occurrence	Acute onset	Acute onset
Radiology	New patchy or homogeneous bilateral lung infiltrates	New patchy or homogeneous bilateral lung infiltrates
Cardiac status	PA wedge pressure <18 mmHg or absence of left atrial hypertension	PA wedge pressure <18 mmHg or absence of left atrial hypertension
Oxygenation status	P_aO_2:FiO_2 <300 mmHg	P_aO_2:FiO_2 <200 mmHg

ALI, acute lung injury; ARDS, adult respiratory distress syndrome; PA, pulmonary artery; P_aO_2, alveolar air concentration; FiO_2, oxygen concentration in inspired air.

Table 2 Common ROS and RNS in biological systems and their half-life

Name	Symbol	Half-life (s)
Hydroxyl radical	$^{\bullet}OH$	1.10^{-6}
Superoxide anion	$O_2^{\bullet-}$	1 to 4.10^{-6}
Hydrogen peroxide	H_2O_2	1.10^{-3}
Peroxidized lipid	$LOO^{\bullet}$	10.10^{-3}
Peroxynitrite	$ONOO^{-}$	1
Nitric oxide	$^{\bullet}NO$	2 to 6
Ascorbyl radical	$Asc^{\bullet}$	1.10^{-3} to days[†]

[†], dependent on temperature, presence of trace metals and oxygen concentration, ascorbyl radical is the most stabile biological radical that may remain detectable for days under low-temperature conditions. ROS, reactive oxygen species; RNS, reactive nitrogen species.

connective tissue with oxygenated blood. It is occluded in anoxic ischemia by complete hilar clamping, but remains open during cardiac bypass. It is debated whether bronchial artery supply is sufficient to meet metabolic demand in the lung during cardiopulmonary bypass (4). Moreover, there is increasing evidence that failure to revascularize the bronchial artery following lung transplantation may lead to loss of airway microcirculation and increased risk of developing the bronchiolitis obliterans syndrome (5).

Ventilated ischemia occurs, for instance, during PA embolism. The alveolar tissue is still receiving oxygen supply because it directly uses inhaled oxygen, but as no erythrocytes pass through the pulmonary circulation, there is no normal gas exchange between the air in the alveoli and the blood in the pulmonary vessels (6).

With increasing numbers of lung transplantation procedures observed during the last years, pulmonary IRI is a clinically relevant phenomenon that warrants extensive study. Here we review the contribution of oxidative and nitrosative stress to LIRI pathogenesis, describe both intracellular and extracellular processes that lead to their production and provide information that is useful to a clinician.

Oxidative and nitrosative stress

Oxidative and nitrosative stress is caused by free radicals. These are molecules that contain an unpaired electron on their outer electron shell. Based on their central atom, they are defined as either reactive oxygen species [ROS, or as reactive nitrogen species (RNS)]. Oxidative and nitrosative stress is a natural byproduct of normal metabolism in the oxygen and nitrogen rich environment we live in (7). ROS and RNS are important in maintaining normal homeostasis as inter- and intracellular signal molecules, both in physiological as in pathophysiological conditions. For instance, phagocytic cells use ROS to destroy pathogens and combat infection, while nitric oxide ($^{\bullet}NO$) plays a pivotal role in flow-mediated vasodilation. However, ROS and RNS remain highly reactive molecules and can damage various cellular components when produced massively. Nucleic acids in DNA or RNA, amino acids in proteins and fatty lipids in the cell membrane are especially at risk. Because of their high reactivity, their half-life is only very short. A number of ROS and RNS with their respective half-life are summarized in *Table 2*.

Intracellular sources of oxidative and nitrosative stress

NADPH oxidase

The NADPH oxidase enzyme complex (NOX) is a membrane-bound protein cluster that oxidizes its substrate

nicotinamide adenine dinucleotide phosphate (NADPH) into $NADP^+$, thereby transferring an electron from the cytoplasm into a phagocytic vacuole (8). In the lumen of the vacuole, also called phagosome, molecular oxygen is reduced by this electron into superoxide anion in massive quantities. This system is highly active in cells that are responsible for pathogen killing, such as neutrophils, macrophages and monocytes. The flip side of the medal is that ROS generated by activated NOX can aggravate certain pathologies, especially inflammatory diseases (9).

There are many NOX homologues which are not necessary involved in pathogen killing, but generate ROS for redox signaling purposes, to regulate cell growth and apoptosis (NOX2), angiogenesis (NOX1), kidney function (NOX3) and even thyroid hormone synthesis (DUOX2) (10). NOX4 is most predominant in smooth muscle cells in the PA and the airway epithelia where it serves as an oxygen sensor. In patients with idiopathic pulmonary hypertension, NOX4 induces vascular remodeling associated with this disease in response to chronic hypoxia (11).

