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*Review article*

# Mechanisms of reperfusion injury after warm ischemia of the liver

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**Abstract:** The review highlights recent advances in our understanding of basic mechanisms of reperfusion injury after warm hepatic ischemia. Kupffer cells play a central role as the initial cytotoxic cell type and as a source of many proinflammatory mediators. Subsequently, neutrophils are activated and recruited into the liver. Factors and conditions are outlined that determine whether neutrophils undergo apoptosis without causing damage or migrate out of the sinusoids and attack parenchymal cells. In addition to the inevitable inflammatory response during reperfusion, microcirculatory perfusion failure, due to an imbalance between the actions of vasodilators and vasoconstrictors, also has a serious impact on reperfusion injury. A better understanding of the basic pathophysiology will reveal potential targets for therapeutic interventions and will show us how to avoid risk factors that may aggravate reperfusion injury.

**Key words:** Kuppffer cells, neutrophils, microcirculation, inflammation, reactive oxygen species, adhesion molecules

## Introduction

Warm hepatic ischemia-reperfusion injury occurs during surgical resections, liver transplantation, and hemorrhagic shock. This can lead to local damage in the liver, but also, if severe enough, to systemic organ dysfunction. This review focuses on the basic mechanisms of reperfusion injury as they have been worked out in a variety of experimental models. Although these mechanisms of injury may be discussed separately, it is important to recognize that each component can contribute to a variable degree to the overall injury. The exact contribution can vary greatly depending on the experimental conditions, e.g., length of ischemia or priming of inflammatory cells by endotoxin/sepsis.

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## Kupffer cells

### *Reactive oxygen species*

Interest in mechanisms of reperfusion injury increased dramatically with the hypothesis that xanthine oxidase-derived reactive oxygen species might be responsible for the pathophysiology.[1] The initial, simplistic view was that these oxygen radicals generated during reperfusion cause cell damage by lipid peroxidation (LPO). A large number of investigations using mainly antioxidant interventions appeared to support this hypothesis.[2] However, more detailed mechanistic studies into the role of reactive oxygen-mediated liver injury clearly argued against LPO as a relevant injury mechanism. Some of the experimental evidence against this hypothesis was that no relevant intracellular oxidant stress could be found in the reperfused liver either in vitro[3] or in vivo.[4] Furthermore, hepatocytes can detoxify a tremendous amount of reactive oxygen[5] even when subjected previously to ischemia.[3] Finally, the extent of LPO necessary to cause significant liver cell damage is by far higher than that ever measured during reperfusion.[6] These data do not exclude the possibility that, under certain pathophysiological conditions, there can be an intracellular oxidant stress in hepatocytes. Indeed, after extended periods of hypoxia or ischemia, intracellular reactive oxygen formation can be detected.[7] Under these circumstances xanthine oxidase and mitochondria contribute to the intracellular oxidant stress.[7,8] However, a prerequisite for intracellular reactive oxygen formation is serious ischemic damage to the hepatocyte.[7] These are conditions under which cells probably will not

be able to survive, with or without intracellular oxidant stress.

The lack of intracellular reactive oxygen formation under pathophysiologically relevant conditions has directed interest towards oxidant stress in the hepatic vasculature.[4] Kupffer cells (KC) were rapidly identified as the critical source of reactive oxygen during the initial phase of reperfusion injury in vivo.[9,10] Reducing the capacity of KC to produce reactive oxygen by gadolinium chloride and methyl palmitate effectively protected against reperfusion injury,[9,11] suggesting an important role of vascular oxidant stress in the pathophysiology. Nevertheless, based on the evidence discussed above, it appears unlikely that oxygen radical-induced LPO is the main injury mechanism. Recent experiments using protease inhibitors suggest that proteases also may play a role in the KC-mediated injury phase,[12] indicating potentially synergistic effects of these cytotoxic mediators.[13] Additional effects of reactive oxygen generated by KC could be to modulate the activation of redox-sensitive transcription factors such as nuclear factor (NF)-κB and activator protein-1 (AP)-1 in endothelial cells (EC) and hepatocytes,[14,15] thereby regulating proinflammatory genes.[16]

