Assessment of Microvascular Integrity in the Isolated Perfused Rat Liver by Contrast-Enhanced MRI. Attenuation of Reperfusion Injury by Conjugated Deferoxamine

Jean-Marie Colet, Erol Cetiner, Bo E. Hedlund, Robert N. Muller

Reperfusion of an ischemic organ can lead to microcirculatory impairment caused, in part, by the generation of reactive free radicals. The iron-catalyzed formation of these deleterious substances can be counteracted by strong metal chelators like deferoxamine. In this study, the protective effect of deferoxamine conjugate was evaluated by assessment of the hepatic microcirculation in the post-ischemic phase. Assessment of the microvasculature was performed by MRI on the isolated perfused rat liver. The restriction of sinusoids subsequent to reperfusion injury was demonstrated by the use of a particulate superparamagnetic contrast agent trapped in the microvasculature. The protective effect of conjugated deferoxamine was evaluated by both MRI and release of alanine aminotransferase. Contrast-enhanced MRI demonstrated a marked impairment of the microcirculation subsequent to the unprotected reperfusion of the ischemic tissue. This injury was attenuated by deferoxamine conjugated to hydroxyethylstarch (HES-DFO).

Key words: contrast-enhanced MRI; perfused liver; deferoxamine.

INTRODUCTION

The protection against injuries induced to organs by a reperfusion subsequent to hypoxic or ischemic periods is of paramount importance in various medical fields. In human medicine, these injuries generally occur as a consequence of severe trauma, heart failure, or an artery thrombosis (1). Reperfusion injury also contributes to organ damage after transplantation (2). In the early phase of ischemia, the prominent alteration of the organ is the formation of protuberances on parenchymal cells. These membranous blebs (3) cross the endothelial fenestrations and invade the sinusoidal lumen (4). Unlike heart or brain, the liver has a high regenerative capacity. Therefore, hepatocytes are able to resorb these blebs if the ischemic insult is not too severe. Although the damage caused by ischemia is less critical for the non-parenchymal cells, Kupffer and endothelial cells do exhibit some morphological changes (5). However, for these cell types, the reperfusion phase can be critically injurious. After reoxygenation, Kupffer and endothelial cells undergo dramatic vacuolization, mitochondrial lysis, and degranulation leading to the rupture of the plasmic membrane (6).

Two phases of liver injury have been identified *in vivo* during the reperfusion period after a hepatic ischemia (7). The initial phase occurs during the first hour and seems to be mainly due to Kupffer cells, which act as a source of reactive oxygen. At the end of this first period, the second phase of injury begins with the accumulation of neutrophils chemoattracted to the liver. Among the metabolites involved during the initial phase, the hydroxyl radical appears to be most deleterious (8). Its formation is catalyzed by transition metals like iron through the Fenton reaction (9). Efficient metal chelators decrease the amount of free iron and therefore prevent formation of the hydroxyl radical. Deferoxamine (DFO) and a macromolecular form of this chelator, hydroxyethylstarch-deferoxamine (HES-DFO) (10), have been proposed as such agents. Their protective effect after ischemia and reperfusion has been nicely demonstrated in the rat liver by fluorescence microscopy (11). However, in the *in vivo* conditions used by the authors, the contribution of neutrophils to the microcirculatory injuries could not be excluded. Therefore, the first objective of the present study was to assess, by magnetic resonance imaging (MRI), the status of the microcirculation of the isolated rat liver after 30 min of normothermic ischemia and 45 min of reperfusion with a neutrophil-free medium.

To better observe the impaired sinusoids by MRI, a colloidal suspension of superparamagnetic microspheres was used. Due to a strong T_2* effect (12), this particulate contrast agent strongly enhances the image contrast. This material is composed of magnetic crystals of about 10-nm diameter embedded in starch to give particles of a mean global diameter of 200 nm. A restriction of the microvasculature could thus provoke the retention of such large particles. To avoid a parallel uptake of the contrast agent by the Kupffer cells, the liver was perfused at 4°C since in these hypothermic conditions, the endocytosis is inhibited (13) and magnetic starch microspheres (MSM, Nycomed, Oslo, Norway) remain in the vascular compartment.

The second objective of this work was to evaluate the protective effect of HES-DFO on microcirculation during the post-ischemic reperfusion period by using MRI and on the release of the hepatic enzyme alanine aminotrans-

MRM 36:753–757 (1996)

From NMR Laboratory, Department of Organic Chemistry, University of Mons-Hainaut, B-7000 Mons, Belgium (J-M.C. E.C. R.N.M.); and Biomedical Frontiers, Inc., Minneapolis, Minnesota (B.E.H.).

Address correspondence to: Robert N. Muller, Ph.D., NMR Laboratory, Department of Organic Chemistry, University of Mons-Hainaut, B-7000 Mons, Belgium.