Xanthine oxidase/dehydrogenase (XO/XDH) system

Xanthine oxidase/dehydrogenase (XO/XDH) is an enzyme that is pivotal in purine catabolism. It breaks down adenosine to hypoxanthine and xanthine with further degradation into uric acid, which can be excreted in urine. In physiological circumstances, the enzyme exists in its XDH form. It can be converted irreversibly by proteolysis or reversibly by sulfhydryl residue oxidation into XO (12). The main difference is that XDH mainly uses NAD^+ as electron acceptor, whereas XO reduces oxygen to form superoxide anion, independent of NAD^+. XO activity is enhanced by hypoxia and concentrations are increased in serum of patients with ARDS (13). During ischemia, XDH is increasingly converted into XO. Higher levels of cellular adenosine due to breakdown of ATP in ischemic cells therefore lead to an increase in substrate for XO, further inducing oxidative stress (14).

NO synthases (NOS)

$^\bullet NO$ is an important intercellular messenger and neurotransmitter, responsible for destruction of pathogens, and flow-mediated vasodilation. It is generated through an enzymatic reaction in which arginine is converted into citrulline by NOS (15).

There are four major NOS isoforms: neuronal NOS (NOS I, nNOS) is primarily found in neuronal cells, inducible NOS (NOS II, iNOS) in cells of the immune system, endothelial NOS (NOS III, eNOS) in the endothelial lining of blood vessels, and mitochondrial NOS (mtNOS) in the inner mitochondrial membrane (16). iNOS is activated by an external trigger, as iNOS transcription is modulated by nuclear factor kappa bèta (NF-κB), and produces vast bursts of $^\bullet NO$ when stimulated by pro-inflammatory cytokines (17). As iNOS induction mostly occurs in an environment with increased oxidative stress, the reaction of superoxide $O_2^{\bullet-}$ with $^\bullet NO$ is also further propagated, leading to the production of peroxynitrite ($ONOO^-$) which rapidly oxidizes fatty acids in the cell membrane, leads to cross-linking of proteins and other harmful reactions (18).

eNOS and nNOS are both constitutive types of NOS, generating a steady and constant amount of NO dependent on cofactors such as tetrahydrobiopterin (BH_4), NADPH, calmodulin and flavins. There is evidence that an imbalance between these cofactors can change eNOS conformation, leading to both a functional and structural eNOS uncoupling (19). In absence of BH_4, eNOS shifts from a dimeric into a monomeric state and catalyzes $O_2^{\bullet-}$ production instead of $^\bullet NO$, leading to increased oxidation of BH_4 and a more oxidative-stress rich environment.

eNOS transcription appears to become upregulated under ischemic conditions, although the promoter of the enzymes mRNA sequence does not contain a specific HIF (hypoxia inducible factor) receptor. It is possible that an alteration in the redox balance, with higher concentrations oxidated NAD^+ and $NADP^+$ has greater influence on the eNOS promoter (20). The mechanisms described here are summarized in *Figure 1*.

Mitochondrial enzymatic chain disruption

Mitochondria are the energy suppliers of the cell. These organelles generate an electron flow during the oxidative phosphorylation reaction for the production of high-energy phosphates through a proton gradient. In physiological conditions, $O_2^{\bullet-}$ is a small but unavoidable byproduct of this respiratory chain (21). Electrons leak from the different chain complexes and are readily accepted by molecular oxygen, leading to $O_2^{\bullet-}$ and H_2O_2 formation. Mitochondrial complex I is the entry point for electrons in the electron transport chain, where NADH is oxidized to NAD^+. Under specific circumstances, such as an abnormally high electrochemical proton gradient during ischemia, the

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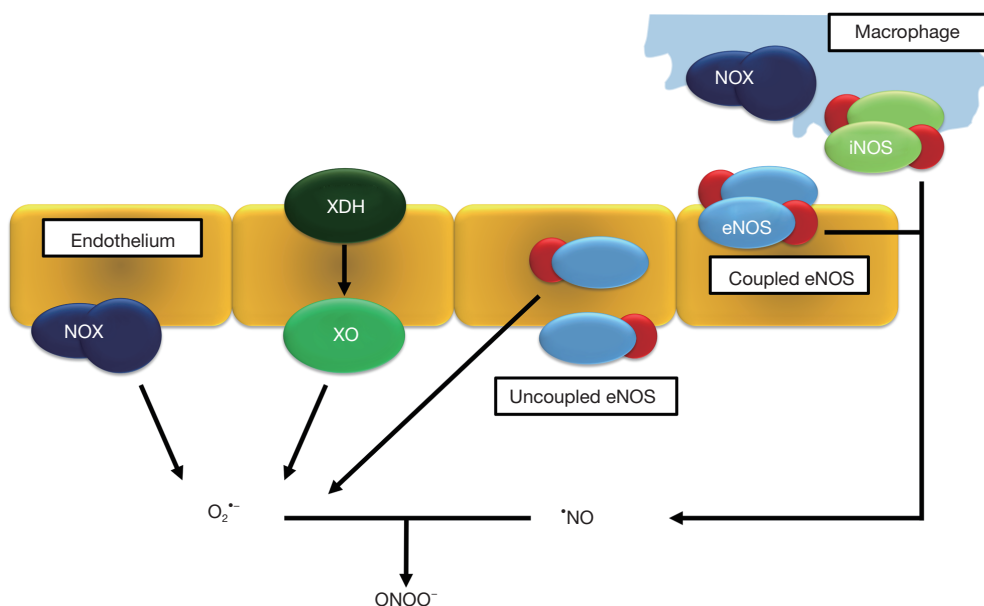Gielis et al. ROS & RNS in pulmonary ischemia-reperfusion injury