Although KC can be activated by subjecting them to hypoxia with subsequent reoxygenation,[17,18] reactive oxygen formation under these conditions is only moderate and of short duration.[18] However, the initial activation in vivo is potentiated by the generation of complement factors, leading to prolonged oxidant stress.[19] Since complement activation also affects neutrophils, the complement cascade represents multiple opportunities to effectively attenuate reperfusion injury during both the early and the later phases.[19] Interestingly, complement factors not only activate inflammatory cells but also initiate defense mechanisms in the liver. Although first recognized during ischemia-reperfusion,[4,9] an increased efflux of glutathione (GSH) from hepatocytes with subsequent oxidation in plasma was also shown during endotoxemia.[20] Since there is no cell damage during the early phase of endotoxemia, it was concluded that the increased GSH levels in plasma were not caused by nonspecific leakage of cellular contents but had to be caused by increased release through the GSH transporter. This conclusion was supported by the finding that depletion of complement eliminated this efflux.[20] Thus, there appears to be a defense system in place that is activated by the same mediators, i.e. complement factors, that also stimulate reactive oxygen formation of KC, thereby protecting the hepatic vasculature from damage by KC. This hypothesis was further supported by the demonstration of a protective effect of extracellular GSH in the sinusoids against reperfusion injury.[21] Furthermore, hydrogen peroxide was identified as the relevant oxidant under these conditions.[21]

*Proinflammatory cytokines*

In addition to reactive oxygen and proteases, KC are the major source of cytokines during reperfusion. Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), are generated during reperfusion.[22,23] Eliminating TNF-α by using neutralizing antibodies effectively attenuated the second phase of hepatic reperfusion injury.[22] Since this injury phase is mediated to a large degree by neutrophils,[24] these findings indicate that the dominant effect of these primary cytokines is to activate neutrophils. Indeed, TNF-α and IL-1, together with complement factors ($C_{5a}$) and platelet activating factor (PAF), can induce neutrophil sequestration in the liver.[25–28] Moreover, TNF-α and $C_{5a}$ can upregulate Mac-1 (CD11b/CD18),[28,29] an adhesion molecule that is critical for neutrophil-mediated liver injury.[30,31] TNF-α and IL-1 are also potent activators of transcription factors and a number of proinflammatory genes, e.g., chemokines and adhesion molecules. Kupffer cells have always been considered the primary source of cytokine formation during reperfusion. However, recent data showed that splenectomy can reduce TNF-α formation and protect against reperfusion injury.[32] These findings suggest that either extrahepatic sources have to be considered or that signals from outside the liver may be involved in KC activation in vivo. In line with these observations is a recent publication demonstrating a critical regulatory role of CD4+ T-lymphocytes in generating proinflammatory cytokines during hepatic ischemia-reperfusion injury.[33] Consistent with the hypothesis that the main effect of these cytokines is neutrophil and EC activation, depletion of CD4+ T-lymphocytes reduced neutrophil accumulation during reperfusion and protected against the later, neutrophil-dependent injury phase.[33] Other recent data suggest that platelet activating factor (PAF) could also play a regulatory role in TNF-α and chemokine production.[34] Thus, the activation of KC during reperfusion, especially in terms of cytokine release, is not well understood and needs further investigation.

