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REVIEW

Ischaemic Preconditioning Protects Against Ischaemia/Reperfusion Injury: Emerging Concepts

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Introduction. *Ischaemic preconditioning (IP) has emerged as a powerful method of ameliorating ischaemia/reperfusion (I/R) injury to the myocardium. This review investigates whether this phenomenon is universally applicable in modulating I/R injury to other tissues.*
Methods. *A Medline search was conducted to identify both animal and human studies that described IP-induced protection from I/R injury in a variety of non-cardiac organ systems. Particular emphasis was placed on elucidation of underlying physiological concepts.*
Results and conclusions. *IP utilises endogenous mechanisms in skeletal muscle, liver, lung, kidney, intestine and brain in animal models to convey varying degrees of protection from I/R injury. To date there are few human studies, but recent reports suggest that human liver, lung and skeletal muscle acquire similar protection after IP. Specifically, preconditioned tissues exhibit reduced energy requirements, altered energy metabolism, better electrolyte homeostasis and genetic re-organisation, giving rise to the concept of 'ischaemia tolerance'. IP also induces 'reperfusion tolerance' with less reactive oxygen species and activated neutrophils released, reduced apoptosis and better microcirculatory perfusion compared to non-preconditioned tissue. Systemic I/R injury is also diminished by preconditioning. IP is ubiquitous but more research is required to fully translate these findings to the clinical arena.*

Keywords: Ischaemic preconditioning; Reperfusion injury; Myocardium; Skeletal muscle; Liver; Lung; Intestine; Brain; Kidney.

Introduction

Ischaemic injury to vital organs such as the heart, brain and kidneys contributes significantly to morbidity and mortality throughout the world.[1] Deprived of oxygen-carrying blood, cellular respiration slows down with irreversible damage occurring within minutes in tissues such as myocardium and liver.[2] Rapid restoration of the circulation, while essential to maintain life, brings its own hazards. Alluded to by Haimovici[3] in the 1970s as the 'myonephropathic metabolic syndrome', the full extent of reperfusion-induced tissue injury was demonstrated with startling effect by Parks and Granger.[4] Whilst ischaemic injury is mainly due to oxygen-deprived cellular necrosis, reperfusion produces an inflammatory response that both heightens local damage and leads to systemic insult as well. Various methods of limiting reperfusion injury have been described including oxygen radical scavenging, leucodepletion, induced hypothermia and controlled reperfusion.[5–8]

In 1986, Murry and colleagues described the phenomenon of ischaemic preconditioning (IP) which proved to be one of the most powerful methods of minimising ischaemia/reperfusion (I/R) injury. Murry *et al.* demonstrated in the canine heart that four cumulative 5 min ischaemic periods, rather than worsening adenosine 5′-triphosphate (ATP) depletion and myocardial necrosis, instead conferred protection from a further prolonged 40 min ischaemic insult.[9] The zone of infarcted myocardium in preconditioned dogs was reduced by 70% compared to controls subjected only to the 40 min coronary occlusion. These experiments galvanised the cardiovascular scientific community and were consistently reproducible across different animal models.[10–12]

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IP was effective within minutes, suggesting that preformed mediators were responsible for its effects. Locally released agonists such as adenosine, bradykinin, catecholamines and opioids trigger this protective response through various cell surface G-protein coupled receptors.[13] Redundancy built into the system initially led investigators to publish conflicting reports on the efficacy of these agonists. Kinases such as protein kinase C, tyrosine kinase and p38MAPKinase participate in the signalling pathway.[14–16] The end-effector of preconditioning remains elusive, although mitochondrial ATP-sensitive potassium (K_{ATP}) channels appear to be involved.[17]

In 1993, it was reported that a second window of protection ('SWOP') developed 12–24 h after the initial preconditioning stimulus.[18] Unlike early IP, which is shortlived, lasting only about 2–3 h, this 'SWOP' remains protective for 48–96 h.[19] However, the magnitude of protection is less and different mechanistic pathways are responsible.[20] Hence IP induces a biphasic protection from I/R injury—an initial strong protective stimulus that is brief and a later, less powerful but longer lasting protection (Fig. 1). Repeated preconditioning episodes are neither additive nor cumulative (Fig. 2).[21]

This stunning experimental data led to successful clinical application of IP to coronary angioplasty and coronary artery bypass grafting.[22–24] The phenomenon of IP is not confined to myocardial protection alone. Recent clinical trials involving pulmonary and hepatic surgery attest to the safety and efficacy of IP as a therapeutic adjunct.[25,26] However, clinical progress is slow.