Figure 1 Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation in the endothelial cells by combined effects of eNOS uncoupling, xanthine dehydrogenase conversion and NOX induction.

direction of the electron transport chain may reverse (22). This implies that succinate is oxidized at complex II, where NAD^+ is subsequently reduced to NADH at complex I. This reverse electron transfer, where NADH oxidation is replaced by succinate oxidation, generates massive amounts of ROS and leads to opening of the mitochondrial permeability transition pore (mPTP) complex in the inner mitochondrial membrane. This process is called uncoupling of mitochondrial function, which results in mitochondrial swelling, membrane rupture and initiation of cell death mechanisms (23). Induction of mtNOS by calcium influx leads to increasing $^\bullet$NO concentrations, which inhibit the cytochrome C reductase complex (complex IV) and causes cytochrome C release, leading to cell death. $^\bullet$NO further reacts with $O_2^{\bullet-}$ to form $ONOO^-$ which inhibits all mitochondrial complexes (24). There is some evidence from small animal models that cyclosporine treatment might prevent assembly of the mPTP, although a larger meta-analysis failed to show any significant effect (25). Other mitochondrial enzymes, apart from the respiratory chain, contribute to mitochondrial ROS generation as well. For instance, the NADPH oxidase NOX2 isoform is responsible for the formation of mitochondrial ROS in human endothelial cells (26). Mitochondrial cytochrome P450 and P2C can generate $O_2^{\bullet-}$ when electrons used to reduce the central heme iron are transferred to molecular oxygen (27,28). This mechanism of mitochondrial chain disruption and processes leading to assembly of the mPTP complex are illustrated in *Figure 2*.

Disruption of iron and calcium metabolism

The lactic acidosis that is associated with ischemia induces iron dissociation from its carrier proteins, such as transferrin, ferritin and different cytochromes. This free iron may serve as a catalyst of hydroxyl ($^\bullet$OH) radical formation in what is called the Fenton and Haber-Weisz reaction. In these reactions, $O_2^{\bullet-}$ and H_2O_2 react with free ferrous iron (Fe^{2+}) to form the detrimental hydroxyl radical. This is further illustrated in *Figure 3*.

Iron also catalyzes tyrosine nitration by $ONOO^-$ (29). Iron is also responsible for glutathione (GSH) oxidation and may activate platelet aggregation in the microvasculature of the lung. Iron chelators such as deferoxamine have been proven to reduce acute pulmonary injury (30). Calcium is an intracellular messenger that is responsible for multiple biochemical pathways in the cell. Calcium stocks are maintained in the sarcoplasmatic or endoplasmic reticulum by calcium pumps and released through calcium channels. During IRI, lipid peroxidation alters Ca^{++} homeostasis leading to calcium overload and activation of Ca^{++} dependent proteases (31). Furthermore, because
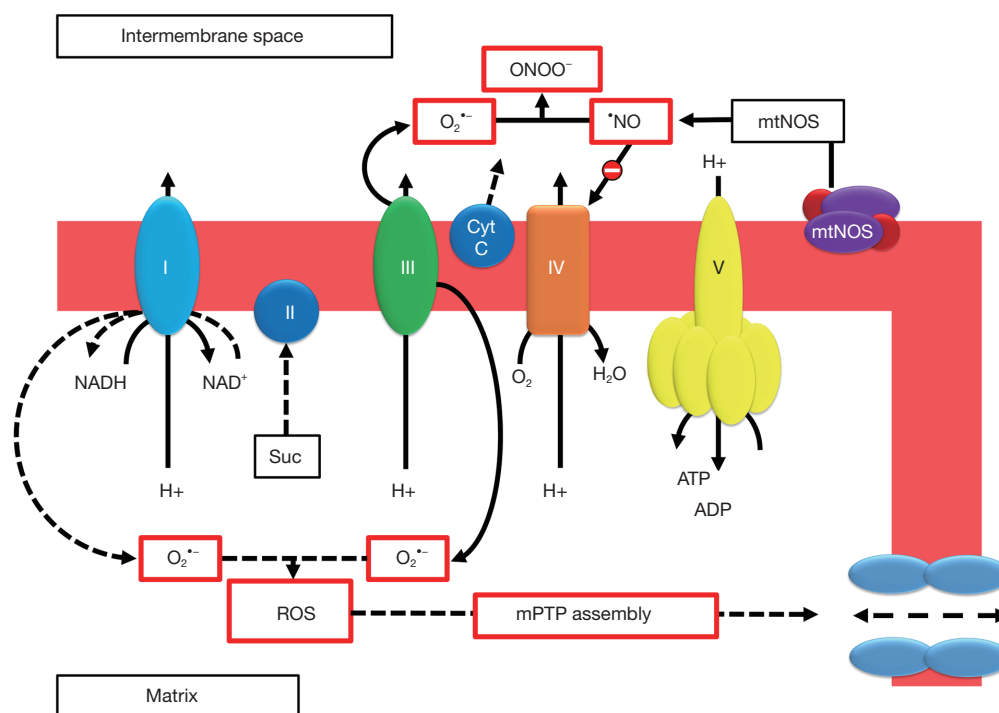
Figure 2 Dashed lines depict the pathways leading to mitochondrial uncoupling, which take place when increased amounts of reactive oxygen species (ROS) are produced, causing the assembly of the mitochondrial permeability transition pore (mPTP) complex and subsequent cell death. Full lines express normal physiological pathways.

