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REVIEW

Ischemic post-conditioning to counteract intestinal ischemia/reperfusion injury

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Abstract

Intestinal ischemia is a severe disorder with a variety of causes. Reperfusion is a common occurrence during treatment of acute intestinal ischemia but the injury resulting from ischemia/reperfusion (IR) may lead to even more serious complications from intestinal atrophy to multiple organ failure and death. The susceptibility of the intestine to IR-induced injury (IRI) appears from various experimental studies and clinical settings such as cardiac and major vascular surgery and organ transplantation. Whereas oxygen free radicals, activation of leukocytes, failure of microvascular perfusion, cellular acidosis and disturbance of intracellular homeostasis have been implicated as important factors in the pathogenesis of intestinal IRI, the mechanisms underlying this disorder are not well known. To date, increasing attention is being paid in animal studies to potential pre- and post-ischemia treatments that protect against intestinal IRI such as drug interference with IR-induced apoptosis and inflammation processes and ischemic pre-conditioning. However, better insight is needed into the molecular and cellular events associated with reperfusion-induced damage to develop effective clinical protection protocols to combat this disorder. In this respect, the use of ischemic post-conditioning in combination with experimentally prolonged acidosis blocking deleterious reperfusion actions may turn out to have particular clinical relevance.

Key words: Acidosis; Intestinal ischemia/reperfusion injury; *In vivo* models; Ischemic post-conditioning

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INTRODUCTION

Reperfusion following ischemia (IR) causes severe injury (IRI) to the intestine that is life threatening. Here we overview methods to prevent or at least diminish these deleterious effects of IR. Special attention is being paid to ischemic post-conditioning (POC) with manipulation of the intracellular pH (pH_i). Ischemia occurs when an organ lacks sufficient blood supply as a result of, for example, shock, vascular disease or organ transplantation. Complete cessation of oxygenation for more than 20 min typically

results in irreversible organ damage causing cell death within hours[1]. The intestine is particularly susceptible to ischemia because its high rate of oxygen use renders it relatively incapable of increasing oxygen transport in the face of hypoxic stress. Intestinal ischemia can result from intestinal intussusception, acute mesenteric arterial occlusion, hemodynamic shock and bowel disease[1,2]. The compromising effects of ischemia concern various aspects of intestinal physiology including impaired capillary blood flow[3,4], acidosis[5], changed villus structure[6,7], increased mucosal permeability[8,9] and reduced mitochondrial activity leading to decreased NADPH production[10,11]. During the ischemia event, aside from inadequate oxygen supply that compromises mitochondrial oxidative phosphorylation, there is an accumulation of metabolites that, directly or through mediators, may lead to cellular injury[12]. Prolonged ischemia (10-12 h) causes the affected intestinal area to die which evokes wide-spread systemic adverse effects due to the intestinal release of toxic substances into the circulation that subsequently affects other organs like the heart, lungs, liver and kidney and eventually results in sepsis and multiple organ failure[13]. Therefore, acute intestinal ischemia can be a devastating disease with a high mortality rate, depending to some extent on the underlying cause: venous thrombosis 32%; arterial embolism 54%; non-occlusive ischemia 73%; and arterial thrombosis 77%[14].

Paradoxically, restoration of blood flow by reperfusion may intensify rather than decrease organ damage ("oxygen paradox")[15] depending on the duration and intensity of the ischemia and on the timing of oxygen reintroduction to the tissues[1,16,17]. The intestine is, with the heart, lungs, brain and kidney, among the organs most sensitive to IR. In the intestinal mucosa, IR induces damage that is characterized by altered microvascular and epithelial permeability as a result of complex interactions between the endothelium and various cell types and cellular necrosis and/or apoptosis of villous cells[18]. In this injury process, activation of neutrophils, mast cells and platelets and increased release of endothelial factors are involved[19,20]. Cytokines such as TNF-α, IL-1 and IL-6 and oxygen free radicals are assumed to be important pathogenic mediators in IRI, as is capillary no-reflow[1,21]. A neurotransmitter believed to be released from the injured intestine and playing a main role in the aggravation of intestinal IRI is serotonin which controls intestinal movement, platelet activity and vasoconstriction[22].

As a result of reperfusion, the injured intestine may increase the release of toxic substances into the circulation that subsequently cause sepsis and multiple organ failure[23-25]. Eventually, IR may cause loss of mucosal barrier function, bacterial translocation and strong activation of inflammatory responses leading to endothelial destruction[23,26]. This inflammatory aspect of IR includes both cellular and humoral components and increasing evidence highlights the role of leukocytes and leukocyte adhesion molecules in intestinal IRI[27,28].

