Ischemia and reperfusion—from mechanism to translation

Holger K Eltzschig & Tobias Eckle

Ischemia and reperfusion—elicited tissue injury contributes to morbidity and mortality in a wide range of pathologies, including myocardial infarction, ischemic stroke, acute kidney injury, trauma, circulatory arrest, sickle cell disease and sleep apnea. Ischemia-reperfusion injury is also a major challenge during organ transplantation and cardiothoracic, vascular and general surgery. An imbalance in metabolic supply and demand within the ischemic organ results in profound tissue hypoxia and microvascular dysfunction. Subsequent reperfusion further enhances the activation of innate and adaptive immune responses and cell death programs. Recent advances in understanding the molecular and immunological consequences of ischemia and reperfusion may lead to innovative therapeutic strategies for treating patients with ischemia and reperfusion–associated tissue inflammation and organ dysfunction.

Ischemia and reperfusion is a pathological condition characterized by an initial restriction of blood supply to an organ followed by subsequent restoration of perfusion and concomitant reoxygenation. In its classic manifestation, occlusion of the arterial blood supply is caused by an embolus and results in a severe imbalance of metabolic supply and demand, causing tissue hypoxia. Perhaps surprisingly, restoration of blood flow and reoxygenation is frequently associated with an exacerbation of tissue injury and a profound inflammatory response (called ‘reperfusion injury’). Ischemia and reperfusion injury contributes to pathology in a wide range of conditions (Table 1). For example, cardiac arrest and other forms of trauma are associated with ischemia of multiple organs and subsequent reperfusion injury when blood flow is restored. Cyclic episodes of airway obstruction during obstructive sleep apnea also lead to hypoxia with subsequent reoxygenation on arousal. Similarly, individuals with sickle cell disease have periodic episodes of painful vaso-occlusion and subsequent reperfusion with many characteristics that resemble ischemia and reperfusion. Exposure of a single organ to ischemia and reperfusion (for example, the liver) may subsequently cause inflammatory activation in other organs (for example, the intestine), eventually leading to multiorgan failure. However, it is important to point out that ischemic syndromes are a heterogeneous group of conditions. Although there are some similarities in the biological responses among these syndromes, there are important differences between a systemic reduction in perfusion (for example, during shock) compared to regional ischemia and reperfusion of a single organ (or differences between warm ischemia—as occurs, for example, during myocardial ischemia and reperfusion—and cold ischemic conditions—such as those that occur during organ transplantation when the organ is cooled with a cold perfusion solution following procurement).

Indeed, a wide range of pathological processes contribute to ischemia and reperfusion associated tissue injury (Fig. 1). For example, limited oxygen availability (hypoxia) as occurs during the ischemic period is associated with impaired endothelial cell barrier function due to decreases in adenylate cyclase activity and intracellular cAMP levels and a concomitant increase in vascular permeability and leakage. In addition, ischemia and reperfusion leads to the activation of cell death programs, including apoptosis (nuclear fragmentation, plasma membrane blebbing, cell shrinkage and loss of mitochondrial membrane potential and integrity), autophagy–associated cell death (cytoplasmic vacuolization, loss of organelles and accumulation of vacuoles with membrane whorls) and necrosis (progressive cell and organelle swelling, plasma membrane rupture and leakage of proteins and lysosomes into the extracellular compartment). The ischemic period in particular is associated with significant alterations in the transcriptional control of gene expression (transcriptional reprogramming). For example, ischemia is associated with an inhibition of oxygen-sensing prolylhydroxylase (PHD) enzymes because they require oxygen as a cofactor. Hypoxia–associated inhibition of PHD enzymes leads to the post-translational activation of hypoxia and inflammatory signaling cascades, which control the stability of the transcription factors hypoxia-inducible factor (HIF) and nuclear factor-κB (NF-κB), respectively. Despite successful reopening of the vascular supply system, an ischemic organ may not immediately regain its perfusion (no reflow phenomenon). Moreover, reperfusion injury is characterized by autoimmunity, including natural antibody recognition of neoantigens and subsequent activation of the complement system (autoimmunity). Despite the fact that ischemia and reperfusion typically occurs in a sterile environment, activation of innate and adaptive immune responses occurs and contributes to injury, including activation of pattern-recognition receptors such as TLRs and inflammatory cell trafficking into the diseased organ (innate and adaptive immune activation).

In this review, we highlight recent studies that provide new insight into the molecular and immunological pathways of ischemia and

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Associated Pathological Process</th>
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<tbody>
<tr>
<td>Myocardial infarction</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Ischemic stroke</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Acute kidney injury</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Trauma</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Circulatory arrest</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Sickled cell disease</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>Sleep apnea</td>
<td>Ischemia-reperfusion</td>
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These receptors can also be activated by endogenous interferon pathways, resulting in the induction of proinflammatory cytokines during the context of cell damage or death, as occurs during ischemia and reperfusion.

Table 2 Examples of ischemia and reperfusion injury

<table>
<thead>
<tr>
<th>Affected organ</th>
<th>Example of clinical manifestation</th>
</tr>
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<tbody>
<tr>
<td>Single-organ ischemia and reperfusion</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>Kidney</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>Intestine</td>
<td>Intestinal ischemia and reperfusion; multigland failure</td>
</tr>
<tr>
<td>Brain</td>
<td>Stroke</td>
</tr>
<tr>
<td>Multiple-organ ischemia and reperfusion</td>
<td></td>
</tr>
<tr>
<td>Trauma and resuscitation</td>
<td>Multiple organ failure; acute kidney injury; intestinal injury</td>
</tr>
<tr>
<td>Circulatory arrest</td>
<td>Hypoxic brain injury; multiple organ failure; acute kidney injury</td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td>Acute chest syndrome; pulmonary hypertension; priapism; acute kidney injury</td>
</tr>
<tr>
<td>Sleep apnea</td>
<td>Hypertension; diabetes</td>
</tr>
<tr>
<td>Ischemia and reperfusion during major surgery</td>
<td></td>
</tr>
<tr>
<td>Cardiac surgery</td>
<td>Acute heart failure after cardiopulmonary bypass</td>
</tr>
<tr>
<td>Thoracic surgery</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>Peripheral vascular surgery</td>
<td>Compartment syndrome of extremity</td>
</tr>
<tr>
<td>Major vascular surgery</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>Solid organ transplantation</td>
<td>Acute graft failure; early graft rejection</td>
</tr>
</tbody>
</table>

Ischemia and reperfusion, as well as discuss examples of innovative therapeutic approaches based on these mechanistic findings (Table 2).