This review examines the mechanisms by which IP ameliorates I/R injury in a variety of organ systems. A Medline search using combinations of the key-words 'ischaemic preconditioning', 'reperfusion injury', 'myocardium', 'brain', 'liver', 'lung', 'kidney', 'intestintestine' and 'skeletal muscle' was carried out. Additional articles were identified from reference lists of recent topical reviews. The mechanisms of protection are grouped under the headings of 'ischaemia tolerance' and 'reperfusion tolerance'. Of course, the sum total of post-ischaemic injury is a combination of these two processes and the relative contribution from each differs between different organs.[27] Indeed, many of the damaging events that take place at the time of reperfusion are secondary to critical changes that have already occurred during the preceding ischaemic period.

IP Induces 'Ischaemia Tolerance'

Metabolic inhibition allows cells to survive hypoxia: lessons from comparative physiology

In hypoxia-tolerant systems, e.g. turtle hepatocytes, anoxia leads to 'metabolic arrest', i.e. 90–100% suppression of non-essential cellular activity, implying that cells can survive 10 times longer on the same amount of high-energy phosphates (HEP) available (Summary Box 1).[28] The dominant energy sink during anoxia is the Na^+ pump which is responsible for maintaining the high transmembrane Na^+ and K^+ potential gradient. Even so, the activity of this pump drops to only one-quarter of its normal rate because the passive drift of ions across the plasma membrane through non-energy dependent ion channels is restricted; i.e. 'membrane arrest' occurs. These 2 key defence strategies allow the hypoxia-tolerant cell to adjust its energy balance to a new, lower steady-state equilibrium.[29] Indeed, 'membrane arrest' is observed in hypoxia-sensitive tissues that receive IP. In a study

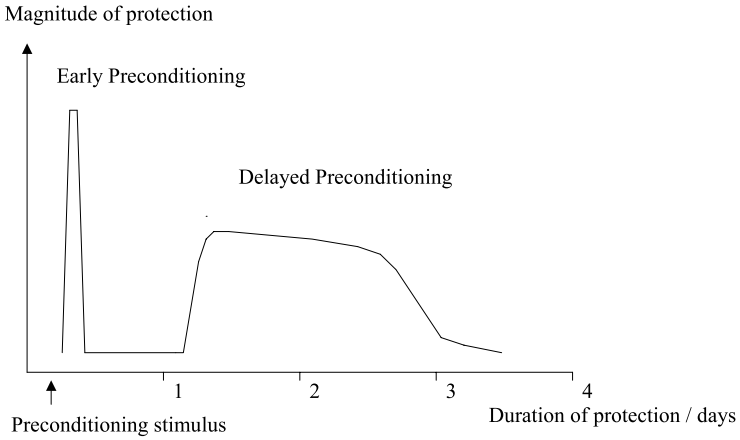

Fig. 1. Biphasic protection induced by a single preconditioning episode.

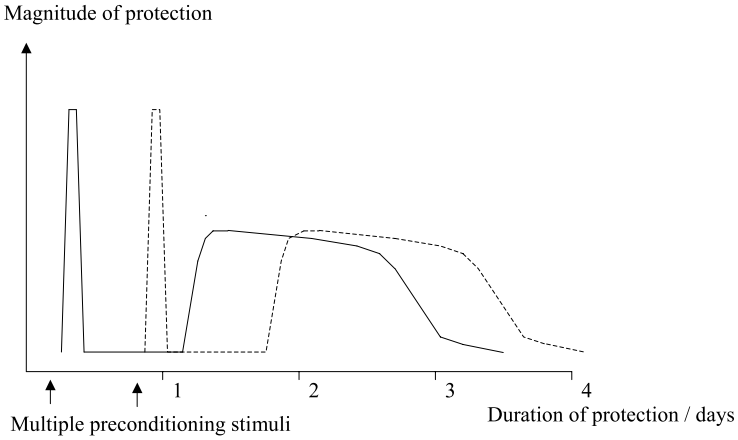

Fig. 2. Multiple preconditioning episodes convert biphasic protection into a monophasic pattern. Note that the magnitude of protection is not increased and the duration of protection is dictated by the last preconditioning stimulus.

on neonatal mice exposed to cerebral ischaemia, preconditioned cerebral tissue had lower whole-cell conductance and potassium-channel activity *in vitro* compared to non-preconditioned controls.[30]

Energy sparing effect of IP

IP reduces cellular energy demand during ischaemia.[31] Preconditioned *in vivo* canine hearts consumed ATP slower than controls during the first 20 min of sustained ischaemia. Although the rate of nucleotide degradation gradually increased in both groups as ischaemia progressed, the adenine nucleotide pool was still greater in preconditioned tissue at the end of 40 min.[32] Slower destruction of adenine nucelotides and reduced glycolysis was concordant with the period of ischaemic preconditioning, i.e. the diminished metabolic rate lasted for the 180 min or so that IP rendered protection from subsequent ischaemia.

