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Review article

Pathophysiology of liver ischemia—Reperfusion injury

José Ángel Ildefonso, and Javier Arias-Díaz*

Departamento de Cirugía, Hospital Clínico San Carlos, Facultad de Medicina, Universidad Complutense, Madrid, Spain

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A B S T R A C T

Hepatic ischemia-reperfusion injury is an underlying complication that occurs in clinical conditions such as hepatic resection surgery, liver transplantation and the states of shock. Such injury has classically been attributed to the joint deleterious action of both neutrophils and reactive oxygen species. However, there is increasing evidence that T lymphocytes are also key players in the acute reperfusion injury of diverse organs. They seem to act mainly by promoting the recruitment of inflammatory cells. The purpose of this review is to summarize the molecular and cellular mechanisms that participate in the pathophysiology of liver reperfusion injury.

Fisiopatología de la lesión hepática por isquemia-reperfusión

Palabras clave:
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R E S U M E N

El fenómeno de isquemia-reperfusión subyace a la lesión hepática que acontece en situaciones clínicas tales como la cirugía de resección hepática, el trasplante hepático y los estados de shock. Esta lesión se ha atribuido clásicamente a la acción deletérea conjunta de neutrófilos y especies reactivas de oxígeno. Sin embargo, diversos estudios llevados a cabo en la última década han mostrado un papel cada vez más relevante de los linfocitos T en los fenómenos de isquemia-reperfusión, que activan el reclutamiento de células inflamatorias y causan daño en los tejidos afectados. El objeto de esta revisión es mostrar los mecanismos moleculares y celulares implicados en la fisiopatología de esta lesión.

*Corresponding author.
E-mail address: joseangelildefonso@hotmail.com (J.A. Ildefonso).

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Introduction

Liver reperfusion ischaemia (R-I) causes significant morbidity and mortality in 3 main situations:

1. In major liver resections (accompanied in many cases by complete reduction of blood supply caused by the Pringle manoeuvre).[1,2]
2. In liver transplant, when it is carried out in livers that have undergone a variable period of cold ischaemia, with temperatures between 1–4 °C during graft preservation.[3,4] R-I lesions are closely related to the development of primary graft failure (which is seen in less than 5% of transplants) and to primary graft dysfunction (seen in 10%–30% of cases)[5] due to cell and extracellular matrix lesions,[6] causing a greater incidence of immune rejections and leading to the loss of the transplanted liver.[7,8]
3. When these take place in situations that cause systemic hypoxia or those related to a low blood flow, the result is insufficient liver perfusion. The following entities can be included in this group: Septic, hypovolaemic or cardiogenic shock,[9] in cardiovascular surgery with heart-lung pump,[10] laparoscopic surgery[11,12] and abdominal compartmental syndrome.[13]

The 2 cell types most affected by ischaemic damage are the following: 1) liver cells, that are more sensitive to hot ischaemia, and 2) sinusoidal endothelial cells that are more sensitive to cold ischaemia, so that after 48 hours of liver preservation followed by reperfusion, 40% of endothelial cells are not viable, causing sinousoidal damage with the consequent alterations of the microcirculation, which results in liver lesions and organ dysfunction.[14]

As a consequence of hypoxia, mitochondrial respiratory chain function is altered and mitochondrial enzymes are reduced, causing inhibition of the oxidative phosphorylation process with the subsequent reduction in ATP[15] synthesis.

This reduction of cell ATP causes alterations in transmembrane ion transport. The inhibition of Na^+-K^+ ATP-ase causes the intracellular entry of sodium which accumulates in the cell causing oedema and death.[16] Furthermore, there is an increase of cytosolic calcium, which activates cell membrane phospholipases causing phospholipid degradation and membrane disruption.[17] Therefore, intracellular accumulation of calcium is closely linked to the development of ischaemic damage and is considered of crucial importance in the evolution towards irreversible damage.[18] This increase of calcium plays a significant role in the production of free oxygen radicals (O_2) subsequent to reperfusion, by activation of xanthine reductase.[19]

The damage to liver cells, after any type of ischaemia, is caused mainly during the reperfusion period, when the blood and O_2 supply are re-established.