Ischemia and reperfusion causes sterile inflammation

With a few exceptions, such as bacterial translocation after intestinal injury, ischemia and reperfusion typically occurs in a sterile environment. Nevertheless, the consequences of ischemia and reperfusion share many phenotypic parallels with activation of a host immune response directed toward invading microorganisms10. This sterile immune response involves signaling events through pattern-recognition molecules such as Toll-like receptors (TLRs), recruitment and activation of immune cells of the innate and adaptive immune system and activation of the complement system (Fig. 2). As these responses can have adverse consequences, targeting immune activation is an emerging therapeutic concept in the treatment of ischemia and reperfusion. In contrast, some aspects of the adaptive immune response—particularly the recruitment and expansion of regulatory T cells (Treg cells)—may be beneficial11.

Innate immune responses. The inflammatory response to sterile cell death or injury has many similarities to that observed during microbial infections. In particular, host receptors that mediate the response to microorganisms have been implicated in the activation of sterile inflammation during ischemia and reperfusion10. For example, ligand binding to TLRs leads to the activation of downstream signaling pathways, including NF-κB, mitogen-activated protein kinase (MAPK) and type I interferon pathways, resulting in the induction of proinflammatory cytokines and chemokines10. These receptors can also be activated by endogenous molecules in the absence of microbial compounds, particularly in the context of cell damage or death, as occurs during ischemia and reperfusion10. Such ligands have been termed ‘damage-associated molecular patterns’ (DAMPs). Many of these ligands (for example, high-mobility group box 1 (HMGB1) protein or ATP) are normally sequestered intracellularly; upon tissue damage, they are released into the extracellular compartment where they can activate an immune response12,13. There is also evidence that extracellularly located damage-associated molecular patterns are generated or released in the process of catabolism10. Such catabolic DAMPs can either activate an immune response12 or function as a safety signal to restrain potentially harmful immune responses and promote tissue integrity during ischemia and reperfusion (examples of the latter type of catabolic DAMP are adenosine and the fibrinogen-derived peptide Bβ(15–42)14,15).

One of the most widely studied pattern recognition receptors is TLR4, which is known to mediate inflammatory responses to Gram-negative bacteria through its activation by lipopolysaccharide. Mice with targeted gene deletion of Tlr4 are hyporesponsive to lipopolysaccharide, as are humans with missense mutations in TLR4 (ref. 16). TLR4 activation may be enhanced by oxidative stress17, which is generated by ischemia and reperfusion and is known to prime inflammatory cells for increased responsiveness to subsequent stimuli. Alveolar macrophages from rodents subjected to hemorrhagic shock and resuscitation express increased surface levels of TLR4, an effect that was inhibited by adding the antioxidant N-acetylcysteine to the resuscitation fluid17. Moreover, H2O2 treatment of cultured macrophages similarly caused an increase in surface TLR4 expression17. Fluorescent resonance energy transfer between TLR4 and the raft marker GM1, as well as biochemical analysis of raft components, showed that oxidative stress redistributes TLR4 to lipid rafts in the plasma membrane, consistent with the idea that oxidative stress primes the responsiveness of cells of the innate immune system17. Other studies have implicated TLR4 signaling in renal ischemia and reperfusion. Mice with a genetic deletion of Tlr4 are protected from kidney ischemia, and experiments using bone-marrow chimeric mice suggest that kidney-intrinsic Tlr4 signaling has the predominant role in mediating kidney injury18. In addition, a study of patients undergoing kidney transplantation revealed a detrimental role of TLR4 signaling in early graft failure19. Indeed, endogenous TLR4 ligands such as HMGB1 and biglycan were induced during human kidney transplantation, thereby supporting a role for TLR4 in sterile inflammation in the kidney19. Kidneys from individuals with a TLR4 loss-of-function allele (as assessed by diminished affinity of TLR4 for its ligand HMGB1) contained lower levels of proinflammatory cytokines in association with higher rates of immediate graft function after transplantation19. Other TLRs may also have deleterious effects. TLR3, implicated in sensing viral RNA, was proposed to sense RNA released from necrotic cells independent of viral activation, and treatment with a neutralizing antibody to TLR3 was protective in studies of intestinal ischemia and reperfusion in vivo20. TLR2 expression on epithelia is induced by hypoxia21 or inflammation22, and renal TLR2 signaling contributes to acute kidney injury and inflammation during ischemia and reperfusion23. Taken together, these studies suggest that inhibitors of TLR signaling could be effective for the treatment of sterile inflammation induced by ischemia and reperfusion. Accordingly, antagonists for TLR receptors are currently under development24 (Table 2). These antagonists are structural analogs of TLR agonists and likely act by binding the receptor without inducing signal transduction24. For example, experimental studies of TAK-242, a small-molecule inhibitor of TLR4, in large animals have shown efficacy in the treatment of acute kidney injury25. A recent randomized clinical

Figure 1 Biological processes implicated in ischemia and reperfusion.
Sterile inflammation during ischemia and reperfusion is also characterized by the accumulation of inflammatory cells. Particularly during the early phase of reperfusion, innate immune cells dominate the cellular composition of the infiltrates. The functional contributions of these cells are not clear; they may contribute to a pathological activation of inflammation and promote collateral tissue injury, or conversely to the resolution of injury. Notably, a recent study showed that monocytes can be recruited from a splenic reservoir to injured tissue after myocardial ischemia and reperfusion to participate in wound healing. Other studies have found that depletion of conventional dendritic cells increases sterile inflammation and tissue injury in the context of sterile inflammation. In a clinically relevant mouse model for transplant-mediated lung ischemia and reperfusion, a recent study showed that expression of the Bcl3 protein by the recipient protects against lung ischemia–reperfusion injury. Elevated levels of IL-17 were found in ischemia and reperfusion of the brain, the heart, and the kidneys. In a clinically relevant mouse model for transplant-mediated lung ischemia and reperfusion, a recent study showed that expression of the Bcl3 protein by the recipient led to inhibition of emergency granulopoiesis and limited acute graft injury. Inhibition of myeloid progenitor cell differentiation may therefore have promise as a therapeutic strategy for the prevention of tissue injury in the context of sterile inflammation.