of ATP depletion, Ca^{++} pumps fail to work adequately which reduces active efflux and decreases reuptake by endoplasmatic and sarcoplasmatic reticulum. This leads to a netto increased calcium release from intracellular stocks during reperfusion. In the heart, this calcium overload may result in severe arrhythmias (32) that complicate post-operative recovery. Increases in cytosolic Ca^{++} also lead to failed Ca^{++} homeostasis in mitochondria, causing a massive influx of Ca^{++} ions and protons in the mitochondrial matrix. This ultimately leads to cell death by necrosis and further impairment of ATP production (33).

Elevated Ca^{++} levels also activate cytokines such as interleukin (IL)-1β that lead to increased iNOS transcription. Ca^{++} further increases eNOS activity through calmodulin activation (34) and pushes the XDH/XO balance further to XO, potentiating the effect of ROS on cellular damage (35).

Cellular mechanisms leading to pulmonary oxidative and nitrosative stress

Macrophages & macrophage polarization

Macrophages activate the innate immune response by producing cytokines and chemokines and assist in cleaning up cellular debris by phagocytosis. Currently, two phenotypes of macrophages are recognized: a Th-1 driven, pro-inflammatory M1 phenotype and a pro-angiogenic, debris-scavenging M2 phenotype. HIF, a transcription factor of which breakdown is inhibited by low oxygen concentrations can shift macrophages either toward the M1 or M2 phenotype (36). Apparently, HIF1-alpha shifts macrophages towards M1 polarization while HIF2-alpha causes a shift towards the M2 phenotype. Also, increased concentrations of ROS and iron overload (37) determine macrophages toward a M1 profile (38). M1 macrophages themselves produce ROS through NADPH oxidase and •NO through iNOS activation. Therefore, M1 macrophages aggravate the reaction to ischemia and are a major determinant of ROS and RNS production in response to hypoxia. M2 macrophages produce lower amounts of inflammatory cytokines and higher amounts of the anti-inflammatory cytokine IL-10. Higher expression of arginase I decreases the availability of the eNOS and iNOS substrate arginine in these cells, resulting in a decrease in inducible •NO production (39). Detection of pharmacological shifting
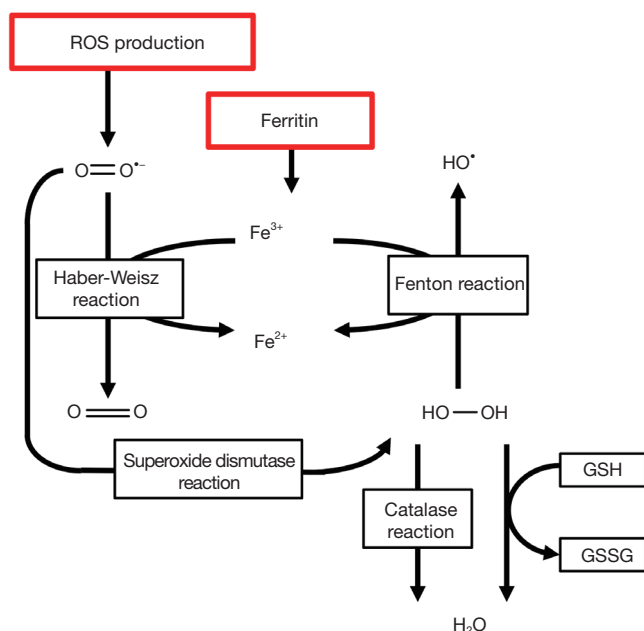
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Figure 3 Biochemical reactions catalyzed by iron leading to increased oxidative stress formation and different enzyme systems responsible for neutralization of free radicals.

of macrophages toward a M2-profile with flowcytometry or qPCR may therefore be an interesting approach to decrease the extent of IRI.