Investigators have shown similar high levels of HEP, adenine nucleotides and glycolytic intermediates, and reduced accumulation of lactate and H^+ after I/R in preconditioned porcine and murine models.[33,34] One possible mechanism by which metabolism can be downregulated is through adenosine 5′-monophosphate-activated protein kinase (AMPK), which inhibits glycolysis in rat hepatocytes.[35] Degradation of ATP during IP leads to accumulation of adenosine 5′-monophosphate (AMP), an allosteric activator of AMPK.[36] Inhibition of AMPK production abolished the protective effects of preconditioning on energy metabolism and hepatocyte injury.[37]

ATP itself exerts allosteric inhibition of pyruvate kinase, an important regulatory enzyme in glycolysis.[38] Maintaining higher levels of ATP during prolonged ischaemia allows preconditioned tissues to reduce the rate of glycolysis. CyclicAMP dependent protein kinase phosphorylation of another regulatory enzyme complex phosphofructo-2-kinase/fructose-

Summary Box 1

Different physiological mechanisms by which ischaemic preconditioning induces 'ischaemia tolerance'. Ischaemia causes injury at a cellular level. Ischaemic preconditioning protects cells during hypoxic stress.

Passive drift of ions across plasma and mitochondrial membranes restricted
Cellular energy demand reduced
ATP consumption reduced
Rate of glycolysis reduced
Intra-cellular energy relays become more efficient
Decreased production of H+ ions
Maintenance of ionic equilibrium during prolonged hypoxia
Transformation to 'hypoxia-tolerant' phenotype

2,6-bisphosphatase also controls glycolytic rates during prolonged ischaemia.[39] In a study on rat liver, brief ischaemia led to increased production of cyclic-cAMP in sinusoidal endothelial cells.[40]

IP enhances efficiency of phosphotransfer networks (energy relays)

The elucidation of the tricarboxylic acid cycle[41] and chemiosmotic theory[42] have directed our understanding of energy transactions, enzyme kinetics and ion transfer across mitochondria. An emerging concept in cellular energetics is the use of phosphotransfer networks to effectively link sources of energy production with sites of utilisation. The cell is not just a 'watery bag of enzymes'.[43] Movement of adenine nucleotides by diffusion is both kinetically and haemodynamically inefficient as it requires a significant concentration gradient.[44] In actual fact, ATP produced in the tight folds of the mitochondrial inner membrane is rapidly shuttled by near-equilibrium enzymatic flux transfer relays and high-throughput contact sites between inner and outer mitochondrial membranes.[45] ATP production is coupled efficiently to sites of utilisation by key enzyme pathways including creatine kinase, adenylate kinase and glycolytic enzymes. Nuclear magnetic resonance (NMR) spectroscopic analysis of the relative distribution of HEP intermediates in rat myocardium demonstrated that IP increased efficiency of these phosphotransfer networks.[46]

IP improves acid–base and ion balance during hypoxia

Depletion of ATP during sustained ischaemia can lead to failure of the Na^+/K^+ ATPase pump and Na^+ influx from the extra-cellular space, resulting in cell swelling and rupture. Production of H^+ from the hydrolysis of glycolytically derived ATP contributes to acidosis in severely ischaemic tissues.[47] Recovery of intracellular pH during reperfusion can give rise to significant influx of Na^+ and Ca^{++} (via the Na^+/H^+, Na^+/Ca^{++} transporters) with disastrous consequences.[48] IP has a direct effect in maintaining intra-cellular ion homeostasis and acid–base balance following prolonged ischaemia. Intracellular Na^+ accumulation and acidosis were prevented in preconditioned rat hepatocytes subject to sustained anoxia.[49]

Ionic equilibrium across the inner and outer mitochondrial membranes is also critical in maintaining cellular integrity.[50] IP allows mitochondrial K_{ATP} channels to remain open during subsequent ischaemia, reducing calcium accumulation in the matrix.[51]

Can mammalian tissues undergo transformation to a hypoxia-tolerant phenotype?