For some time, necrosis has been considered to be the main effect of ischemia on intestinal epithelial cells but, to date, apoptosis seems to be the principal contributor to IR-induced cell death[29]. The main executors of this "programmed cell death" are the endoprotease cysteines called caspases[30].

TREATMENTS TO COUNTERACT IRI

For the reasons given above, there is a strong and increasing interest in understanding and counteracting the damaging effects of IR on the intestinal mucosa. Various approaches to diminish the deleterious consequences of IR have been tested in animal models and *in vitro*. Many of these involve pretreatment (before the start of experimentally induced ischemia) with exogenous substances to interfere with the various processes that underlie the IRI syndrome such as intracellular signaling pathways, free radical dynamics and inflammation. The vast number of drugs tested in such pretreatment studies is steadily increasing and include anti-cytokine-induced neutrophil chemoattractant antibody[31], propofol[32], curcumin[33], NMDA receptor antagonists[34], carnitine[35], peroxisome proliferator-activated receptor-gamma ago nist[36] and erythropoietin[37]. Although these pre-treatment studies do not have immediate clinical applications, they have increased our understanding of the IRI disease process. This is exemplified by pretreatment studies with nitroglycerin as follows. Due to its strategic location at the luminal surface of vessels, the vascular endothelium is particularly sensitive to IR. Endothelial functioning is impaired by the sudden increase in oxygen free radical species upon reperfusion. Paradoxically, free radicals (including oxygen free radicals and nitric oxide) are also involved in the protective process of ischemic preconditioning whereby a given stimulus increases tissue tolerance to IR damage[38]. Interestingly, it has been shown in both human and animal studies[39,40] that nitroglycerin can induce a protective phenotype that limits tissue damage by IR. It appears that nitroglycerin protects the endothelium against post-ischemic endothelial dysfunction via a mechanism that is mediated by oxygen free radical release and opening of mitochondrial permeability transition pores[41].

However, no pretreatments have found clinical application yet because treatment should be initiated shortly before the onset of ischemia, a moment that, obviously, cannot be precisely anticipated in a clinical setting. For this reason, attention is being increasingly focused at other protective treatments of the intestine, namely directly after ischemia, of which we will summarize the most characteristic ones below.

Melatonin, applied intraperitoneally in rat at the start of reperfusion, appears to exert a strong antioxidant effect that prevents intestinal IRI in a dose-dependent manner[42] and the administration before mesenteric reperfusion of allopurinol, a xanthine-oxidase inhibitor[43], offered protection against IRI as well. Although use of allopurinol to inhibit xanthine oxidase prior to intestinal reperfusion is utilized by some clinicians, substantial clinical evidence to support this practice has not been generated. Intestinal

regional hypothermia applied during mesenteric ischemia reduced the pro-inflammatory responses induced by IR[44]. Also, whereas no significant effect was found on microvascular barrier function during reperfusion in dogs, it has been claimed that hypothermia does protect the rat intestine against IRI. Apparently, this protection is associated with diminished NF-kB activity, induction of iNOS and expression of heme oxygenase-1[45]. After mesenteric IR, moderate hypothermia may have beneficial effects on the intestine, heart and liver[2].

Reperfusion of ischemic tissue is generally associated with intense inflammation-mediated tissue injury. In animal models, the *in vivo* anti-inflammatory actions of physalins, natural steroidal compounds, appear to be mostly due to the activation of glucocorticoid receptors[46]. Following IR, dexamethasone and physalin B and F markedly prevented neutrophil influx and increased vascular per meability in the intestine and the lungs. Moreover, hemorrhage was prevented in the intestine of reperfused ani mals. Dexamethasone and physalins effectively suppressed the increase in organ (intestine and lungs) and serum concentrations of TNF-alpha. Interestingly, treatment with these compounds was associated with enhancement of IL-10. The anti-inflammatory effects of dexamethasone and physalins were reversed by pretreatment with the corticoid receptor antagonist RU486. Therefore, the *in vivo* anti-inflammatory actions of physalins, natural steroidal compounds, appear to be mostly due to the activation of glucocorticoid receptors. Compounds derived from these so-called secosteroids may represent novel therapeutic options for the treatment of inflammatory diseases including IRI[46].

Another way to reduce neutrophil infiltration during IRI has been pursued by luminal treatment with special amino acid-based solutions of rodent small bowel throughout reperfusion after 60 min ischemia. This reduced neutrophil infiltration while electrophysiology and histology revealed good preservation of mucosal structure and barrier function[47]. Finally, post-treatment with simvastatin, which has anti-inflammatory and antioxidant actions, resulted in a significant increase in bowel and mucosal weight in ileum and in villus height and crypt depth in jejunum and ileum in rat. Moreover, simvastatin reduced intestinal injury score as well as the apoptosis index, indicating that this drug inhibits programmed cell death following intestinal IR[48].