Adaptive immune response. Ischemia and reperfusion elicits a robust adaptive immune response that involves, among other cell types, T lymphocytes. The mechanisms by which antigen-specific T cells are activated during sterile inflammation are not well understood, but emerging evidence indicates a contribution of both antigen-specific and antigen-independent mechanisms of activation. Several studies have shown that T cells accumulate during ischemia and reperfusion. For example, T cells are localized to the infarction boundary zone within 24 h of reperfusion of the ischemic brain, accumulate further at 3 and 7 d after reperfusion and are decreased in number after 14 d. Studies of mouse lines deficient in specific populations of lymphocytes showed that both CD4+ and CD8+ T cells have a detrimental role in ischemia and reperfusion of the brain, the heart, and the kidneys. Further, a recent study suggested a pivotal role for IL-17 produced by γδ T cells in ischemia and reperfusion injury of the brain. Elevated levels of IL-17 were found in virtually all inflammatory cells, contributes to the formation of one such cytotoxic ROS, peroxynitrite. In addition, H$_2$O$_2$ derived from O$_2$ dismutation gives rise to highly toxic hydroxyl radicals through the Haber-Weiss reaction, facilitated by the increased availability of free iron in ischemia. Peroxynitrite and other reactive species induce oxidative DNA damage and consequent activation of the nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP-1), the most abundant isoform of the PARP enzyme family. Accordingly, PARP inhibitors are in clinical development for the treatment of ischemia and reperfusion injury.

At sites of sterile inflammation, the accumulation of granulocytes has to be tightly controlled, as too few granulocytes may not allow for adequate tissue repair, whereas too many granulocytes can promote uncontrolled inflammation and tissue injury. In a clinically relevant mouse model for transplant-mediated lung ischemia and reperfusion, a recent study showed that expression of the Bcl3 protein by the recipient led to inhibition of emergency granulopoiesis and limited acute graft injury. Inhibition of myeloid progenitor cell differentiation may therefore have promise as a therapeutic strategy for the prevention of tissue injury in the context of sterile inflammation.

### Table 2: Examples of promising therapeutic approaches targeting ischemia and reperfusion

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Target</th>
<th>Potential downside</th>
<th>Stage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAK-242</td>
<td>Inhibition of TLR4</td>
<td>Immune suppression, worsening of bacterial infections</td>
<td>Phase 2 clinical trial in acute respiratory failure; preclinical studies in ischemia and reperfusion</td>
<td>25,26</td>
</tr>
<tr>
<td>T cell–based approaches</td>
<td>Suppression of γδ T cells; expansion of T$_{reg}$ cells</td>
<td>Unclear</td>
<td>Preclinical</td>
<td>37,39,40,42</td>
</tr>
<tr>
<td>Fibrinogen split product Bp$_{15–42}$</td>
<td>Unclear</td>
<td>Unclear</td>
<td>Phase 2 clinical trial</td>
<td>15,56,57</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>Inhibition of apoptosis</td>
<td>Immune suppression; worsening of bacterial infection</td>
<td>Phase 2 clinical trial</td>
<td>66</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Activation of autophagy</td>
<td>Bone marrow toxicity (bone marrow suppression or aplastic anemia)</td>
<td>Preclinical (large animal study)</td>
<td>69</td>
</tr>
<tr>
<td>PHD inhibitors</td>
<td>Inhibition of the oxygen sensing PHD enzymes resulting in HIF stabilization</td>
<td>Unclear</td>
<td>Phase 2 clinical trial in renal anemia; preclinical studies in ischemia and reperfusion</td>
<td>79,92–94</td>
</tr>
<tr>
<td>Ischemic preconditioning</td>
<td>Multiple (for example, adenosine signaling, HIF stabilization and attenuation of inflammation)</td>
<td>Unclear</td>
<td>Phase 2 clinical trial</td>
<td>79–83</td>
</tr>
<tr>
<td>Ischemic postconditioning</td>
<td>Multiple</td>
<td>Unclear</td>
<td>Phase 2 clinical trial</td>
<td>87,88</td>
</tr>
<tr>
<td>Remote ischemic conditioning</td>
<td>Multiple</td>
<td>Unclear</td>
<td>Phase 2 clinical trial</td>
<td>89</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>Multiple</td>
<td>Elevation of methemoglobin</td>
<td>Phase 2 clinical trial</td>
<td>106,107</td>
</tr>
<tr>
<td>Apyrase</td>
<td>ATP breakdown (attenuation of ATP signaling and promotion of adenosine generation and signaling)</td>
<td>Unclear</td>
<td>Preclinical</td>
<td>81,120</td>
</tr>
<tr>
<td>Nucleotidase</td>
<td>AMP conversion to adenosine; enhanced adenosine generation and signaling</td>
<td>Unclear</td>
<td>Preclinical</td>
<td>80,122,123</td>
</tr>
<tr>
<td>Regadenoson, ATL146e</td>
<td>Specific adenosine receptor agonists targeting Adora2a</td>
<td>Unclear</td>
<td>Phase 1 trial ongoing</td>
<td>3,131</td>
</tr>
<tr>
<td>Bay 60-6583</td>
<td>Specific adenosine receptor agonist targeting Adora2b</td>
<td>Sickling of red blood cells in individuals with sickle cell disease</td>
<td>Preclinical</td>
<td>80,129,130</td>
</tr>
<tr>
<td>Inhibitors of miR-92a</td>
<td>Promotion of angiogenesis</td>
<td>Unclear</td>
<td>Preclinical</td>
<td>137</td>
</tr>
<tr>
<td>Activators of miR-499 or miR-24</td>
<td>Inhibition of apoptosis</td>
<td>Unclear</td>
<td>Preclinical</td>
<td>138,139</td>
</tr>
</tbody>
</table>
both in individuals suffering from stroke\textsuperscript{18} as well as in mice exposed to brain ischemia and reperfusion\textsuperscript{37}. Notably, subsequent studies identified $\gamma\delta$ T cells (as opposed to CD4\textsuperscript{+} T helper cells) as the main source of IL-17 (ref. 37). In addition, genetic and pharmacologic approaches targeting IL-17 or $\gamma\delta$ T cells led to reduced inflammation and robust neuroprotection, indicating that $\gamma\delta$ T cells that produce IL-17 are an attractive therapeutic target for ischemic stroke (Table 2).