Brief periods of ischaemia initiate transformation to a hypoxia-tolerant state in mammalian tissues. Transcription factors such as NF-κB and AP-1 and the immediate early genes c-fos and c-jun are upregulated after hypoxia.[52–54] Perhaps, as if having received an early-warning signal, hypoxia-sensitive cells carry out crucial genetic rearrangements to not only withstand further assault, but also to continue optimal performance of vital functions.

IP reprogrammed the genomic response of neonatal mouse brain to ischaemia, reflecting endogenous neuroprotective responses similar to that seen in hibernation and hypoxia-tolerant organisms.[30] Nf-κB activation, acting via p38 MAPkinase allowed preconditioned hepatocytes to enter the cell cycle upon exposure to prolonged ischaemia.[55] This development of an adaptive phenotype is speculated to be a critical biological step favouring survival against further I/R injury.

Transcriptional targets include heat shock proteins(HSP)—cellular chaperones that maintain structural and functional integrity of nuclear and cytoplasmic proteins—which increase 50-fold 48 h after preconditioning ischaemia in rat hepatocytes.[56] In a recent report, 30 min of bilateral preconditioning ischaemia to mouse kidneys improved post-ischaemic renal function and reduced post-ischaemic leucocyte infiltration and actin cytoskeleton disruption for 12 weeks.[57]

IP Induces 'Reperfusion Tolerance'

Protection from oxidative stress

Reperfusion, although essential to restore oxidative phosphorylation in hypoxic tissues and remove accumulated waste products, has serious local and systemic consequences (Summary Box 2).[58] Free oxygen radicals, produced during the degradation of xanthine by xanthine oxidase (XO), are a major contributor to reperfusion injury. In healthy tissue, 90% of this enzyme is in the dehydrogenase (XD) form. Calcium activated proteases catalyse the conversion of XD to XO in endothelial cells during hypoxia.[59] This sets the stage for generation of reactive oxygen species (ROS). Bulkley[27] suggested that these ROS are intended to be signalling molecules that trigger the reticulo-endothelial system as part of a protective immune response against microbes. (That this

Summary Box 2
Different physiological mechanisms by which ischaemic preconditioning induces 'reperfusion tolerance'. Reperfusion can paradoxically result in worsening of the pre-existing ischaemic injury to not only the entire organ but the organism as a whole. This is partly due to induction of the inflammatory cascade. During reperfusion, ischaemic preconditioning renders protection at the cellular, organ and systemic level.

Mechanism of protection	Level of protection
Reduced production of free oxygen radicals	Cellular, organ, systemic
Maintenance of intra-cellular redox potential	Cellular
Reduction of reperfusion-induced apoptosis	Cellular
Preservation of mitochondrial integrity during reperfusion	Cellular
Diminution of the 'no-reflow' phenomenon	Organ, systemic
Reduction of activated leucocytes and cytokine production	Organ, systemic

conversion occurs more readily in the gut than in the heart[60] may lend some credence to this hypothesis.) However, the return of oxygenated blood produces large amounts of ROS that, in addition to activating leucocytes, also directly contribute to tissue injury by lipid peroxidation of membranes.

Preconditioned rat livers subjected to cold ischaemia had reduced conversion of XD to XO,[61] attenuating oxidant stress during reperfusion. Not only did preconditioned grafts have reduced hepatic damage after liver transplantation, but the recipient rats also suffered less pulmonary injury. Similar moderation of oxidative stress was observed in murine skeletal muscle and intestine.[62,63] In a clinical trial of patients undergoing pulmonectomy, those randomised to IP had significantly higher levels of superoxide dismutase, an enzyme that reduces free radicals, following surgery.[25]

Kupfer cells are the dominant producers of ROS in the liver.[64] IP reduced Kupfer cell activation and leucocyte-endothelial interaction, and preserved mitochondrial redox potential in rat livers.[65] IP also exerts a direct cytoprotective effect from oxidant stress. Preconditioned rat hepatocytes had greater resistance to hydrogen peroxide-mediated cytotoxicity.[66] In separate experiments, hepatocytes were exposed to stimulated Kupfer cells and exogenous hydrogen peroxide infusion (both of which produced abundant ROS). In both studies, preconditioned cells sustained less injury and preserved their intra-cellular thiol status (an indicator of the anti-oxidant potential).

