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Review Article

MICROVASCULAR DYSFUNCTION INDUCED BY REPERFUSION INJURY AND PROTECTIVE EFFECT OF ISCHEMIC PRECONDITIONING

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Abstract—Hepatic ischemia/reperfusion injury has immediate and deleterious effects on the outcome of patients after liver surgery. The precise mechanisms leading to the damage have not been completely elucidated. However, there is substantial evidence that the generation of oxygen free radicals and disturbances of the hepatic microcirculation are involved in this clinical syndrome. Microcirculatory dysfunction of the liver seems to be mediated by sinusoidal endothelial cell damage and by the imbalance of vasoconstrictor and vasodilator molecules, such as endothelin (ET), reactive oxygen species (ROS), and nitric oxide (NO). This may lead to no-reflow phenomenon with release of proinflammatory cytokines, sinusoidal plugging of neutrophils, oxidative stress, and as an ultimate consequence, hypoxic cell injury and parenchymal failure. An inducible potent endogenous mechanism against ischemia/reperfusion injury has been termed ischemic preconditioning. It has been suggested that preconditioning could inhibit the effects of different mediators involved in the microcirculatory dysfunction, including endothelin, tumor necrosis factor-α, and oxygen free radicals. In this review, we address the mechanisms of liver microcirculatory dysfunction and how ischemic preconditioning could help to provide new surgical and/or pharmacological strategies to protect the liver against reperfusion damage. © 2002 Elsevier Science Inc.

Keywords—Reperfusion injury, Microcirculatory dysfunction, Free radicals, Ischemic preconditioning

INTRODUCTION

What is hepatic microvascular dysfunction?

Hepatic injury secondary to warm ischemia and reperfusion is an important clinical issue. It has been implicated in the pathogenesis of a variety of clinical conditions including trauma, thermal injury, hypovolemic and endotoxin shock, reconstructive vascular surgery, liver transplantation, and liver resectional surgery [1–7].

A considerable number of experimental studies have indicated that ischemia/reperfusion-induced liver injury occurs in a biphasic manner [5,8,9]. Data obtained by several different research groups suggest that in both early and late phases of reperfusion injury, oxidative stress is one of the main pathogenic mechanisms [5,10,11]. The early phase, which occurs between 0.5 and 4 h from the onset of reperfusion, appears to be associated with the generation of reactive oxygen species (ROS) by the activated Kupffer cell and by the reduced

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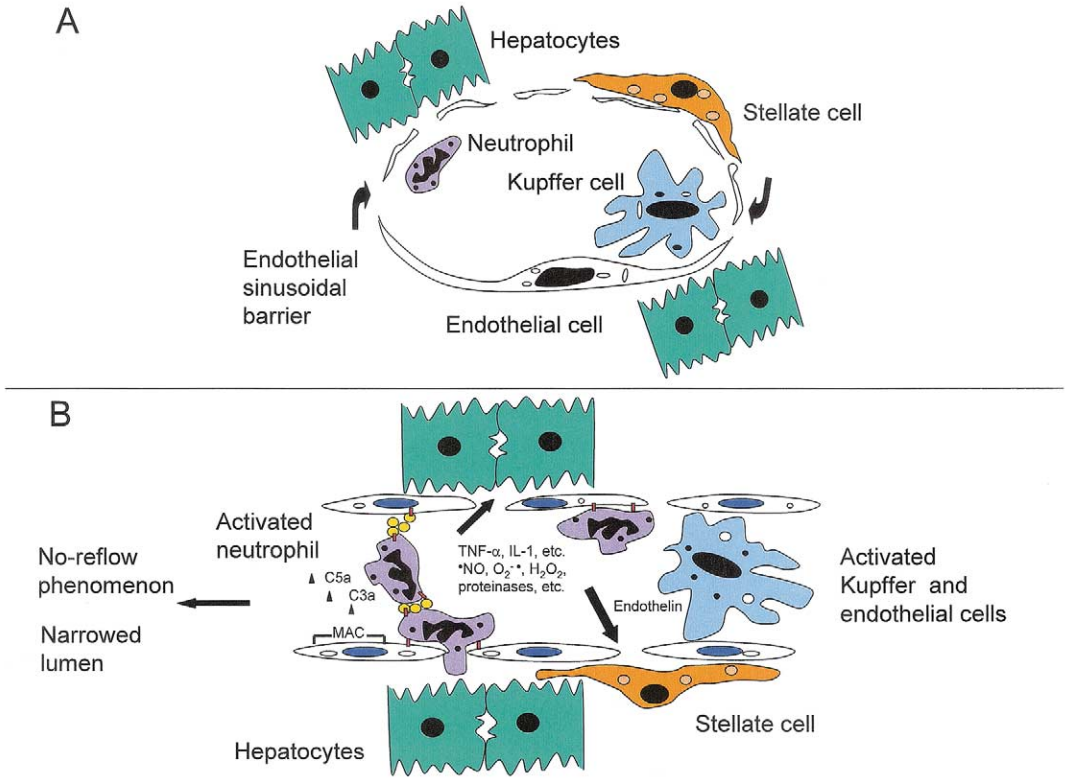