During the last 20 years there has been constant discussion on the molecular mechanisms of reperfusion lesions. It is well-known that any post ischaemic oxidative stress leads to cellular death by lipid peroxidation. However, lipid peroxidation is quantitatively insufficient to explain severe cellular damage suffered during reperfusion.[20]

Stages of reperfusion liver damage

A complex network of intra- and extrahepatic mechanisms is involved in the pathophysiology of R-I liver damage. Experimental evidence shows that there are 2 distinct phases in reperfusion liver lesions.

1. *Early or acute phase*: during the first 3 to 6 hours post-reperfusion. The main event during this phase is the *activation of Kupffer cells*.[21,22] This activation is carried out due to the prior action of activated components of the complement system, and recruitment and activation of $TCD4^+$ lymphocytes.[23]
2. *Late or subacute phase*: it is characterized by *massive neutrophil infiltration*, which reaches a peak 18-24 hours post-reperfusion. These activated neutrophils release oxygen reactive species (ORS) and proteases, both of which cause oxidative stress and liver cell lesions during this phase of reperfusion damage,[24,25] which is more severe than damage during the early phase. The sequestration of polymorphonuclear cells (PMN) in the liver after R-I is so marked that the acute reduction in their peripheral count has been proposed as an early intraoperative marker of reperfusion damage to liver grafts.[26]

PMN recruitment is due to a complex series of mechanisms that are secondary to ischaemia, both in the liver parenchyma and vessels, which alter the adherence characteristics of PMN. Among these changes there are some that play an important role:

1. The release of chemotactic factors such as ORS by the endothelium or liver cells[27] and the activated PMN themselves,[28] which perpetrate the chemotactism of proinflammatory cells.
2. The production of inflammatory mediators such as tumour necrosis factor (TNF)-alpha,[28-30] interleukins (IL)-1[31] and platelet activating factor[32] by Kupffer cells or the liver cells themselves.[33]
3. Changes in the expression of surface antigens such as intercellular adhesion molecules and class II major histocompatibitility complex (MHC).[34]
4. Damage to the microvascular bed, with phenomena such as “absence of reflux”, which can trap PMN and prolong ischaemia.[35]

On the other hand, it is well-known that PMN play a central role in R-I. Endothelial activation produced by cytokines and ORS increases endothelial adherence of PMN, which increases the local production of proteases and more ORS that alters the microcirculation and increases the damage.[36,37]

Although different studies have been designed with the object of determining the mechanisms of PMN selection after liver R-I, this phenomenon is still not completely understood.

Molecular mechanisms of reperfusion damage

Liver damage induced by tumour necrosis factor-alpha

In the liver TNF-α possesses a dual role, it not only acts as a cell death mediator, but also induces liver cell proliferation and liver regeneration.[38]

In mice, inhibition of the TNF-α signal by anti-TNF serum or by genetic inactivation of the TNF 1 receptor decreases liver damage due to reperfusion and prolongs survival. After R-I several intracellular signal pathways are induced, including the nuclear factor κ-B and the Jun N-terminal Kinase (JNK). It has been shown that if liver production of ORS is blocked due to over-expression of superoxide dismutase-1 the activation of liver JNK can be almost completely prevented as also the corresponding damage, which indicates how important the role of ORS is in JNK activation and R-I lesion.[29,39] Therefore, after liver R-I, the Kupffer cells would generate ORS, which, in turn, would activate JNK and increase the secretion of different chemokines and cytokines including TNF-α. This TNF released by the Kupffer cells may, in turn, activate liver cell TNF receptors to induce JNK and kinases of I κ B and also the production of more ORS. Whereas ORS and JNK cause liver cell death, I κ B kinase activation causes leukocyte liver infiltration.[30]