In contrast, T\textsubscript{reg} cells appear to have a protective role in ischemia and reperfusion. For example, a recent study using an experimental stroke model showed that depletion of T\textsubscript{reg} cells substantially increased delayed brain damage and caused a deterioration in functional outcome\textsuperscript{39}. Based on results including the finding that transfer of wild-type but not IL-10–deficient T\textsubscript{reg} cells attenuated ischemic brain injury, the authors proposed that T\textsubscript{reg} cell–dependent production of IL-10 decreases tumor necrosis factor $\alpha$ abundance at early time points and delays interferon $\gamma$ accumulation\textsuperscript{39}. Although not tested in the setting of ischemia and reperfusion, administration of ex vivo expanded human T\textsubscript{reg} cells had beneficial effects in a model of transplant atherosclerosis, providing evidence that such an approach is feasible\textsuperscript{40}. Other strategies to enhance T\textsubscript{reg} cell function following ischemia and reperfusion could involve boosting expression of FOXP3, the key transcription factor for T\textsubscript{reg} cell differentiation. Previous studies have shown that Foxp3 levels are subject to epigenetic regulation\textsuperscript{41}. Indeed, pharmacological inhibitors of histone/protein deacetylases are effective in treating experimentally induced inflammatory bowel disease and in improving cardiac and islet graft survival in mouse transplantation models through increasing T\textsubscript{reg} cell numbers and function\textsuperscript{42}.

**Innate autoimmunity, complement, platelets and coagulation.**

During ischemia and reperfusion, innate recognition proteins can be self reactive and initiate inflammation against self tissue in a manner similar to the response triggered by pathogens (known as ‘innate autoimmunity’\textsuperscript{3}). A series of studies has linked reperfusion injury to the occurrence of so-called ‘natural’ antibodies, leading to activation of the complement system. Natural antibodies are produced in the absence of deliberate immunization and are a major component of the repertoire of B1 cells, which produce IgM and, in some cases, IgG\textsuperscript{43}. For example, a single type of natural antibody prepared from a panel of B1 cell hybridomas (IgM\textsubscript{CM}–22) restored reperfusion injury in antibody-deficient mice\textsuperscript{44}, suggesting that reperfusion injury can be considered to be an autoimmune type of disorder. Using mouse models of skeletal muscle and intestinal reperfusion injury, a highly conserved region within nonmuscle myosin heavy chain type II; DC, dendritic cell; EC, endothelial cell; Poly P, polyphosphate; TF, tissue factor; VSMC, vascular smooth muscle cell.
the complement protein C5, would not affect C3b, which is “upstream” of C5 in the complement cascade and is a key mediator of bacterial opsonization and immune complex solubilization and clearance. In addition, the complexity of the complement system and incomplete mechanistic insight into the functional consequences of manipulating individual components of the cascade may contribute to difficulties in therapeutic targeting of complement pathways. A recent study of hepatic ischemia and reperfusion injury in mice indicated a dual role of the complement system, although excessive complement activation is detrimental, a threshold of complement activation is crucial for liver regeneration, and impaired regeneration due to inadequate complement activation can lead to acute liver failure following hepatic resection or liver transplantation.

Excessive platelet aggregation and release of platelet-derived mediators can exacerbate tissue injury following ischemia and reperfusion. Platelet activation can occur through integrin-mediated endothelial interactions. In addition, platelets can be transported by inflammatory cells across epithelial barriers (by ‘piggybacking’ on polymorphonuclear leukocytes) to sites of injury or inflammation. A recent study showed a central role for a FERM domain-containing protein (Fermt3, also known as Kindlin-3) in mediating integrin-dependent platelet activation and aggregation. Fermt3−/− mice were protected in a model of ischemia and infarction after mesenteric arterial injury with virtually no firm adhesion of platelets to the injured vessel wall. Other studies have shown that platelets release inorganic polyphosphates, polymers of 60–100 phosphate residues that directly activate plasma protease factor XII and thereby function as proinflammatory and procoagulant mediators in vivo. Ischemia and reperfusion triggers coagulation by inflammatory mediators and platelet activation in many ways, but several natural anticoagulant mechanisms can inhibit clot formation following ischemia and reperfusion, such as those mediated by antithrombin-heparin, tissue factor inhibitor and protein C. Furthermore, fibrin degradation following ischemia and reperfusion, resulting in the formation of fibrin D fragments, including the peptide Bp15–42, has been implicated in attenuating inflammation and preserving vascular barrier function during shock and in dampening ischemia-reperfusion injury. Administration of an intravenous bolus of Bp15–42 attenuated myocardial injury in mice, and a subsequent randomized clinical trial of patients with acute myocardial infarction with ST-segment elevation showed that treatment with intravenously-administered Bp15–42 upon reperfusion reduced the size of the necrotic core zone, as assessed using magnetic resonance imaging 5 days after infarction (ref. 57, Table 2).