IP can inhibit apoptotic cell death

Apoptosis or 'programmed cell death' is increasingly recognised as an important form of cell death after reperfusion.[67] The mechanism of this type of self-regulation has been remarkably conserved down the eukaryotic line. Caspases, or cysteine aspartyl-specific proteases, are the central components in a complex process of 'ritual suicide'.[68] Members of the Bcl-2 family of proteins, on the other hand, are crucial integrators of survival and death signals, somehow registering diverse forms of intracellular damage and integrating these competing signals to determine whether the cell is 'to be or not to be'.[69] Post-ischaemic tissues produce tumour necrosis factor-α (TNF-α), which is a crucial inducer of apoptosis in the liver.[70]

IP decreased reperfusion-induced hepatic apoptosis by lowering TNF-α levels and modulating the caspase dependent pathway.[71] Apoptosis was attenuated in the preconditioned rat kidney, intestine and brain after I/R injury.[72–74] In a non-randomised study of 24 patients undergoing hepatic resection, the preconditioned group had dramatically lower levels of apoptotic sinusoidal lining cells.[75] In another clinical trial of 16 patients undergoing pulmonectomy, patients randomised to receive IP had significantly increased expression of Bcl-2 in lung tissue exposed to I/R injury and consequently, a reduced apoptosis index.[76] Although the interrelationship between cell death promoters and inhibitors is complex, mitochondria appear to be critical in coordinating these events.

IP preserves mitochondrial integrity during reperfusion

Mitochondria play an important part in energy production through oxidative phosphorylation, intracellular ROS generation, calcium homeostasis and release of apoptosis-inducing factors (AIF).[77] These functions give mitochondria a central role in determining cellular fate after stress,[78] but exactly how they induce cytoprotection is unclear. Mitochondrial K_{ATP} channels regulate apoptosis induced by oxidative

stress.[79] These channels play a key role in regulating calcium[51] and ATP levels,[80] both in the mitochondrial matrix and in the cytoplasm.

Preconditioned rat hearts released less mitochondrial AIF after I/R injury.[81] Activation of mitochondrial K_{ATP} channels after IP was protective in murine, rabbit and canine organs exposed to subsequent I/R.[17,82,83] Another possible mechanism of protection is by inhibition of mitochondrial permeability transition pores (MPTP).[84] Opening of these pores within a few minutes of reperfusion in conditions of increased Ca^{++} and Pi, ATP depletion, oxidative stress and increased matrix pH, can initiate apoptotic cell death.[85] In a Langendorff-perfused rat heart model, IP inhibited initial MPTP opening in hearts reperfused after 30 min of global ischaemia.[86]

Preventing 'no-reflow': IP protects the microcirculation during reperfusion

The no-reflow phenomenon upon reperfusion of ischaemic tissue can inhibit the full return of nutritive perfusion.[87] Up to 30% of the vascular bed can remain non-perfused, further potentiating ischaemic injury. Post-ischaemic endothelial cell swelling, up-regulation of adhesion molecules, reduced nitric oxide (NO) bioavailability and platelet and neutrophil activation are contributary factors.[88]

Preconditioning reduced arteriolar vasospasm and capillary no-reflow in animal models.[33,83] Preconditioned rats exposed to experimental transient ischaemic attack (TIA) had enhanced arachidonic acid metabolism, with increased production of vasodilating eicosanoids, enabling better brain perfusion and reduction of cerebral infarcts by more than 50%. Kharbanda *et al.* demonstrated preservation of endothelial vasoregulatory function in preconditioned human forearm skeletal muscle following tourniquet ischaemia.[89]

NO produced by endothelial cells plays an important physiological role in maintaining the calibre of blood vessels and its reduced release is one of the earliest signs of reperfusion induced endothelial dysfunction.[90] High levels of NO following preconditioning inhibited the effects of vasoconstrictor peptides such as endothelin released after hepatic I/R injury.[91]

Reduced 'stickiness' of post-ischaemic neutrophils in preconditioned tissue has been attributed to diminished I/R-induced P-selectin expression[92] and downregulation of inter-cellular adhesion molecule(I-CAM)-1 release.[93] Intra-vital microscopy demonstrated attenuated leucocyte adhesion and emigration in preconditioned rodent skeletal muscle and feline mesentery compared to controls subjected only to I/R.[94,95]

Systemic Protective Effects of IP: Defence Against Re-circulation of Activated Cells and Toxic Metabolites