Fig. 1. Hepatic microvasculature. Cell components and their relationships under the basal condition (A) and during the course of microvascular dysfunction (B).

respiratory chains in hepatocytes and endothelial sinusoidal cells [5,8,12–14]. Furthermore, during this initial period, neutrophils are primed and activated. In the late phase of injury, between 6 and 24 h from reperfusion, an evolving inflammatory process occurs, which is mediated by oxidants of extrahepatic cellular origin [8,12,15].

Although the concept of lethal reperfusion damage of parenchymal cells is not universally accepted, it is becoming widely recognized that the hepatic microvasculature, i.e., the sinusoidal space with the lining endothelium, is particularly vulnerable to the deleterious consequences of ischemia and reperfusion [16–19].

CELLULAR AND MOLECULAR EVENTS MAINLY RESPONSIBLE FOR THE HEPATIC MICROVASCULAR DYSFUNCTION

Microvascular dysfunction results from a series of events that involve the interaction of intravascular blood cells, i.e., neutrophils, with nonparenchymal cells, endothelial and Kupffer cells, and are mediated by the synthesis and release of adhesion proteins, cytokines, reactive oxygen species (ROS), nitric oxide (NO), and endothelins (ET) [20–22] (Fig. 1A, B).

It is important to note that many of the ischemia-dependent cell changes, i.e., depletion of energy stores, increase of cellular volume due to altered ion homeostasis, reduced production of certain bioactive molecules (e.g., prostacyclin, nitric oxide), and increased formation of others (e.g., endothelin, thromboxane A2), are exacerbated by reoxygenation. Changes include: increase of swelling, lifting of endothelial cells from the underlying basement membrane, vasoconstriction as a result of a net imbalance between NO and endothelin-1 production, primed-neutrophil entrapment, and platelet aggregation. The entrapment of neutrophils favors homo-type (neutrophil-neutrophil) and hetero-type (neutrophil-platelet) aggregations with further worsening of flow hindrance in the sinusoidal space and prolongation of hypoxic conditions [20,22,23].

In this scenario, Kupffer cells become activated. These resident phagocytes produce ROS and proinflammatory mediators, such as interleukins and TNF-α, enhancing the expression of endothelial cell adhesion molecules, such as ICAM-1 and P-selectin, and priming circulatory neutrophils [24–27]. On the other hand, activated Kupffer cells also polarize and protrude into the sinusoidal lumen, where they come into close contact with circulating blood cells and interfere with the movement of primed and stiffened neutrophils.

Another mechanism involved in the pathogenesis of the microvascular dysfunction is the local complement activation with the formation of chemotactic and cytotoxic products. Whereas C5a contributes to cell damage by means of neutrophil recruitment and activation, platelet accumulation, and Kupffer cell activation, MAC deposition at the cell surface aggravates membrane damage by its direct cytolytic effect [28–33].

Accordingly, the ischemia/reperfusion-induced microvascular dysfunction can be substantially explained by means of: (i) imbalance between the rate of NO production and the release of endothelin-1; (ii) alteration of the ratio between NO and superoxide anion; (iii) degree of neutrophil adhesion and activation.