Cell death during ischemia and reperfusion

Ischemia and reperfusion activates various programs of cell death, which can be categorized as necrosis, apoptosis or autophagy-associated cell death. Necrosis, characterized by cell and organelle swelling with subsequent rupture of surface membranes and the spilling of their intracellular contents, is a frequent outcome of ischemia and reperfusion. Necrotic cells are highly immunostimulatory and lead to inflammatory-cell infiltration and cytokine production. In contrast, apoptosis involves an orchestrated caspase signaling cascade that induces a self-contained program of cell death, characterized by the shrinkage of the cell and its nucleus, with plasma membrane integrity persisting until late in the process. Although this process has traditionally been viewed as less immunostimulatory than necrosis, recent studies have shown that extracellular release of ATP from apoptotic cells through pannexin hemichannels acts as a ‘find-me’ signal that attracts phagocytes. Inhibition of apoptosis may have promise as a therapeutic strategy for ischemia-reperfusion injury. For example, a study in a mouse model of acute kidney injury identified the matricellular protein thrombospondin 1 (THBS1, also known as TSP-1), produced by injured proximal tubular cells, as an inducer of apoptosis and found that Thbs1−/− mice are protected from injury. Other studies have focused on platelet-derived growth factor CC (PDGF-CC), a potent neuro-protective factor that acts by modulating glycogen synthase kinase 3β (GSK-3β) activity. PDGF-CC gene or protein delivery protected neurons from apoptosis in both the retina and brain in various animal models of neuronal injury, including ischemia-induced stroke. PDGF-CC treatment resulted in increased levels of GSK-3β Ser9 phosphorylation and decreased levels of Tyr216 phosphorylation, consistent with previous findings that Ser9 phosphorylation inhibits and Tyr216 phosphorylation promotes apoptosis.

The transcription factor NF-κB may also modulate apoptosis during ischemia and reperfusion. Limited oxygen availability is associated with activation of NF-κB through a mechanism involving hypoxia-dependent inhibition of oxygen sensors. Mice with disruption of the gene encoding IKK-β, the catalytic subunit of IKK that is essential for NF-κB activation, offer an opportunity to study the consequences of preventing canonical NF-κB pathway activation. This manipulation, however, results in embryonic lethality owing to massive apoptosis of the developing liver driven by tumor necrosis factor-α (ref. 64). To circumvent this difficulty, studies from the laboratory of Michael Karin examined mice with selective ablation of IKK-β. Study of intestinal ischemia and reperfusion revealed that although IKK-β deficiency in enterocytes is associated with reduced inflammation, severe apoptotic damage occurred in the reperfused mucosa. NF-κB inhibition can therefore be viewed as a ‘double-edged sword’, in that it is associated with the prevention of systemic inflammation but increased local injury. These results underscore the need for caution in using NF-κB inhibitors for treating intestinal ischemia-reperfusion injury.

The commonly used immunosuppressant cyclosporine is an inhibitor of mitochondrial permeability transition pore opening, an important step in programmed cell death. In a randomized clinical study of 58 individuals with acute myocardial infarction with ST-segment elevation, treatment with an intravenous bolus of cyclosporine immediately before percutaneous coronary intervention was associated with smaller infarct sizes compared to the saline control (Table 2). Although these findings are very encouraging, given the small sample size of this trial, confirmation in a larger clinical trial will be important. In addition, the development of more specific and safer inhibitors of the mitochondrial permeability transition pore could enhance the potential of this approach.

There is strong evidence supporting the idea that autophagy is an adaptive response to sublethal stress, such as nutrient deprivation, and the deletion of key autophagic genes accelerates rather than inhibits cell death. The transcription factor HIF, a central mediator of hypoxic responses, also seems to regulate autophagy. The process of mitochondrial autophagy is induced by hypoxia and requires HIF-dependent expression of autophagic genes, indicating a crucial role for HIF in the metabolic adaptation of hypoxic or ischemic tissues during conditions of limited oxygen. From a therapeutic perspective, a recent study showed that chloramphenicol, traditionally used to treat bacterial infections but limited oxygen. From a therapeutic perspective, a recent study showed that chloramphenicol, traditionally used to treat bacterial infections but more recently recognized as an inducer of autophagy, is protective in a swine model of myocardial ischemia and reperfusion (Table 2).

Microvascular dysfunction

Ischemia and reperfusion is associated with a vascular phenotype that includes increased vascular permeability, endothelial cell inflammation, an imbalance between vasodilating and vasoconstricting factors and activation of coagulation and the complement system. Microvascular dysfunction following ischemia and reperfusion in humans can lead to...
Figure 3 Therapeutic gases for the treatment of ischemia and reperfusion. CO, NO and H2S are considered to be endogenous gas transmitters. The predominant pathway for endogenous CO production involves the conversion of the erythrocyte-derived porphyrin molecule heme to biliverdin by the action of heme oxygenase, liberating CO as a byproduct141. CO has been implicated in attenuating inflammation and tissue injury through the stabilization of HIF. NO is produced predominantly from the endogenous metabolism of l-arginine to citrulline by NO synthase, which is expressed in multiple cell types, including vascular endothelia and neurons (not shown)105. Inhaled NO has been therapeutically used to attenuate hypoxic pulmonary vasoconstriction or to dampen apoptosis during ischemia and reperfusion142. H2S is produced endogenously through the metabolism of l-cysteine by the action of either cystathionine β-synthase (CBS) (expressed predominantly in the brain, nervous system, liver and kidney) or cystathionine γ-lyase (CSE) (expressed predominantly in liver and in vascular and nonvascular smooth muscle)143. Therapeutic use of inhaled H2S has been shown to induce a suspended-animation–like state characterized by hypothermia and stable cardiovascular hemodynamics, and to have protective effects during ischemia and reperfusion. In contrast to endogenous gas transmitters, no biological pathway for the generation of H2 has been described in mammalian cell systems. Therapeutic use of inhaled H2 has been shown to attenuate ischemia and reperfusion–associated accumulation of ROS and to preserve mitochondrial function. EC, endothelial cell; VSMC, vascular smooth muscle cell.