## Neutrophils

A role of neutrophils in the pathophysiology of warm hepatic ischemia-reperfusion injury was first recognized by demonstrating a beneficial effect of neutropenia.[24] However, evidence quickly accumulated suggesting that the neutrophil-induced injury phase starts hours after the KC-mediated injury. For example, neutropenia only during the first 5–6 h of reperfusion did not protect, despite reducing the number of neutrophils in the tissue by 60%.[24] These results were supported recently with a study showing that only about 30% of all neutrophils in

liver during endotoxemia transmigrate and contribute to the injury.[35] Furthermore, despite the priming of early neutrophils in the liver,[10] neutrophils contribute to the oxidant stress[36] and to mediator formation, e,g., of leukotriene $B_4$[37] and 12-hydroxyeicosatetraenoic acid,[6] only at later times. Moreover, antibodies against TNF-α[22] and chemokines[38] protected only against the secondary injury phase (>6 h). In line with these findings are reports showing that antibodies against adhesion molecules such as Mac-1[31] or intercellular adhesion molecule (ICAM)-1[39] are still beneficial, even if administered during the KC-mediated injury phase. These data together support very strongly the hypothesis that neutrophils contribute to the injury several hours after initiation of reflow. Based on these findings, it is clear that interventions directed against KC have to be initiated before, or at the latest, at the beginning of reflow, whereas interventions against neutrophils can be administered later. The apparently delayed response of neutrophils is caused by the multiple steps required in that mechanism. For a neutrophil to damage a hepatocyte, mediators have to be generated that recruit neutrophils into the liver, adhesion molecules have to be upregulated, and mediators have to be formed that induce neutrophil transmigration and adherence to parenchymal cells.

*Recruitment of neutrophils into the liver vasculature*

As discussed in part above there are a number of acute inflammatory mediators that cause neutrophil accumulation in sinusoids. These include the complement factor $C_{5a}$,[19,29] TNF-α,[25,27,28,40] IL-1,[28] PAF,[26,34] and C-X-C chemokines.[38,41,42] Although some factors may act directly, e.g., $C_{5a}$, others may act by inducing the generation of other mediators.[34] Although accumulated neutrophils were shown to be activated and primed,[19,26,40] there is limited evidence that these leukocytes actually cause relevant injury while sequestered in sinusoids. In vitro experiments using an isolated liver system showed that virtually all isolated neutrophils, even without stimulation, get stuck in the liver vasculature during their first pass through the liver.[43,44] However, these neutrophils do not cause injury under these circumstances.[43,44] Only if these leukocytes are artificially stimulated with phorbol ester can a neutrophil-mediated injury be detected.[43,44] These data demonstrate the safety margin needed to avoid unnecessary organ damage. The mediators discussed above are generated quite frequently in response to trauma, infection, and endotoxemia. Based on the animal data one would expect that in each case neutrophils accumulate in the vasculature of the liver and other organs. However, in most cases these neutrophils will undergo apoptosis and will be removed by KC without harm.[45]

Still, a controversial topic is the question of how neutrophils actually accumulate in the liver. In the general vasculature, various families of adhesion molecules, e.g., selectins, integrins, and the immunoglobulin super family, are responsible for neutrophil rolling and firm adherence in postcapillary venules.[46] Although P-selectin[47] and ICAM-1[48] appear to be relevant for neutrophil adherence in postsinusoidal venules, recent data strongly suggest that, at least during endotoxemia and ischemia-reperfusion, the neutrophils relevant for the injury are actually accumulating in sinusoids.[35] In these capillaries, neutrophil sequestration does not depend on $\beta_2$ integrins[27] or on ICAM-1[27,48] or selectins.[47] These results were obtained at time points when neutrophils do not contribute to the injury. During the neutrophil-mediated injury phase antibodies to adhesion molecules may well protect and reduce the number of cells in the liver. However, since mediators generated during the injury contribute to neutrophil recruitment at later phases, these data do not allow the conclusion that adhesion molecules are actually necessary for neutrophil sequestration in sinusoids. In contrast, mechanical factors appear to be involved in this process.[49] These include cell swelling and injury in sinusoids during an inflammatory response,[49] generation of vasoconstrictors,[50] and reduced deformability of neutrophils exposed to inflammatory mediators.[51]