Imbalance between NO and endothelin levels in the endothelial sinusoidal cells

ET has been reported to provoke contraction of the stellate cells in the sinusoid, via phospholipase C activation and interactivations with membrane ion channels, and subsequently to cause sinusoidal constriction [34–36]. On the other hand, NO, especially that derived from endothelial nitric oxide synthase (eNOS) in sinusoidal endothelial cells, is able to counter the vasoconstriction effects of stellate cell activation, thus limiting perfusion deficits [37,38]. However, in the early stages of reperfusion, the increased concentration of ET reported in both plasma and liver parenchyma, as well as the low concentration of NO, most probably due to the low intracellular levels of NADPH and oxygen after the ischemic period [20], contribute both with a decrease in liver blood flow [39,40].

Imbalance between NO and superoxide anion production by the endothelial sinusoidal cells

Under normal physiological conditions, both NO and superoxide anion are produced by endothelial cells, with NO production exceeding superoxide anion generation by two/three orders of magnitude. This allows NO to (i) effectively scavenge intracellular superoxide anion, (ii) prevent platelet aggregation, and (iii) minimize adhesive interactions between neutrophils and the endothelial cell surface, increasing microvascular permeability [38].

Following ischemia and reperfusion insult, the formation of superoxide anion exceeds NO because of the overproduction of superoxide anion and/or the dramatically reduced bioavailability of NO due to (i) low intracellular levels of cofactors for the synthesis of NO (NADPH and oxygen), (ii) L-arginine breakdown by the large amount of arginase released after ischemic insult, (iii) inhibition of endothelial cell NO synthase activity, and (iv) rapid inactivation of NO by superoxide anion. Thus, under these conditions many, if not all, of NO beneficial physiological actions are lost [38,41–46].

Neutrophil activation

Activation of neutrophils has been implicated in the hepatic microvascular dysfunction and parenchymal damage associated with ischemia/reperfusion [8,9,11,12,15,47,48]. This is based on the observation that preventing neutrophil influx into tissues, either by depleting the number of circulating neutrophils or by preventing neutrophil adhesion, significantly reduces microvascular dysfunction and organ injury in animal models of ischemia/reperfusion damage [49,50].

Activated neutrophils cause endothelial and hepatocellular cell damage through the release of oxidants and proteases. The primary neutrophil oxidant-generating pathway involves NADPH oxidase. Although NADPH oxidase-derived superoxide anion and its dismutation product hydrogen peroxide are the reactive oxygen species primarily formed, the concomitant release of myeloperoxidase results in the formation of hypochlorous acid as the major oxidant. Due to the high levels of reactive oxygen species occurring in the proximity of the sinusoidal endothelial and parenchymal cells, oxidative breakdown of the membrane PUFAs is one of the main molecular mechanisms of liver cell injury during reperfusion after ischemia [51–54]

In addition to the generation of ROS, activated neutrophils release a number of proteases and hydrolytic enzymes by granule exocytosis, which may be directly cytotoxic to liver cells. It has been reported that serine proteases, such as elastase and cathepsine G, are mainly responsible for the injury [55–57].

ISCHEMIC-PRECONDITIONING AS A STRATEGY FOR ATTENUATING HEPATIC MICROVASCULAR DYSFUNCTION RESULTING FROM ISCHEMIA/REPERFUSION INJURY

Over recent years, a surgical strategy known as ischemic-preconditioning has been developed to reduce ischemia/reperfusion injury. By this procedure, an organ is made resistant to the deleterious effects of sustained ischemia and reperfusion by previous exposure to repeated short periods of ischemia, separated by intermittent reperfusion. The protective effect of ischemic-preconditioning was first described in the heart, and has since been demonstrated in many other organs [58–65].