Therapeutic approaches to enhance ischemia tolerance
Therapeutic approaches to render organs more resistant to ischemia could have important clinical uses. Such therapies could be used in a preventive manner during organ transplantation or other types of major surgery associated with ischemia and reperfusion, or after ischemic injury in patients during an intervention aimed at the restoration of blood flow and reperfusion (for example, percutaneous coronary intervention in patients with acute myocardial infarction).

Ischemic conditioning (preconditioning, postconditioning and remote conditioning).
Ischemic preconditioning is an experimental strategy in which exposure to short, nonlethal episodes of ischemia results in attenuated tissue injury during subsequent ischemia and reperfusion. Numerous studies have investigated the underlying mechanisms with the goal of finding pharmacological approaches that would imitate ischemic preconditioning. For example, combinations of genetic and pharmacologic studies have implicated oxygen-dependent signaling pathways79 and purinergic signaling80,81. Other studies have directly applied this experimental strategy to dampen tissue injury from ischemia and reperfusion, for example, by ischemic preconditioning of a transplant graft before liver transplantation or after major liver resections82,83. Although these studies have shown some benefit, they have not been able to reproduce the profound tissue-protective effects of ischemic preconditioning observed in animal studies, perhaps because it is very difficult to systematically identify the most effective preconditioning protocol for a clinical study84–86. Similar to ischemic preconditioning, short episodes of ischemia applied during reperfusion are associated with a reduction in myocardial infarct size, called postconditioning1. A prospective, randomized, controlled, multicenter study investigated whether postconditioning in 30 patients protects the human heart during coronary angioplasty after acute myocardial infarction found beneficial effects87. After reperfusion by insertion of a stent into the occluded coronary artery, postconditioning was initiated within 1 min of reflow by applying four episodes of 1-min inflation and 1-min deflation of the angioplasty balloon. Another clinical study showed that postconditioning was associated with improved cardiac function up to 1 year after an acute myocardial infarction88. Remote ischemic conditioning—induced by repeated brief periods of limb ischemia—was recently found to be effective in myocardial salvage in patients with acute myocardial infarction89. In this study, 333 patients were randomly assigned to remote ischemic conditioning (four cycles of 5-min inflation and 5-min deflation
of a blood pressure cuff) or no treatment during transport to the hospital, where they were treated with a percutaneous intervention to achieve reperfusion. Thirty days later, myocardial perfusion imaging revealed increased myocardial salvage with remote conditioning.

### Metabolic strategies to increase ischemia tolerance

During ischemia, energy metabolism switches from fatty acid oxidation to more oxidation-efficient glycolysis, allowing tissues to sustain cellular viability during ischemia for a longer amount of time. This metabolic switch is under the direct control of the HIF transcription factor, whose stabilization when oxygen levels fall is responsible for the transcriptional induction of glycolytic enzymes.\(^8,90\) The stability of HIF is regulated by the oxygen-sensing PHD enzymes, of which there are three isoforms, PHD1–PHD3. Loss of Phd1 lowers oxygen consumption in ischemic skeletal muscle by reprogramming glucose metabolism to a more anaerobic route of ATP production through activation of a peroxisome proliferator-activated receptor-α pathway.\(^91\) Moreover, treatment with pharmacological PHD inhibitors results in increased ischemia tolerance of the kidneys\(^92\) and in cardioprotection similar to that seen with ischemic preconditioning in the heart.\(^79\) To date, PHD inhibitors seem to be well tolerated in humans,\(^93\) suggesting that they could be readily tested in larger clinical trials (Table 2).

In addition to these more canonical effects of PHD inhibitors, they could also have other potentially desirable effects, including on vascular normalization of tumors. In mice with heterozygous deletion of the gene encoding the oxygen-sensing PHD2, tumor vessel leakiness and vascular distortion is attenuated, an effect called ‘vascular normalization’—for example, normalization of the architecture of sharply demarcated boundaries and branching points of the tumor vessels. This effect can be mimicked pharmacologically by PHD inhibitors via its stabilizing effects on HIF.\(^84\)

Among the most well known of HIF target genes is erythropoietin (EPO), encoding erythropoietin, the major regulator of red blood cell formation, whose production and secretion are regulated by tissue oxygen levels.\(^90\) In addition to its role in stimulating red cell production, preclinical studies implicated erythropoietin in tissue protection from ischemia and reperfusion by metabolic adaptation, inhibition of apoptosis or stimulation of angiogenesis.\(^95–98\) Recently, a prospective, randomized, double-blind, placebo-controlled trial was carried out in patients with acute myocardial infarction with ST-segment elevation to address the efficiency of intravenous treatment with erythropoietin in a clinical setting.\(^99\) In contrast to many preclinical studies, this trial did not find a protective effect of treatment with intravenous erythropoietin. In fact, erythropoietin treatment of such patients who had successful reperfusion within 4 h of percutaneous coronary intervention did not show reduced infarct size but, rather, higher rates of adverse cardiovascular events.\(^99\)

Other studies of metabolic adaptation during ischemia and reperfusion have shown that activation of mitochondrial aldehyde dehydrogenase 2 (ALDH2) is associated with robust cardioprotection in rat models,\(^100\) indicating that pharmacological enhancement of ALDH2 activity applied preventively might increase ischemia tolerance in patients subjected to cardiac ischemia, for example during coronary bypass surgery. Individuals with a genetic defect of ALDH2, as occurs in up to 40% of the Asian population, may particularly benefit from this therapy. Other studies have focused on AMP-activated protein kinase (AMPK), which orchestrates the regulation of energy-generating and energy-consuming pathways and whose activation has been shown to protect the heart against ischemic injury.\(^101,102\) AMPK activation seems to be an endogenous protective mechanism, as the proinflammatory cytokine macrophage migration inhibitory factor (MIF), whose production is stimulated by ischemia, stimulates AMPK and thereby promotes glucose uptake and cardioprotection.\(^103\) These results are consistent with findings that human fibroblasts containing a low-activity MIF promoter polymorphism show diminished MIF release and AMPK activation during hypoxia.\(^104\)

### Therapeutic gases

Several therapeutic gases have been used for the treatment of ischemia and reperfusion (Fig. 3), including hydrogen (H₂), nitric oxide (NO), hydrogen sulfide (H₂S), and carbon monoxide (CO). H₂ is a highly diffusible gas and can combine with hydroxyl radicals to produce water, thereby acting as an antioxidant. In a rat model in which oxidative stress damage was induced in the brain by focal ischemia and reperfusion, production of ROS by mitochondria was shown to trigger the mitochondrial permeability transition pore, leading to mitochondrial swelling, rupture and release of cytochrome c, and finally to apoptotic cell death.\(^103,104\) Inhalation of H₂ gas markedly suppressed brain injury by buffering these effects of oxidative stress.