Neutrophils

Neutrophilic granulocytes expand and potentiate the immunologic reaction which is initiated by macrophages and activated endothelial cells (40). Neutrophils are responsible for the so-called respiratory burst that is part of an inflammatory reaction. This is the rapid and sudden release of ROS such as O_2^-, which is synthesized by NOX2, and OH, generated by the combined effect of the reaction catalyzed by superoxide dismutase and the Fenton reaction (41) as shown in *Figure 3*. In the neutrophil, these ROS are combined with Cl^- by the enzyme myeloperoxidase to form highly toxic hypochlorous acid (HOCl) (42). Neutrophils are recruited during IRI by of macrophages toward a M2-profile with flowcytometry or qPCR may therefore be an interesting approach to decrease the extent of IRI.

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Bronchial epithelia

Alveolar type II cells appear to be quite susceptible to the effect of ROS and RNS. These cells are responsible for the release of inflammatory mediators and cytokines such as lymphocyte activation by IL-1β, and increased acute phase protein synthesis via IL-6 (45). Type II cells also contribute to TNF-α production in response to oxidative and nitrosative stress and neutrophil recruitment through synthesis of IL-8. Transcription factors such as HIF1-alpha and NF-κβ further propagate the inflammatory responses that are activated in the bronchi and alveoli. Furthermore, there is increasing evidence that iNOS, NOX2 and NOX4 are also active in bronchial cells (46), which additionally marks the bronchial epithelium as a potential source of ROS and RNS during IRI.

Surgical procedures leading to pulmonary ischemia-reperfusion injury

Lung transplantation

Every transplant procedure, by definition, incorporates

Table 3 Pulmonary transplantation donor selection criteria (49)

<55 years old
<20 pack-years tobacco use
No infiltrations on chest radiograph
No thoracic trauma
No earlier cardiopulmonary procedures
P_aO_2/FiO_2 ratio >300 mmHg; PEEP 5 cmH_2O
Compatible blood group and HLA match
Compatible size of donor lung to recipient thoracic cavity
Absence of primary lung disease or active pulmonary infection
No aspiration pneumonia, patient not septic
Bronchoscopy specimens: non-purulent, with negative gram stain

a period of ischemia, followed by reperfusion. Many methods have been developed to reduce reperfusion injury and keep the ischemia time as short as possible. Still, lung transplantation is a surgical procedure with many per- and postoperative risks. Death still occurs in 20% of patients undergoing lung transplantation procedure, most often following primary graft failure (47). Primary graft failure is a more specific term to describe the most severe and possibly lethal form of IRI, requiring more than 72 hours of continuous mechanical ventilation, development of radiologic shadowing on chest X-ray, pulmonary edema and occasionally extracorporeal membrane oxygenation. Moreover, severe IRI may also cause problems in the long term, as it is a determinant of graft dysfunction and may lead to the bronchiolitis obliterans syndrome after several years (48). Thorough assessment of donor organs (*Table 3*) also selects those lungs that can sustain a longer period of ischemia with minimal reperfusion injury. New techniques of donor lung preservation and organ management are being perfected, such as *ex-vivo* lung perfusion (EVLP) (49,50). EVLP allows the evaluation of donor lungs before transplantation, keeps the lung under physiological conditions for extended time, and maintains an active cellular metabolism. It is also possible to recondition less performing pulmonary grafts by reduction of pulmonary edema, remove toxic waste products such as cytokines, ROS and RNS, and potentially damaging graft inflammatory cells such as activated M1 macrophages and neutrophils (51).

Cardiopulmonary bypass

During coronary artery bypass graft (CABG) procedure, circulation is temporarily derived from the right atrium directly into the aorta, with a perfusion pump in between. The pulmonary circulation is thus completely bypassed and the delivery of oxygenated blood to the lung parenchyma occurs solely through the bronchial arteries. If this bronchial circulation is not sufficient enough to provide enough blood flow, some degree of ischemia occurs (52). The effects of anesthesia, hypothermia, medication and peroperative transfusion may aggravate injury (53). Fortunately, ARDS only occurs in two to three percent of cardiac surgical patients. There is some controversy as to cardiopulmonary bypass is the main cause of lung dysfunction in patients undergoing CABG, as off-pump CABG produces a similar incidence of postoperative impaired pulmonary function (54).