Recent studies using *ex vivo* perfused livers have shown that, in clinical situations usually associated with hyperproduction of liver TNF-α, the most likely cause of the endogenous release of cytokines is the change of blood flow through the liver vessel bed, rather than reperfusion or ischaemia of these vessels. Furthermore, the increase of TNF-α has, in fact, been associated with a transient improvement of liver function[40] and would indicate that liver dysfunction secondary to reperfusion is due to alternative molecular mechanisms, probably the generation of ORS. Therefore, in spite of the abundance of experimental results in this sense, the true role of TNF-α in liver damage due to R-I has yet to be defined.

Oxygen reactive species in reperfusion-ischaemia liver damage

Although O_2 is the most important molecule to maintain life, it is also the main source of free radicals due to its high availability. Super oxide anion (O2-), hydroxile (OH-) and nitric oxide (NO) are the biologically most relevant radicals. In normal conditions about 1-3% of the O_2 metabolized in the mitochondria becomes super oxide anion.[41]

There are other intermediate products of O_2 and NO metabolism, but they are not radicals since they do not contain unpaired electrons. These intermediate products together with free radicals are known respectively as ORS and nitrogen reactive species (NRS).[42]

The most representative examples of non-radical ORS are hydrogen peroxide and hypochlorous acid. In the case of ORSs the most well-known non-radical is peroxynitrite.[43]

ORS and NRS are partly, at least, the cause of liver damage induced by R-I, with concomitant consumption of endogenous antioxidants.[25,44]

Under conditions of oxidative stress, mitochondria are the main site of production of large amounts of superoxide. This stress may lead, in its final phase, to the formation of mitochondrial permeability transition pores (mPTP) and cause rupture of the mitochondrial membrane and cell death.[45]

The accumulation of activated neutrophils within the liver parenchyma causes tissue damage by production of ORS and the release of proteases by azurophile granules, mainly elastase and catepsin G. Under normal conditions, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is present as inactive subunits located in the cell membrane and cytoplasm. Cell activation causes translocation of the cytosolic subunits to the cell membrane and results in a multimeric complex that has oxidase activity. The active enzyme oxidises NADPH and the released electron reduces molecular O_2, forming a superoxide anion.[46] The reduction of the superoxide anion gives rise to hydrogen peroxide that can diffuse into the interior of the liver cells causing oxidative stress, unless detoxified by glutathione peroxidase that uses glutathione as an electron donor.

On the other hand, myeloperoxidase secreted by neutrophil azurophile granules generates hypochlorous acid, that, after diffusing into the liver cells, can form tyrosine chlorinates, intracellular protein residues or other proteins modified by hypochlorous acid.[36]

In mammals, the xanthine-oxidase (XO) system, very abundant both in the liver and the intestine,[48] is considered one of the major sources of ORS after R-I damage.[47] During the hypoxic phase of R-I, hypoxanthine accumulates due to ATP depletion, since the total level of energy decreases. In a parallel process, hypoxia activates the proteolytic enzymes that convert xanthine-dehydrogenase to XO.[49] Once blood flow is recovered during the reperfusion phase the increasing levels of XO oxidise hypoxanthine to urate. In the course of this molecular reaction, O_2 becomes superoxide radicals.[50]

Although most XO is found in endothelial cells, XO has been found in other parts of the body, a fact which contributes to the formation of ORS at sites distal to that of the initial R-I lesion. The importance of the role of circulating XO is made apparent by the protective effect of free radical scavengers, such as catalase and superoxide dismutase.[51]

Lastly, it is estimated that peroxisomes cause 10%-30% of the total consumption of cell O_2 in the liver and are important sites of ORS production. Both antioxidant systems (catalase/superoxide dismutase enzyme) and ORS producing systems (XO and cytocrome P450 hydroxilation) are located in the peroxisomes,[52,53] and these granules could play a significant role in modulation of the cell redox status.[54]