*Adhesion molecules and transmigration*

If the sinusoidal endothelial cell layer is intact, transmigration of the neutrophil requires adhesion molecules, e.g., ICAM-1[28] and vascular cell adhesion molecule-1 (VCAM-1),[52] but not platelet endothelial cell adhesion molecule-1 (PECAM-1).[53] Even under conditions of ischemia-reperfusion with significant endothelial cell damage, only denudation of the sinusoids would make the parenchymal cell accessible without transmigration. This difference is reflected by observations that antibodies against lymphocyte function associated antigen (LFA-1, CD11a/CD18) only protect against endotoxin-induced neutrophil injury, but not during ischemia-reperfusion.[54] In contrast, antibodies against the adhesion molecule Mac-1 (CD11b/CD18), which is mainly responsible for inducing a cytotoxic response (i.e., degranulation, reactive oxygen formation) protected in both models.[30,31,54] Moreover, antibodies against ICAM-1, the counterreceptor for both $\beta_2$ integrins, are protective in both situations.[28,39,55] Nevertheless, even with damaged but still present endothelial cells, transmigration may still be required.

Adhesion molecule pairs critical for transmigration of neutrophils in the liver include $\beta_2$ integrins/ICAM-1[28] and $\beta_1$ integrins/VCAM-1.[52] ICAM-1 is constitutively expressed on endothelial cells and KC, but not on hepa-

tocytes, and can be upregulated on all liver cell types.[56] Cytokines, i.e., TNF-α, IL-1, and IFN-γ, have been identified as the most potent stimuli.[56] In contrast, VCAM-1 is not expressed on any control liver cells, but can be transcriptionally induced on all endothelial cells and KC.[52] PECAM-1, which is critical for neutrophil transmigration in many organs, is not expressed on sinusoidal endothelial cells and appears to play no role in hepatic neutrophil transmigration.[53]

Although the expression of adhesion molecules is important for neutrophil migration, a gradient of chemotactic factors is necessary for a neutrophil to move. The recent discovery of several families of chemokines provided significant progress in our understanding of the pathophysiology of reperfusion injury. The C-X-C chemokine family, which represents members that are selectively chemotactic for neutrophils, includes IL-8, growth-related oncogene (Gro), cytokine-inducible neutrophil chemoattractant (CINC, rat), epithelial neutrophil activating protein-78 (ENA-78, rat, human), and KC (mouse). It is interesting that hepatocytes can generate large amounts of these C-X-C chemokines[41,57] and therefore can provide a chemotactic gradient for neutrophil transmigration. In support of this hypothesis it was shown that overproduction of CINC in hepatocytes causes hepatic neutrophil sequestration, transmigration, and injury.[42] There is increasing evidence in models of hepatic warm ischemia-reperfusion of enhanced generation of C-X-C chemokines during reperfusion and that antibodies against these mediators attenuate the neutrophil-mediated reperfusion injury.[38,58,59] In addition to chemokines, there appear to be other injury-related mediators (e.g., lipid peroxidation products) that may act as chemoattractants, especially at later time points when proinflammatory mediators are exhausted.[60] Unidentified factors released by parenchymal cells undergoing apoptosis can also induce transmigration.[61]

### *Adherence to parenchymal cells and mechanism of injury*

Experiments with isolated cells suggest that reactive oxygen formation and injury to hepatocytes involves Mac-1 on neutrophils and ICAM-1 on hepatocytes.[62] A more detailed analysis of adherence indicated that both $\beta_2$ integrins participate in the binding to hepatocytes.[63] Surprisingly, only LFA-1 used ICAM-1 as a counterreceptor; Mac-1 appeared to bind to a constitutively expressed ICAM-1-independent ligand. The binding of neutrophils to hepatocytes was dependent on the presence of low levels of IL-8.[63] One of the most controversial topics is the molecular mechanism of neutrophil-mediated killing of hepatocytes. For an in-depth discussion, the reader is referred to a recent review.[64] Briefly, in vivo there is substantial evidence for a role of reactive oxygen as well as proteases in the pathophysiology. However, isolated neutrophils appear to kill hepatocytes predominantly through proteases.[65] One reason for the necessity to generate reactive oxygen in vivo is to inactivate plasma anti-proteases.[65] Other proinflammatory effects of reactive oxygen are to activate redox-sensitive transcription factors such as NF-κB and AP-1, which are involved in the transcriptional regulation of cytokine and chemokine formation and adhesion molecule expression.[14] In addition to these proinflammatory effects, it appears that reactive oxygen can activate intracellular signalling pathways, leading to cell death.[66] These pathways can be blocked by hormones such as atrial natriuretic peptide (ANP) and cyclic guanosine monophosphate (cGMP).[67] Both ANP and cGMP also protect against hepatic reperfusion injury.[68]