Pulmonary thromboembolectomy

There are three different mechanisms that contribute to pulmonary ischemia-reperfusion injury in patients undergoing surgical treatment for PA embolism.

First, a thrombo-embolic event of the PA or any of its branches renders the lung segments that are perfused by that part of the PA ischemic. Second, cardiopulmonary bypass is part of the standard surgical treatment protocol, with the same possible influences on reperfusion injury as mentioned in "Cardiopulmonary bypass" section. Third, revascularization results in the diffusion of toxic mediators such as ROS and RNS in both the pulmonary and systemic circulation (55). The lung tissue itself may be protected from ischemic injury during pulmonary thrombo-embolic events when bronchial circulation remains intact. However, lack of sufficient oxygenation during embolism creates hypoxia in the entire circulation and subsequent reperfusion injury when oxygenation is adequately restored. An important risk during and after thromboembolectomy is massive endobronchial hemorrhage caused by reperfusion injury during re-establishment of the pulmonary blood flow (56-58).

Isolated lung perfusion

Isolated lung perfusion is an experimental technique for treatment of pulmonary metastases (59). Trials are currently running, investigating the applicability of this technique to treat pulmonary metastases from primary tumors

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such as sarcoma and colorectal carcinoma. High doses of chemotherapy, which would be toxic when administered systemically, are pumped through the pulmonary circulation for thirty minutes with the aid of a bypass pump. During the procedure, the treated lung is isolated from the circulation and oxygenation depends solely on the contralateral lung. The treated lung is thus completely isolated from the body's circulation, as the bronchial artery and vein are temporarily occluded during the procedure, as well. This implies a period of 30 minutes of complete pulmonary ischemia in the treated lung. When the isolated lung perfusion procedure is terminated, reperfusion injury may occur. Recent findings show that this reperfusion injury can be prevented by addition of carbon dioxide to and removal of molecular oxygen from the perfusate. The type of pump used for the procedure may also contribute to the extent of injury (60). The addition to the perfusate of ROS and RNS scavengers or stabilizing enzymatic cofactors, such as BH_4 to keep eNOS coupled, is still the subject of ongoing research.

Ischemia-reperfusion injury of another organ leading to lung injury

The lung is also prone to reperfusion injury originating from ischemia elsewhere in the body. There is an abundance of research models of intestinal (61), hepatic (62), renal (63) and hindlimb ischemia (64) that cause lung injury through oxidative and nitrosative stress mechanisms available in literature (65). It appears that the extension of lung injury is more dependent on the volume of tissue that is rendered ischemic, rather than on the duration of ischemia. The activation of intestinal, hepatic and renal inflammatory cascades induce oxidative and nitrosative stress that contributes to multiple organ failure, including the lung. Liver reperfusion injury, for instance, induces a downregulation of eNOS and increase of iNOS expression in lung tissue and an increase in $\cdot OH$ in the peripheral circulation (66). Clinical examples of procedures that cause peripheral ischemia and reperfusion injury are restoration of circulation after acute ischemic colitis due to superior mesenteric artery thrombosis, partial hepatectomy and liver or kidney transplantation.

Possible therapeutic strategies in tackling pulmonary ischemia-reperfusion injury

Dietary restrictions

Caloric restriction is not quite the same as malnutrition.

It implies a dietary reduction of calory intake without a shortage of essential nutrients. It is thought to extend the lifespan of many species and is therefore considered an "anti-aging" strategy, as it may prevent senescent changes in the cell (67). A small, constant stressor for a longer period of time may "prime" the organism to better cope with larger stressors in the future.

Caloric restriction reduces production of oxidative and nitrosative stress, although the exact mechanisms still remain to be elucidated (68). Furthermore, it is not certain when, how and how long dietary modifications need to be initiated in order to prevent damage from ischemic events. In rats, caloric restriction for 2 weeks already showed a significant reduction in myocardial ischemia-reperfusion injury (67). Rats subjected to caloric restriction for 12 months showed an earlier reduction in superoxide dismutase and catalase expression during reperfusion compared to normally fed animals, indicating an earlier return to baseline oxidative stress levels (69).

Antioxidants & enzymatic cofactors

Antioxidants are molecules that terminate or alternate the chain reactions that are induced by free radicals and therefore may attenuate the effect of oxidative and nitrosative stress. Some antioxidants are enzymatic, such as superoxide dismutase and catalase, while others are small molecules like vitamins and GSH (70). Hydrophilic antioxidants, such as vitamin C, react with free radicals in the cell cytosol while lipid-soluble antioxidants, such as vitamin E, inhibit lipid peroxidation of the cell membrane. Iron binding enzymes such as transferrin and ferritin inhibit radical generation through the Fenton reaction (*Figure 3*) (14). Selenium, zinc and manganese ions are often mentioned as "antioxidant ions" but they do not have any anti-oxidative properties themselves. These ions are essential cofactors to cytosolic and mitochondrial SOD, catalase and the GSH reductase system. Other cofactors include arginine, BH_4 and flavins which are essential to physiological NOS function and prohibit NOS uncoupling (15).