In liver damage due to R-I, ORS causes the following:

1. Increase of expression of pro-inflammatory genes (TNF-alpha, IL-1, IL-8 or cell adhesion molecules).[46,55]
2. Induction of transcription factors: nuclear kappa-B factor and protein activator-1.[56,57]
3. Direct cell damage through protein oxidation and degradation, lipid peroxidation and DNA damage.[25]

4. Direct induction and regulation of cell death, both apoptotic and necrotic.[58]
5. Inactivation of antiproteases.[25]
6. Induction of stress protective genes in liver cells.[59]
7. Formation of mediators involved in sinusoidal blood flow regulation and liver regeneration.[60]

NO is a radical synthesized by oxidation of L-arginine by nitrogen synthase (NOS). There are 2 isoforms of NOS in the liver: Endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is expressed constitutively and its activity is dependent on calcium and calmodulin.[61] Endothelial cells, liver cells and Kupffer cells synthesize iNOS and their activity is independent of calcium levels. iNOS is not constitutively present in normal liver, but it can be induced by pro-inflammatory mediators such as cytokines and lipopolysacharides or during R-I, shock, trauma or infection and can give rise to the production of large amounts of NO.

In physiological conditions, only eNOS is present in the liver and the low levels of NO produced regulate liver perfusion, prevent thrombus and platelet adhesion, the secretion of inflammatory modulators and the accumulation of PMN cells.[62] NO induces vasodilatation at sinusoidal and presinusoidal level and maintains a balance with vasoconstrictors such as endothelin.[63]

The induction of iNOS may have both protective and toxic effects. The effects depend on the type of aggression, the level and duration of expression of iNOS and the simultaneous production of a superoxide anion.[64]

In liver R-I, the expression of messenger RNA iNOS begins an hour after reperfusion, with an increase of iNOS activity 5 hours post-reperfusion[65]. Different studies have shown that, by iNOS inhibition by means of a specific inhibitor, it was possible to prevent liver R-I damage in the rat's liver.[66,67]

Some of the published results on the effects of iNOS on R-I liver damage are contradictory. Whereas some studies indicate that the expression of iNOS has harmful effects on liver function,[68] other authors indicate that it is beneficial,[69] or even that it has no effect.[70]

Role of lymphocytes in reperfusion-ischaemia liver damage

It has been calculated that the human liver contains about 10^{10} lymphocytes, which are dispersed among liver cells as is seen in the portal tracts. There is evidence of a pathogenic role of these passenger lymphocytes in reperfusion damage after cold ischaemia.[71] However, the role of resident lymphocytes vs. these peripheral lymphocytes is not yet clear, nor the interrelation with these. It is known that, in the rat, circulating lymphocytes have similar properties to human PMN, in the sense that they release proteases and ORS.[72] Early adherence of circulating lymphocytes to liver sinusoids has been seen after reperfusion, possibly these lymphocytes were recruited by an increase in the expression of endothelial adhesion molecules.[73] These lymphocytes are considered to play an important role in the deterioration of liver function after prolonged periods of cold ischaemia[72] Furthermore, rats splenectomised prior to liver ischaemia, show a decrease of PMN infiltration and liver protection against reperfusion effects.[74]