While originally described as an immediate adaptation to brief vascular occlusion, ischemic preconditioning actually affords two types of protection, which differ in time frame and mechanisms. For this reason, distinction is made between early and delayed preconditioning. The effect of early preconditioning develops within min-

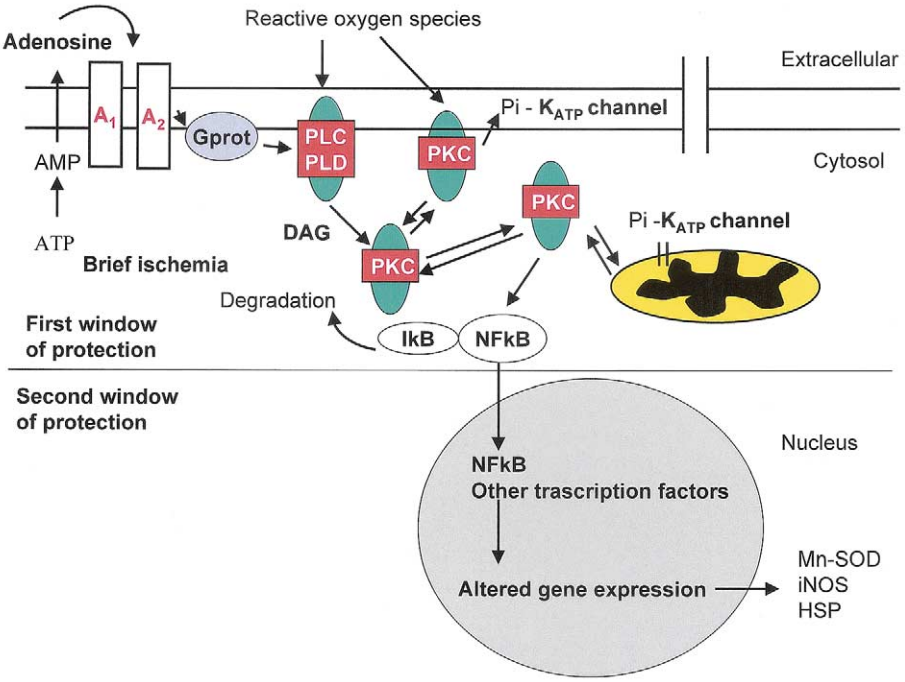

Fig. 2. The molecular basis of ischemic preconditioning. In this diagram are represented the mechanisms thought to be responsible to induce the first and second windows of protection. See text for explication.

utes of reperfusion and lasts for 2 to 3 h. Delayed preconditioning becomes apparent 12–24 h after reperfusion and the effect lasts 2 to 3 d. Further, the protective effects of early preconditioning are independent of protein synthesis, while the effects of delayed preconditioning are dependent on altered gene expression and synthesis of new proteins [66,67].

Protective mechanisms of early ischemic preconditioning

The molecular basis for ischemic preconditioning consists of an ordered series of events. In response to the *triggers* of ischemic preconditioning, a *signal* must be rapidly generated that is *transduced* into an intracellular message and amplified to influence the *effector* mechanism of protection (Fig. 2).

It seems likely that substances released from the ischemic organ act in a paracrine fashion to activate the protective mechanism. Experiments, mainly performed in the heart, suggest a number of substances as potential ligands, including adenosine, bradykinin, catecholamines, reactive oxygen species, opiods, angiotensin II, and NO [68]. With regard to the liver, adenosine has thus far been considered the major player in triggering preconditioning [62,63,65,69,70]. Adenosine is a breakdown product of ATP that is released in large quantities into the extracellular space within seconds of the onset of ischemia.

Adenosine exerts its physiological effect through the interaction with four distinct types of purinergic receptors designated A_1, A_{2a}, A_{2b}, and A_3.

A_1 and A_2 receptors have been chiefly implicated in the ischemic preconditioning of the myocardium and liver, respectively [62,63,65,69,71].

The mechanisms by which adenosine, released during ischemic preconditioning, can limit reperfusion injury are still uncertain. Adenosine has been shown to inhibit neutrophil oxidative metabolism and adhesion to endothelial cells, to increase membrane stability and energy production by promoting glucose transport, and to reduce Ca^{2+} influx through the activation of ATP-dependent K^+ channels [72–75]. However, the role of all biochemical effects in adenosine-afforded protection is yet to be defined.

An increasing mass of data points to the activation of adenosine receptors (I and II) coupled to G proteins as the pathway that initiates the preconditioning response. After activation, the receptor-coupled G protein dissociates and in turn activates a membrane-bound phospholipase (phospholipase C or D). These phospholipases cleave phosphatidylinositol biphosphate into two intracellular second messengers, inositol triphosphate, which releases Ca^{2+} from nonmitochondrial intracellular stores, and diacylglycerol (DAG), which activates specific isoforms of protein kinase C (PKC) [76,77].