The "antioxidant paradox"

An important sidenote to the possible beneficial effect of vitamin and cofactor supplementation is that although free radicals appear to be involved in many inflammatory pathologies, extensive doses of dietary antioxidants do not seem to offer much preventative or therapeutic benefit. This observation is called the antioxidant paradox (71). The antioxidant barriers of normal cellular

physiology are complex and interweaved, and free radicals may offer beneficial effects themselves, for example as a neurotransmitter. Free radicals are part of the communication system between cells, in a process dubbed redox-signaling (72). *In vitro* studies cannot be extrapolated immediately to *in vivo* situations. Flavonoids are plant-derived polyphenols that show radical-absorbing properties *in vitro* (73) but offer little benefit *in vivo* (74).

Medication

Pharmacological strategies to tackle damage caused by free radicals can consist of prevention, by inhibiting radical-generating systems, or treatment on a post-hoc basis, by attenuating the possibly detrimental effects of free radicals.

N-acetyl-cysteine (NAC)

NAC is a molecular precursor of reduced GSH, a thiol with intracellular and extracellular antioxidant capacity (70). GSH concentration in the lung rises significantly following intravenous NAC administration. GSH is the main cofactor of gluthathione peroxidase, an enzyme that prevents formation of hydroxyl radicals by catalyzing the reduction of hydrogen peroxide into water. GSH scavenges $O_2^{\bullet-}$, too. In pigs undergoing isolated ventilation and reperfusion, NAC aerosol administration immediately before and after EVLP prevented warm ischemic damage compared to animals without treatment (75).

NO donors

$^\bullet$NO is a versatile molecule; it is the crucial molecular messenger in flow-mediated vasodilation, and an inhibitor of neutrophil activation, vascular adhesion and platelet aggregation. Nitroglycerin is a NO donor that has been frequently subscribed to patients suffering from transient myocardial ischemia. Adding nitroglycerin to lung preservation solutions results in better pulmonary function after transplantation (76), and continuous intravenous administration during the entire reperfusion period markedly improved gas exchange and reduced neutrophil activity in non-heart beating donor procedures in pigs (77). Nitroglycerin infusion is also more cost-effective than continuous $^\bullet$NO inhalation and immediately targets the endothelial cell, without the necessity for specialized devices.

Corticosteroids

Patients undergoing surgery that includes an episode of pulmonary ischemia-reperfusion injury, especially cardiopulmonary bypass, benefit from pre-treatment with methylprednisolone before the procedure. Glucocorticoids act on a transcriptional level and increase production of anti-inflammatory cytokines such as IL-10 while lowering levels of pro-inflammatory molecules such as Il-6, IL-8, and TNF-α. They also decrease injury by inhibiting neutrophil CD11b expression and neutrophil complement-induced chemotaxis, thereby decreasing neutrophil activation (53).

Enzymatic inhibitors

Specific inhibitors of the enzymes that were mentioned earlier may offer a pharmacological therapeutic strategy to combat reperfusion injury. NADPH oxidase inhibitors such as apocynin which have shown little effect on the rest of the immune system are useful candidates, because they avoid any "collateral damage". The exact mechanism of action of apocynin is yet unknown, but there is evidence that it attenuates LIRI in sheep by lowering oxidative status and altering thromboxane metabolism while not interfering with leukocyte migration (78). Allopurinol is a well-known inhibitor of the XO/XDH system that is used to treat patients suffering from gout. Allopurinol does not seem to work solely by inhibition of XO/XDH and offer only partial protection from IRI. Moreover, it has more effect during anoxia and reoxygenation than when only ventilated ischemia is induced (6). Inhibiting iNOS may be another strategy, which reduces vascular dysfunction, oxidative stress and pulmonary edema following lung transplantation (79). Reducing iNOS mRNA expression with nicotinamide may equally result in a decrease in proinflammatory cytokines and a reduction in observed hydroxyl radical concentration (80).

Adenosine A2A receptor agonists

Adenosine has cytoprotective properties and attenuates organ ischemia-reperfusion injury. All subtype classes of this receptor (A_1, A_{2A}, A_{2B} and A_3) are expressed in lung tissue and work through adenyl cyclase or phospholipase C activation. Some studies attribute an anti-inflammatory role to A_1, A_{2A} and A_3 (81) with reduction in oxidative stress by decreasing neutrophil-mediated $O_2^{\bullet-}$ production (82). A_{2B} activation, on the contrary, may have a pro-inflammatory effect in the lung (83). Specific agonists of these adenosine receptors appear to offer more benefit than adenosine alone, probably because these agonists selectively activate the A_1, A_{2A} and A_3 receptor without additional effect on the A_{2B} receptor (84).