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**Table 2**

| Blood | EC | VSMC | EC
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><strong>Inflammation</strong></td>
<td><strong>Tissue injury resolution</strong></td>
<td><strong>Metabolic adaptation</strong></td>
<td></td>
</tr>
<tr>
<td>Necrotic cell</td>
<td>Apoptotic cell</td>
<td>Inflammatory cell</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine</td>
<td>Ischemia-reperfusion injury</td>
<td></td>
</tr>
<tr>
<td>P2Y7</td>
<td>NLRP3 inflammasome</td>
<td>Chemoattraction</td>
<td></td>
</tr>
<tr>
<td>Macrophage</td>
<td>IL-1β, IL-18, TNF</td>
<td>AMP</td>
<td></td>
</tr>
<tr>
<td>Connexin hemichannels</td>
<td>Connexin hemichannels</td>
<td>A2A</td>
<td></td>
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<tr>
<td>Pannexin hemichannels</td>
<td>Pannexin hemichannels</td>
<td>A2B</td>
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**Figure 4** Nucleotide and nucleoside signaling during ischemia and reperfusion. Multiple cell types release ATP during ischemia and reperfusion (for example, spillover from necrotic cells or controlled release through pannexin hemichannels from apoptotic cells or connexin hemichannels from activated inflammatory cells).\(^95,114,118\) Subsequent binding of ATP to P2 receptors enhances pathological inflammation and tissue injury, for example, through P2X7-dependent Nlrp3 inflammasome activation,\(^13\) and P2Y6-dependent enhancement of vascular inflammation.\(^117\) ATP can be rapidly converted to adenosine through the ecto-apyrase CD39 (conversion of ATP to AMP) and subsequently by the ecto-5′-nucleotidase CD73 (conversion of AMP to adenosine). Adenosine signaling dampens sterile inflammation, enhances metabolic adaptation to limited oxygen availability and promotes the resolution of injury through activation of A2A adenosine receptors expressed on inflammatory cells and activation of A2B adenosine receptors expressed on tissue-resident cells (for example, cardiac myocytes, vascular endothelia or intestinal epithelia). EC, endothelial cell; VSMC, vascular smooth muscle cell.
In contrast to \( \text{H}_\text{2}, \text{NO}, \text{H}_\text{2}S \) and \( \text{CO} \) are produced endogenously by enzymatically controlled pathways. NO, a soluble gas continuously synthesized in endothelial cells by endothelial NO synthase, regulates basal vascular tone and endothelial function and maintains blood oxygenation through hypoxic pulmonary vasoconstriction. Multiple studies have implicated the endogenous production of NO or its therapeutic application in attenuating ischemia-reperfusion injury\(^{105}\). In a small (\( n = 10 \) individuals per group), randomized, placebo-controlled clinical trial using inhaled NO for the treatment of graft ischemia and reperfusion during liver transplantation, NO improved the restoration of liver function and lowered hepatocyte apoptosis\(^{106}\). In contrast, a randomized, placebo-controlled trial for the use of inhaled NO in the acute treatment of sickle cell pain crisis did not find an improvement in the time until crisis resolution\(^{107}\). As the beneficial effects of NO administration may depend on its conversion to nitrite\(^{105}\), a possible explanation for the lack of a beneficial effect in the sickle cell trial is that systemic nitrite was insufficiently generated, perhaps due to the way in which NO was delivered (as a pulse of pure NO in nitrogen at the ‘front’ of the tidal volume).

\( \text{H}_2\text{S} \) has also been reported to have therapeutic effects in animal models of ischemia and reperfusion\(^{108,109}\). For example, similar to AMP\(^{110}\), \( \text{H}_2\text{S} \) can induce a reversible state of hypothermia and a suspended-animation-like state in rodents\(^{111}\), and treatment with \( \text{H}_2\text{S} \) reversibly depresses cardiovascular function without changing blood pressure\(^{112}\). In addition, endogenously produced \( \text{CO} \) or the administration of \( \text{CO} \)-releasing molecules have anti-inflammatory and cytoprotective effects that involve HIF stabilization and activation of a HIF-dependent transcriptional response\(^{113}\).

**Nucleotide and nucleoside signaling.** Nucleotides, particularly in the form of ATP, have been strongly implicated in promoting tissue inflammation during ischemia and reperfusion (Fig. 4). Ischemia and reperfusion results in the release of ATP—whose intracellular concentrations are relatively high (5–8 mM)—into the extracellular compartment. ATP can be spilled by necrotic cells\(^{10}\) or can be released in a controlled fashion from apoptotic cells\(^{58,59}\) or activated inflammatory cells\(^{114,115}\). When it accumulates in the extracellular compartment, ATP acts to recruit phagocytes\(^{58,59}\), activates the Nlrp3 inflammasome during ischemia and reperfusion\(^{13} \) and promotes the chemotaxis of inflammatory cells\(^{116}\). ATP-elicted activation of nucleotide receptors can enhance vascular inflammation; for example, through P2Y6 receptors\(^{117} \) or, following spinal cord injury, through P2X7 receptors\(^{118}\).