## Microcirculatory failure

The development of delayed perfusion failure in the hepatic microcirculation during reperfusion has been well documented.[69–71] In general, the longer the ischemic episode, the more severe the microcirculatory disturbances, which correlate well with cell injury during reperfusion.[70,71] Because of the accumulation of neutrophils in sinusoids, it was initially hypothesized that, similar to the "no-reflow" phenomenon in the heart, sinusoidal plugging was responsible for this problem. However, recent evidence clearly argues against this concept.[43,72] Large numbers of neutrophils stuck in sinusoids did not affect sinusoidal perfusion in an isolated perfused rat liver system.[43] During reperfusion in vivo, most sinusoids that contained neutrophils were still conducting flow.[72] The passage of cells through sinusoids with stagnant neutrophils was delayed but not blocked,[72] suggesting that sinusoidal neutrophil sequestration did not directly cause perfusion failure. The more likely reason for the microcirculatory perfusion failure is an increase in the formation of vasoconstrictors and vasodilators and potentially disease-mediated altered vascular responsiveness, which can lead to local imbalance and, consequently, ischemic injury.[73] Mounting experimental evidence supports this concept. First, removal of the vasodilator nitric oxide (NO) aggravated reperfusion injury, which could be reversed by adding exogenous NO.[74] Moreover, stimulating NO production with L-arginine during reperfusion attenuated portal hypertension and reperfusion injury.[75] Other vasodilators were also shown to be beneficial.[76] On the other hand, the potent vasoconstrictor endothelin-1 (ET-1) is produced during reperfusion.[77] ET-1 can cause sinusoidal constriction and perfusion failure by contraction of Ito cells.[50,78] Consequently, ET-1 antiserum,[79] a mono-

clonal anti-endothelin antibody,[80] or an ET receptor antagonist,[81] improved hepatic microvascular blood flow during reperfusion, attenuated tissue damage, and improved survival. Similarly, a receptor antagonist of the vasoconstrictor thromboxane attenuated reperfusion injury.[82] In addition to the imbalance between vasodilator and vasoconstrictor formation, microcirculatory disturbances can also be triggered by activation of the coagulation cascade and fibrin deposition during reperfusion.[83]

## Clinical implications

A number of different injury mechanisms have been discussed that can contribute to various degrees to the overall pathology of reperfusion injury in the liver and may also cause distant organ damage. Based on these advances in our understanding of the pathophysiology, it is critical to limit microvascular disturbances and to minimize the inevitable inflammatory response during reflow to the point where it does not develop into a self-aggravating process. Reducing the warm ischemia time as much as possible will be beneficial. However, even short periods of ischemia can activate and prime inflammatory cells. As long as these cells are in a hyperactive state, the patient is at risk that during a second insult (e.g. endotoxemia, sepsis, or additional trauma) there is massive aggravation of the inflammatory response and injury. This was demonstrated in an animal model, where a 20-min period of ischemia-reperfusion caused only mild oxidant stress and injury. However, in combination with a low dose of endotoxin, a massive potentiation of oxidant stress and injury was observed.[84] In contrast, if the inflammatory cells are in a hypoactive (refractory) state, the defense mechanisms against bacterial infection are impaired and the patient is also at risk. Because of the documented importance of the inflammatory response for the pathophysiology of reperfusion injury, the state of the immune system before ischemia, as well as additional insults affecting the immune system during reperfusion, will ultimately determine the severity of reperfusion injury.

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