PKC is well placed to play a key role in cellular protection. The kinase is known to regulate a number of biological processes such as metabolism, ion transport, and gene expression. PKC is a family of at least 12 serine/threonine kinases. Each protein contains four constant regions responsible for activation and enzymatic action, as well as five variable regions responsible for translocation and substrate binding. PKC isoenzymes can be divided into three broad categories: conventional, novel, and atypical. The conventional PKCs (α, β_I, β_{II}, and γ) require Ca^{2+}, DAG and phospholipids for activation. The novel PKC isoforms (δ, ε, η, θ) lack the calcium-binding region, so these subtypes are not dependent on Ca^{2+} for activation. Activation of isoenzymes of the atypical PKC group (ζ, ι, λ, μ) is also independent from calcium; however, atypical isoenzymes lack the Zn^{2+} finger region required for binding DAG and 3'-phosphoinositides may instead be the activators of these atypical PKCs. Recent reports indicate that the novel PKC isoforms, particularly δ, ε, and η, may be implicated in the protective effect of ischemic preconditioning, whereas the classical isoforms contribute to the detrimental effects of ischemia/reperfusion [78–80]. Any way they are activated, novel PKCs afford phosphorylation of effector molecules, most probably ATP-sensitive K^+ channel (K_{ATP}), 5'-nucleotidase, and cAMP-protein kinase A activation (PKA).

It has been proposed that in the mitochondria potassium channel openers target an ATP-sensitive K^+ channel implicated in the regulation of mitochondrial ion and volume homeostasis. Under normoxic conditions, K^+ channel opening is regulated by ATP levels. Activated PKC isoforms phosphorylate K^+ channels, changing the stoichiometry of the ATP binding sites and favoring their opening to the ATP concentration seen in early ischemia. Opening of these channels leads to preservation of intramitochondrial calcium homeostasis and ATP levels, thus improving viability.

The PKC-dependent translocation of 5'-nucleotidase from the cytosol to the plasma membrane may also play an important role in the protective effects induced by ischemic preconditioning. Indeed, 5'-nucleotidase has been shown to stimulate production of adenosine from AMP. A cAMP increase is thought to reduce postischemic injury by preserving the endothelial barrier function. In addition, it has been reported to block leukocyte adhesion by reducing the expression of adhesion molecules, superoxide radical production, and phagocytic activity of neutrophils [71,77–80].

On the other hand, during the ischemic preconditioning procedure, cellular consumption of ATP leads to accumulation of AMP [81]. The involvement of AMP in the stimulation of AMP-activated protein kinase (AMPK) is well known. Once activated, AMPK responds by phosphorylating multiple downstream substrates, with the purpose of switching on catabolic pathways that generate ATP, while switching off anabolic pathways that consume ATP. Thus, through the cAMP-activated PKA, ischemic preconditioning could promote energy-saving mechanisms [81–83].

Protective mechanisms of delayed ischemic preconditioning

While the triggering and amplification of signals for protection seem to be as in early preconditioning, the effectors of this second window are probably different, but still far from certain. The time frame within which the second window confers protection lends itself to the theory that altered gene expression, with the consequent synthesis of new proteins, is the protection method. However, it is not possible to exclude posttranslational modifications or changes in compartmentalization of existing proteins as the mechanisms involved in the delayed protective response [66–68].

The transcription-dependent synthesis of proteins, which plays a significant role during the second window of protection, remains practically unknown; however, it has been demonstrated that after preconditioning, the PKCε isomer migrates to the nucleus, inducing nuclear transcription factors. On the other hand, a cytoplasmic transcription factor, nuclear transcription factor B (NFκB), has also been implicated in the delayed preconditioning. Activation and nuclear translocation of these transcriptional factors govern the expression of protective genes responsible for late preconditioning [84–87]. Thus, unlike early preconditioning, late preconditioning requires increased synthesis of new proteins. The time frame of the enhanced tolerance to reperfusion damage, which requires 12 to 24 h to develop and lasts for 3–4 d is also consistent with the synthesis and degradation of protective proteins. Several proteins have been proposed as possible effectors, including nitric oxide synthase, cyclooxygenase-2, aldose reductase, antioxidant enzymes (particularly Mn-SOD), and heat shock proteins [67,68] (Fig. 2).