There is plenty of information available on how T and B cells may interact during immune response and it has been observed that B lymphocytes participate in R-I damage in skeletal muscle,[75] intestine[76] and kidney.[77,78] However, in 1997 a contribution was made to the knowledge of the pathophysiology of these lesions by I-R. Zwacka et al[79] described the beginning of the acute phase with T $CD4^+$ lymphocyte activation. This activation triggers a series of events that cause the first stage of damage and leads to the subsequent subacute phase. Using nude, nu/nu, mice, genetically defined as deficient in T lymphocytes, a significant reduction was seen in inflammatory response in comparison with BALB/c lymphocyte T competent mice in which BALB/c tumour generating strains had been injected, and in which lower serum levels of glutamate-pyruvate aminotransferase and a lower percentage of liver cell necrosis and neutrophil infiltration were seen than in the nude mice. In the same study it was possible to see that the protective effect was replicated with depletion of T $CD4^+$ lymphocytes, but this effect was not seen with depletion of T $CD8^+$ lymphocytes. Furthermore, the entry into the liver of T $CD4^+$ lymphocytes after ischaemia, took place during the first hour of reperfusion, which indicates that this type of cell acts as a mediator in the initial processes that activate the subacute inflammatory cascade. This effect was not seen on entry of T $CD8^+$ cells into the liver, which indicated that T $CD4^+$ cells were important mediators in R-I induced inflammatory response.

The authors of the study described[79] presented a hypothesis on the succession of events after liver R-I, which would explain their findings. First, the stimulation of the R-I lesion itself causes direct activation of liver resident T $CD4^+$ lymphocytes. Once activated, lymphocytes may secrete a series of cytokines, such as IFN-γ (gamma interpheron), TNF-β (tumour necrosis factor beta) and GM-CSF (granulocyte-macrophage colony stimulating factors), that both directly and indirectly (by means of secondary cytokines secreted by Kupffer cells) will activate neutrophils to infiltrate the liver. R-I stimulation could directly activate the Kupffer cells in the liver, which would, in turn, activate the T $CD4^+$ cells by means of secreted cytokines and, therefore, there would be reciprocal activation between Kupffer cells and T $CD4^+$ lymphocytes during liver R-I.[80]

Subsequently, different studies have appeared that confirm the importance of T $CD4^+$ lymphocytes in the recruitment of neutrophils in R-I liver damage. Le Moine et al[71] found that liver resident T lymphocytes play a fundamental role in the early events after reperfusion of livers preserved at low temperatures. Anselmo et al[81] observed a reduction in liver damage due to R-I when infiltration of T lymphocytes in hot ischaemia was reduced by prior treatment with FTY720 (2-amino-2[2-(-4-octylphenyl)ethyl]propane-1,3-diol hydrochloride).

Caldwell et al[82] observed that after R-I, T CD4+ lymphocytes were rapidly recruited in the liver, with a maximum peak during the first post-reperfusion hour and the number of T CD4+ lymphocytes remained high after 4 hours, which indicates infiltration or entrapment of these cells in the liver. However, they did not find any evidence of recruitment of T CD8+ lymphocytes at any time during R-I, which had, however, been found by other authors.[79]

Classically T CD4 lymphocytes functionally differentiate into Th1 cells (that produce INF-γ, lymphotoxin and TNF-α) and Th2 (that produce IL-4, IL-5, IL-6, IL-10 and IL-13).[83,84] Subsequently, a new population known as Th17,[85,86] with effects on PMN infiltration, has been added. This subpopulation has been involved especially in defending epithelial surfaces from pathogens, and presumably plays a secondary role in reperfusion liver damage. There is data that indicates that an inflammation pattern with predominance of Th1 increases reperfusion damage, whereas a Th2 pattern shows a protective effect in the presence of Th1.[87] Therefore Th1/Th2 balance is what, in great measure, determines the consequences of R-I.[88]

Conclusion

Liver damage by R-I is a global process that affects several pathways, both molecular and cellular. Although some aspects will have to be reconsidered, inhibition of production of pro-inflammatory cytokines and ORS will continue to be the main strategies in the development of treatments against liver R-I damage.

The delicate balance between lymphoid cells that activate inflammatory processes and other cells capable of inhibiting these, seems to play a decisive role in damage secondary to reperfusion in several organs, including the liver. Studies with the aim of precisely defining these events will be of vital clinical importance, since they will make it possible to design protection strategies that will potentiate and improve current treatment, and they will improve prevention and avoid serious consequences.

Conflict of interests

The authors state they have no conflict of interests.

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