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Surfactant therapy

The alveolar surfactant system reduces the surface tension that needs to be overcome before the alveoli can inflate completely. Surfactant therapy can compose of administration of animal-derived surfactants (typically a mixture of phospholipids and proteins) or synthetic surfactants which consist of synthetic lipids and recombinant peptides. Surfactant can be administered before ischemia (85), and before (86) or during (87) reperfusion. Inhalation of synthetic surfactant was shown to reduce LIRI, increase arterial oxygen tension and lower levels of oxidative stress after lung transplantation in pigs (88).

Perfusion liquids

EVLP is an attractive strategy to improve graft survival and even improve the quality of donor lungs that would previously be rejected. It might be an answer to meet the high need and long waiting lists with the current low availability of donor organs. EVLP reduces LIRI and may attenuate the oxidative stress response that is generated during brain death and ICU-related complications when compared to standard cold-ischemic storage. There is evidence that the perfusion solution developed by Steen *et al.* may provide antioxidant protection by inhibiting NOX2 activation in the donor lung during lung perfusion (89).

Anesthesia gases

NO

Adding NO to ventilation gases during 20 minutes of EVLP in pigs resulted in significantly better transplant oxygenation, reduced vascular resistance and reduced airway pressure. Ventilation with ˙NO also decreased neutrophil sequestration in the donor lung (90). In another porcine model, ˙NO inhalation in the first 4 hours of reperfusion after lung transplantation improved gas exchange, pulmonary vascular resistance and tissue oxygenation while lowering pulmonary neutrophil counts after 24 hours of reperfusion. These results demonstrate that even a short period of ˙NO administration in the immediate post-operative period might provide additional protective effects after lung surgery (91).

Noble gases

Anesthesias with noble gases such as xenon and argon have organoprotective effects and may provide a useful addition to the standard transplantation anesthesia protocol. These gases can also be added to the gas mixture used during EVLP (92). Attenuation of organ damage following ischemia and reperfusion has been attributed to an increase in apoptosis, an increase in HIF1-alpha or modification of the Akt-pathway (93). However, not all evidence supports this possibly beneficial role (92). There is still a lack of evidence concerning the pathways that are involved in the protective effect of these noble gases, and timing of administration and correct dosage are still uncertain. Moreover, these gases are quite expensive and have a low cost-benefit ratio.

Fluranes

Fluranes are nowadays commonly used for anesthesia during surgical procedures. Anesthetic preconditioning with isoflurane or sevoflurane may stabilize mitochondria and reduce the amount of oxidative stress that is generated during ischemia and reperfusion in the cell. It means that organ function is preserved and ischemic damage is reduced by exposing the target organ to volatile anesthetics before the ischemic insult. These anesthesia gases inhibit the electron transport chain at complex I and III (94), which causes tiny amounts of ROS to be formed. This small preconditioning impulse triggers different organoprotective intracellular pathways, such as tyrosine kinase phosphorylation and activation of mitochondrial K_{ATP} channels. Extra potassium ions stabilize membrane depolarization and decrease the risk of calcium overload in the mitochondria. Administration of sevoflurane appears to reduce oxidative stress but maintain NO production by eNOS and iNOS in a porcine autotransplant model (95).

Ischemic preconditioning

A brief period or a train of short periods of ischemia before the actual ischemic insult can significantly reduce ischemic damage and provide additional protection from additional hypoxic episodes. This was first demonstrated in myocardial tissue, but appears to be valid in lung tissue as well (96). There is currently no clear data available concerning the applicability of pulmonary ischemic preconditioning in the setting of a lung transplantation. Practical issues arise as to how pulmonary ischemia should be induced, and patients are often too weak to undergo such short periods of hypoxia. Many different cellular pathways have been suggested, such as increased transcription of heat shock proteins, triggering of a small burst of ROS as a "training" for further insult, or opening of K_{ATP} channels that stabilize the mitochondrial membrane. A more feasible approach is to apply remote ischemic preconditioning, for instance temporary

occlusion of circulation in an arm or a leg with a blood pressure cuff. This technique has been applied successfully in patients undergoing a lobectomy and reduced oxidative stress levels in the peripheral circulation, decreased ALI incidence and resulted in earlier patient discharge (97).

Conclusions

Oxidative and nitrosative stress are umbrella terms for the highly reactive molecules called ROS and RNS that are generated during inflammatory processes. Free radicals are an important subset of these molecules. This review focuses on the involvement of free radicals in LIRI, or LIRI. Many surgical procedures can lead to LIRI. It is critical that clinicians are aware of the risks and have a notion of the pathophysiological mechanisms that lead towards it. It helps to provide adequate treatment and may even contribute to preventive strategies in the future.

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Footnote

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