The precise mechanism by which ischemic preconditioning exerts its hepatoprotective effect is still under investigation. Recent studies have demonstrated that ischemic preconditioning induces activation of adenosine A_2 receptors, which in turn, by induction of NO synthesis, confer cytoprotection against postischemic damage [63,65]. Along these lines, it has been shown that inhibition of NO synthesis with *N*-nitro-L-arginine methylester, an L-arginine analogue that competitively blocks NO synthase, abolished the protective effect of preconditioning, whereas administration of a NO donor or L-arginine to ischemic and reperfused livers simulated

the protective effect of preconditioning against hepatic injury [63,65].

Ischemic-preconditioning has also been observed to prevent the TNF-α release from Kupffer cells, as well as preventing generation of hepatic ET, in response to ischemia/reperfusion, thus attenuating the microvascular dysfunction of the liver; the mechanism is probably NO modulated. Moreover, in remote organs like the lung, small intestine, and pancreas, hepatic preconditioning cancelled the increase in P-selectin upregulation, preventing recruitment of circulating neutrophils and thus reducing oxidative stress and microvascular disorders in these organs, after one cycle of hepatic ischemia/reperfusion. Because administration of antibodies against P-selectin or TNF-α prior to ischemia was seen to have the same effect as preconditioning, it has been suggested that the blockade of P-selectin upregulation probably results from inhibition of systemic TNF release from Kupffer cells [88]. Moreover, recently it was reported that ischemic preconditioning was able to increase the hepatic tolerance against reperfusion injury by attenuating the release of ET [89] and the production of ROS, either by blocking the xanthine-oxidase pathway or by preserving the mitochondria structure [90,91]. In this last work it was also reported that well-preserved mitochondria were associated with an attenuated release of cytochrome *c* to the cytoplasm, as well as with a low *index* of caspase-3 activity [91]. Therefore, ischemic preconditioning appears to be a useful strategy to downregulate the molecular pathways involved in liver apoptosis.

Preconditioning also appears to be a useful strategy against the deleterious effects of cold storage-reperfusion injury, since it was observed that ischemic-preconditioned grafts had reduced levels of transaminases and TNF-α, as well as augmented bile flow and improved tissue blood flow [92–94]. Accordingly, in a recent publication it was observed that preconditioned rat livers were more tolerant against cold-storage-reperfusion injury, most probably due to a decreased production of $O_2^{-\bullet}$ by Kupffer cells. Moreover, the authors showed also that ischemia to half the liver confers protection to the other half, providing that heterologous preconditioning could be a tool to protect liver tissue against ischemia-reperfusion injury without imposing ischemia on the target tissue [95].

In liver surgery, the reperfusion injury is one of the most serious insults that affect the organ viability and ultimately postsurgical results. Hepatic ischemic preconditioning here described could be of clinical interest because this surgical approach appears as a potential therapeutic strategy to attenuate the postreperfusion damage.

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ABBREVIATIONS

AMPK—AMP-activated protein kinase
DAG—diacylglycerol
eNOS—endothelial nitric oxide synthase

ET—endothelin
HSP—heat shock proteins
H_2O_2—hydrogen peroxide
iNOS—inducible nitric oxyde synthase
IκB—inhibitor of kappa B
ICAM-1—intercellular adhesion molecule 1
IL-1—interleukin-1
$O_2^{-\bullet}$—superoxide anion
Pi-K_{ATP} channel—phosphorylated ATP-sensitive potassium channel
Mn-SOD—manganase superoxide dismutase
MAC—membrane attack complex
NO—nitric oxide
NF-κB—nuclear factor kappa B
PLC—phospholipase C
PLD—phospholipase D
PKC—protein kinase C
PUFAs—polyunsatured fatty acids
ROS—reactive oxygen species
TNF-α—tumor necrosis factor alpha