Unfortunately, up to now these animal studies have not resulted in protocols that can be reliably applied in the clinic. Below we will consider the implications of the particularly interesting recent finding that brief intermittent episodes of ischemia and reperfusion after a prolonged period of ischemia could reduce the deleterious effects of IR on the intestine, a phenomenon called ischemic "post conditioning" (POC).

ISCHEMIC POSTCONDITIONING AND pH_i

Post-conditioning has been shown to protect against various pathological events including necrosis, apoptosis and microvascular injury[49]. More specifically, it attenuates IRI in the heart, spinal cord, brain, kidney, liver, muscle and lung in the experimental setting[50,51]. Recently, POC was tried as a protection paradigm against IRI in the intestine[52,53]. However, semiquantitative histopathological evaluation and measurement of wet-to-dry weight ratios did not reveal a significant difference between the ischemic and post-conditioned rabbit intestine in the degree of necrosis, tissue wet-to-dry weight ratios or blood flow[54], a negative result casting doubt on the potential efficacy and reliability of POC applications in the clinical setting. These ambiguous effects of POC on the animal intestine may be due to the high complexity by which POC changes ischemic tissue including delaying realkalinization of tissue pH, triggering release of auta coids, modulating the activity of ion channels and activa ting kinases[51]. Since these processes together may act on multiple cellular and molecular targets and may severe ly affect intestinal functioning. However, at the same time, these multiple actions of POC differ from the monotherapy approach by drugs that have failed to consistently reduce IRI and therefore might be promising in human trials provided that current POC protocols are adequately improved[51]. Such an improvement might be obtained by strengthening the delaying action of POC on realkalinization by combining POC with prolonged acidosis. This approach appears to substantially limit heart infarct size in animal models[41,55], raising the possibility that this modification of the POC protocol might also be effective in blocking the deleterious effects of IR in the (human) intestine. Lowering the pH of the reperfusion medium might prevent activation of Na^+/H^+ exchange processes, as was shown for reperfusion of ischemia-exposed astrocytes[56] and heart[57]. This notion opens avenues to protocols preventing or curing IRI. Therefore, here we will pay attention to the way the intracellular pH (pH_i) is controlled by cellular factors and can be manipulated *in vivo*.

The pH_i is essential for maintaining cellular homeostasis in the intestinal mucosa. Even small changes in pH_i (less than 0.1 units) may alter ion channel properties and depress the activity of key enzymes involved in glycolysis and ATP synthesis. Consequently, mucosal cells possess homeostatic mechanisms to stabilize their pH_i. If such mechanisms become impaired by prolonged ischemia, cell death will be the result. As we have shown for the mouse jejunum[58], cellular inactivation and subsequent cell death are concomitant with intracellular acidosis. Consequently, restoration of the pH_i by reperfusion will help intestinal epithelial cells to recover. However, this recovery will only take place when ischemia-induced acidosis is not too severe; otherwise acidosis will remain and reperfusion will cause cells to die[58]. This condition of recovery might be improved by experimentally intervening with the pH_i.

A particular role in the maintenance of the pH_i is played by short-chain fatty acids (SCFAs) produced by the bacterial flora in the intestinal lumen and by the various types of Na^+/H^+ exchanger protein (NHEs) res-

ponsible for controlling pH_i homeostasis during the continuously changing luminal environment. SCFAs include N-butyrate, acetate, propionate and isobutyrate and induce acidification of the rat colon, probably by non-ionic diffusion and other means of cellular absorption of the acid moiety[59]. After exposure to acetate, propionate or n-butyrate, all colonocytes acidify rapidly and over 90% reveal pH_i alkalinization[60]. NHE proteins exchange extracellular sodium ions for intracellular protons with a 1:1 stoichiometry. Identified functions of NHE include regulation of the pH_i, in particular during recovery from an acid load, maintenance of cell volume in response to an osmotic load and transepithelial Na^+ absorption[61,62]. Out of the 10 known NHE isoforms, NHE2 and NHE3 are present in the brush border of the duodenum[63-65], in the apical membrane of villus cells of the small intestine and in surface cells of the colon. NHE1 is found in the basolateral membrane of epithelial cells in all intestinal segments[66]. A primary role of mammalian NHE is to regulate the cytosolic pH. NHEs are activated by decreased pH_i and upon activation raise the pH_i to normal value. The ubiquitous NHE1 is a major regulator of pH_i. Through its coordinate functions in H^+-efflux, actin anchoring and scaffolding, NHE1 is assumed to promote protein activities and interactions, assembling signaling complexes in specialized plasma membrane domains and coordinating divergent signaling pathways[67]. NHE3 is an important contributor to the regulation of pH_i, Na^+ and water homeostasis of the organism as it catalysis Na^+ and fluid (re)absorption across epithelia. NHE3-null mice show a disturbed acid-base balance[65].