Pharmacological strategies to block ATP release or ATP receptor signaling may therefore have promise for attenuating sterile inflammation during ischemia and reperfusion. In the extracellular compartment, ATP is enzymatically converted to the nucleoside adenosine\(^{119}\). In animal models of ischemia and reperfusion, pharmacological strategies for spurring ATP breakdown to adenosine—for example, treatment with apyrase, which converts ATP or ADP to AMP, followed by treatment with nucleotidase, which converts AMP to adenosine—are effective in attenuating tissue injury and sterile inflammation (Table 2)\(^{82,83,120–125}\). Beyond alleviating the detrimental effects ATP, ATP conversion to adenosine may be desirable because of the beneficial effects of adenosine itself\(^{126}\). Pharmacological and genetic studies in mouse models of ischemia and reperfusion have shown that signaling through adenosine receptors is protective; for example, through activation of the adenosine A2A receptor (Adora2a) on inflammatory cells\(^{3,127,128}\) or Adora2b on vascular endothelia, epithelia or myocytes\(^{72,80,129,130}\). For example, studies in mouse models of myocardial ischemia and reperfusion\(^{80}\), acute kidney injury\(^{130}\) or intestinal ischemic injury\(^{129}\) have shown promising results for the selective Adora2b agonist BAY 60-6583 in the treatment of ischemia and reperfusion. Moreover, Adora2a activation on invariant natural killer T cells attenuates ischemia and reperfusion in mouse models of sickle cell disease\(^{131}\). Due to its potent vasodilatory properties\(^{132}\), the ADORA2A agonist regadenoson (CVT-3146) was approved by the US Food and Drug Administration as a coronary vasodilator for patients requiring pharmacologically-induced stress echocardiography\(^{133,134}\). An ongoing multicenter, dose-finding and safety trial of infused regadenoson has been initiated to study its safety and efficacy in the treatment of ischemia and reperfusion–related tissue injury in patients with sickle cell disease (Table 2)\(^{131}\). Complicating this approach, a recent study showed that adenosine signaling through ADORA2B induces hemoglobin S polymerization, promoting red blood cell sickling, vaso-occlusion, hemolysis and organ damage\(^{135,136}\).

**MicroRNAs (miRNAs) as therapeutic targets.** Several studies have suggested a functional role for miRNAs in ischemia and reperfusion (Fig. 5). For example, a recent study showed that the miR-17–92 cluster is highly expressed in human endothelial cells and that miR-92a, a
component of this cluster, controls angiogenesis.\textsuperscript{137} Systemic administration of an oligonucleotide antagonist designed to inhibit miR-92a led to enhanced blood vessel growth and functional recovery of damaged tissue in mouse models of limb ischemia or myocardial infarction. MiR-92a seems to target mRNAs corresponding to several proangiogenic proteins, including the α5 integrin. Another study reported that miR-499 administration diminishes apoptosis and the severity of myocardial infarction during ischemia and reperfusion. Inhibition of cardiomyocyte apoptosis by miR-499 was ascribed to direct targeting of a catalytic subunit of the phosphatase calcineurin, attenuating calcineurin-mediated dephosphorylation of dynamin-related protein-1 (Drp1) and thereby decreasing activation of the mitochondrial fission program.\textsuperscript{138} Expression of another miRNA, miR-24, can also be protective in ischemia. miR-24 expression in a mouse model of myocardial ischemia inhibited cardiomyocyte apoptosis, attenuated infarct size and reduced cardiac dysfunction.\textsuperscript{139} In this case, the effect on apoptosis was attributed, in part, through direct repression of the BH3-only domain-containing protein Bim.

Pharmacological approaches to inhibit miRNAs seem likely to become treatment modalities for patients in the near future. For example, liver-expressed miR-122 is essential for hepatitis C virus RNA accumulation in cultured liver cells. Recent studies have shown that administration of a locked nucleic acid complementary to the 5’ end of miR-122 (SPC3649) is effective in silencing miR-122 in nonhuman primates and in the treatment of primates with chronic hepatitis C infection.\textsuperscript{140} Clinical trials in humans are currently being conducted to address the safety and efficacy of SPC3649 in humans (www.clinicaltrials.gov). Similar pharmacological approaches could be developed using locked nucleic acids to target detrimental miRNAs (for example, miR-92a)\textsuperscript{137} during ischemia and reperfusion (Table 2).

Conclusions
Although rapid reperfusion is needed after ischemia, this reperfusion can paradoxically contribute to tissue injury and destruction. The past decade has seen strong progress in understanding the mechanisms of reperfusion injury and in developing strategies to render tissues more resistant to ischemia or to dampen reperfusion injury. For example, experimental studies of hypoxia-elicited adaptive responses have provided strong evidence for new treatment approaches during ischemia and reperfusion, such as PHD inhibitors or adenosine receptor agonists. Specific therapeutic interventions are now under consideration for clinical safety and efficacy trials, and indeed some agents have already provided promising results in small clinical trials and now require larger follow-up studies to confirm the initial results (Table 2). Unfortunately, other clinical studies have failed to provide evidence for a protective effect of specific therapeutic approaches. It is important to keep in mind that a clinical trial is always based on a specific treatment strategy (for example, the dosage used and the timing of drug delivery), which may lead to the failure of a drug to achieve its desired effect despite its inherent efficacy. Moreover, there remains an urgent need to gain additional mechanistic insight into the molecular events that are triggered by ischemia and reperfusion and that could be exploited therapeutically. For example, highly effective pharmacologic tools to manipulate microRNAs are soon to become available for the treatment of humans. However, our biologic understanding of how microRNAs alter gene expression in ischemic tissues remains rudimentary, and additional mechanistic studies to identify miRNA targets that could be used to treat ischemia and reperfusion will be essential for taking advantage of such pharmacological approaches. Despite the challenges ahead, we are hopeful that new therapies for ischemia and reperfusion will soon be integrated into clinical practice.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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106. Gladwin, M.T.
108. Eltzschig, H.K.
109. Grenz, A.
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115. Eltzschig, H.K.
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117. Riegel, A.K.
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