Reperfusion allows endothelial and inflammatory cytokines released during hypoxic stress to be circulated to the entire body. For example, TNF-α produced after hepatic I/R injury can lead to adhesion molecule expression and leukocyte infiltration in the pulmonary circulation.[96] Potentially toxic amounts of metabolites accumulated during ischaemia also enter the circulation, e.g. K^+, H^+, lactate and bradykinin.[97] Neutrophils which become activated in the I/R bed have the potential to cause serious injury in the lung and kidney, providing the basis for systemic complications such as the adult respiratory distress syndrome and acute renal failure.[98]

Preconditioned rat liver had significantly reduced production of TNF-α from Kupffer cells.[96] In remote organs this led to reduced P-selectin expression and hence decreased neutrophil adhesion and infiltration. IP before acute limb ischaemia in pigs led to less severe systemic effects, i.e. reduced circulating interleukin-6 levels, primed phagocytes, leukosequestration, pulmonary oedema and respiratory failure.[99] IP of murine mesenteric circulation 24 h prior to induced haemorrhagic shock attenuated the systemic inflammatory response syndrome and reduced bacterial translocation from post-ischaemic intestine.[100,101] Kharbanda *et al.* demonstrated attenuated neutrophil activation in healthy human volunteers who received IP before tourniquet-induced I/R injury to forearm muscle.[89]

Clinical Implications

Pioneering use of ischaemic preconditioning in cardiac surgery by Yellon *et al.* brought this technique into the clinical arena as a surgical adjunct (Table 1).[23,24] This group demonstrated that two 3 min periods of IP prior to coronary artery bypass grafting could reduce myocardial injury (measured by troponin levels) caused by the procedure. During hepatic-resectional surgery, Clavien *et al.* and Nuzzo *et al.* have demonstrated similar protection for residual liver.[26,75,102] The other specialty in which IP has been used operatively is pulmonary surgery. In separate clinical trials, Chen *et al.* applied clamps to the main pulmonary artery prior to pulmonectomy and intra-operative

Table 1. Current evidence for ischaemic preconditioning in human organs

Organ	Level of evidence	References
Heart	Clinical trial	19,24
Liver	Clinical trial	26,75,102
Lung	Clinical trial	25,76
Brain	Epidemiological evidence	104–107
Skeletal Muscle	*In vivo* experimental studies	89,103

chemotherapy for unresectable lung cancer.[25,76] In all of the clinical situations in which IP has been utilised, a reduction in tissue injury and improvement in post-operative organ function has been demonstrated.

Skeletal muscle is well known to be relatively tolerant of ischaemia, but Kharbanda *et al.* have shown that both local and remote IP reduce neutrophil activation and endothelial dysfunction in healthy human volunteers subjected to forearm I/R injury.[89,103] No clinical studies have been carried out in patients with peripheral arterial disease, although epidemiological evidence points towards IP conferring protection following cerebral ischaemia. Patients who experienced transient ischaemic attacks (TIAs) prior to sustaining strokes, were found to have more favourable neurological recovery in three studies from Germany.[104–106] Despite suffering similar perfusion deficits, magnetic resonance imaging (MRI) confirmed that patients who experienced a preceding TIA developed smaller-volume cerebral infarcts.[107]

This evidence raises intriguing possibilities about effecting endogenous ischaemia-protection in humans. For example, perhaps carotid endarterectomy surgery is best carried out within a few days of a TIA? More clinical trials are clearly required to investigate these issues. Indeed, one of the more profound influences of research into the phenomenon of preconditioning is likely to be a deeper understanding of the various pathways by which the insults of ischaemia and reperfusion cause injury.

Conclusions

The phenomenon of ischaemic preconditioning is ubiquitous. Both early and late forms of IP utilise endogenous pathways in various organ systems to render protection from subsequent I/R injury. Preformed agonists such as adenosine, bradykinin, catecholamines and opioids trigger the early protective response, while the delayed preconditioning effect is initiated by genetic re-arrangements resulting in altered cell physiology. Neurogenic pathways are also important in transmitting protective signals. Preconditioned tissues are therefore better equipped than ischaemia-naïve organs to withstand the two-pronged assault of I/R injury.

During ischaemia, IP-treated tissues exhibit reduced energy requirements, conservation of energy substrates, diminished metabolism and tighter regulation of acid–base and ion balance. Upon exposure to reperfusion, preconditioned tissues demonstrate a reduction in oxidative stress, neutrophil activation, cytokine production and apoptosis, and enhanced microcirculatory perfusion. Appreciating the enthralling mechanisms that organisms previously thought to be highly sensitive to hypoxia, including our own genotype, have evolved to deal with this two-pronged assault can allow the early promise of IP to become full clinical reality.

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