MECHANISM OF POC WITH PROLONGED ACIDOSIS

The mechanism by which POC with prolonged acidosis would protect organs against IRI is largely unknown. In the rat heart, the beneficial effect of delaying the recovery of pH_i during reperfusion is possibly due to inhibition by low pH of the activity of calpain[55], the calcium-binding protein that leads to cardiac contractile dysfunction following ischemic insult. Whereas IR causes endothelial dysfunction, reoxygenation at low pH (6.4) enhances the recovery of acetylcholine-induced vasorelaxation, an improvement of endothelial functioning that likely acts through preservation of cGMP signaling[68]. Recently, a comparative proteomics approach revealed that the IR-protective action of POC on the rat intestinal mucosa involves the expression of proteins functionally concerned with cellular energy metabolism, anti-oxidation and anti-apoptosis[69,70].

Therefore, lowering the pH_i during POC might protect against the deleterious effects of intestinal IR. In view of this latter data, it might be investigated if SCFAs, as natural regulators of the pH_i and inhibitors of NHEs[71], could be used to fine-tune the pH_i of intestinal epithelial cells as a component of POC.

In the heart, recovery from ischemia is enhanced by reintroducing blood flow through repeated intermittent occlusions and reperfusions. The mechanism by which this "IR cycling" protects against IRI is not known but it has been supposed that the intermittent ischemia prevents formation of mitochondrial permeability transition pores by maintaining an acidic myocardial pH_i for several minutes until survival kinases can be activated[41]. This is another argument for assuming that regulating the pH_i may be of importance in preventing IRI.

CONCLUSIONS AND PERSPECTIVES

IRI is a major clinical problem because of the sequelae of a number of clinical conditions. To protect against IRI, some compounds successfully tested in animals and *in vitro* systems might give rise to the development of novel IRI therapeutics but most of them may not have substantial clinical relevance as they have to be administered well before ischemia has started. Therefore, post-ischemic treatments seem to be more promising. Among these, of the various approaches under study, the most promising one may be POC, especially when combined with manipulation of the pH_i. Since POC with prolonged acidosis was recently shown to protect the heart against IRI, it would be interesting to use an intestinal *in vivo* model and real-time imaging techniques to study the action of POC on intestinal structure and functioning[58,72].

In general, the development of successful POC protocols would strongly benefit from better knowledge about the molecular mechanisms of action and optimal ways of the application of POC. For some organs, such information is now becoming available. Ischemic POC probably reduces myocardial apoptosis by increasing BCL-2 protein expression via activation of opioid receptors and the JAK-STAT signaling pathway[73] and neuroprotective POC action on the spinal cord seems to involve phosphatidylinositol 3-kinase and ERK pathways[74]. Furthermore, cardioprotective effects of POC depend critically on the duration of reperfusion and reocclusion episodes[75]. More information of this kind may support the development of sophisticated POC protocols in which fine-tuned periods of intermittent ischemia and reperfusion are combined with drugs that have proven their potential use in pretreatment studies and act specifically on molecular POC targets at the proper cell physiological conditions (pH, temperature *etc*).

Another interesting perspective offered is the intriguing fact that transient non-lethal IRI of one organ confers resistance to a subsequent episode of lethal IRI in a remote organ ("remote preconditioning")[76]; a POC protocol successfully applied to the intestine might exert remote, protective effects on other organs such as the heart, lungs, kidney and brain, finding widespread clinical application in protecting the whole body against the devastating effects of ischemia and IR.

Finally, while emphasis in this review has been placed on the potential protective effects against IRI of POC, especially in combination with pH_i manipulation, a quite

different, but nevertheless promising, approach to solve at least partly the IRI problem is worth mentioning. As inflammation is a main aspect of IRI, controlling the attraction of leukocytes would be a clinically highly relevant tool in counteracting the damaging effects of IRI. Such control could be effectuated by pharmacological manipulation of Toll-like receptors (TLRs) which regulate the organism's defense against infections and sense host tissue injury by recognizing products of dying cells. Better understanding of TLR involvement in IRI may enable the invention of novel TLR-based therapies for IRI in the intestine[77,78].

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