Received October 4, 1995; revised April 15, 1996; accepted May 10, 1996.

This research was supported by the ARC Program 90/94-142 of the French Community of Belgium.

0740-3194/96 $3.00

ferase (ALT). The combination of decreased retention of MSM and reduced release of ALT would be indicative of a protective role of HES-DFO.

MATERIALS AND METHODS

Liver Perfusion

Livers were isolated from Wistar male rats (±100 g) anesthetized with an intraperitoneal injection of 1.2 g/kg of urethane (U-2500, Sigma, St. Louis, MO) and injected with 500 U.I. of heparin (Novo Nordisk, Bagsvaerd, Denmark) into the vena cava. The anterograde perfusion was achieved by catheterization (Angiocath 18GA ; Becton Dickinson, Sandy, UT) of the portal vein as described in ref. 14 by using 200 ml of a phosphate-free Krebs-Henseleit solution (K-H) saturated with carbogen (95%O_2/5% CO_2). A recirculating flow of 1.5 ml × min^{-1} × g^{-1} liver was induced by a Cole-Parmer peristaltic pump (type 7521–10, Chicago, IL). The pressure in the portal vein was continuously recorded by a pressure transducer (Hewlett-Packard 1290C, Böblumgen, Germany) connected to a Hewlett-Packard monitor (H-P 78353b, Böblumgen, Germany).

A lobar ischemia was induced on the isolated perfused rat liver by clamping the vessels irrigating the right lobe, leaving the other lobes normally perfused during the experiment. The perfusion was restored by removing the microsurgical clamp. The periods of ischemia and reperfusion applied in our protocol were shorter than those used by Drugas *et al.* (11) because of the fragility of the isolated perfused liver. As observed by ^{31}P NMR spectroscopy, the non-ischemic lobes remain metabolically stable for 2 h (15). All these animal experiments fulfill the requirements of the Committee of Ethics of our Institution.

MR Imaging

MRI imaging of the perfused rat liver was performed at 4.7 T on a MSL 200 system (Bruker Spectrospin, Karlsruhe, Germany) by using a 2D FT multislice spin-echo sequence (TR = 1200 ms, TE = 30 ms, matrix size 128 × 128, slice thickness 3 mm, slice interval 1 mm). As the absolute signal intensity can vary from one series of images to another one, the contrast-to-noise ratio (CNR) was used to standardize the data. The image intensity was recorded both in the ischemic and non-ischemic lobes, while the noise was measured outside of the liver image. The intensity of each region of interest was computed and the mean of three values calculated. CNR was calculated as in Eq. [1].

$$\text{CNR} = (\text{ischemic lobe intensity} - \text{non-ischemic lobe intensity})/\text{Noise}$$

Assessment of the microcirculation was achieved with MSM (16), a superparamagnetic agent constituted of magnetite crystals of about 10 nm diameter coated with starch. Considering the submicronic overall size of these magnetic particles (ca 200 nm), the material should be better named nanospheres. The contrast agent was injected in the perfusate at the dose of 20 μmoles of iron/liter. Relative image signal intensities were evaluated before and after the administration of MSM.

Relaxometric Dosage of the Contrast Agent in the Perfusate

The MSM concentration in the perfusion fluid was followed by proton relaxometry on aliquots of 0.3 ml at 0.47 T and 37°C on a spin-analyzer (Minispec PC-120, Bruker, Karlsruhe, Germany) by the Carr-Purcell-Meiboom-Gill technique, as described in ref. 13. Briefly, knowing the transverse relaxivity r_2 in these conditions (177 $s^{-1}mM_{Fe}^{-1}$), the concentration was calculated from the relaxation rate R_2 of the perfusion medium.

Hydroxyethylstarch-deferoxamine (HES-DFO) Treatment

To evaluate the protective effect of HES-DFO on the microvasculature during the post-ischemic reperfusion, one group of livers was perfused with a K-H solution containing 0.165 mmol.$liter^{-1}$ of HES-DFO (Biomedical Frontiers, Inc., Minneapolis, MN). The drug was added as a 2-ml saline solution (16.5 mM) directly to the perfusion fluid 10 min before the end of the ischemia and was allowed to recirculate during the whole duration of reperfusion. The absence of interaction between the MSM and the iron chelator has been checked by the constancy of the relaxation rate of a K-H solution containing DFB-B mesylate (Aldrich, 85H0843) and MSM at a concentration of 10 mM and 1 mM_{Fe}, respectively.

Alanine Aminotransferase (ALT) Activity Measurement

The ALT content was determined in the perfusion fluid of three groups of livers. In the first group, the livers were normally perfused for 2 h (SHAM, n = 4), and the ALT dosage was achieved at the end of the experiment. The second group of livers (DFO(−), n = 4) were normally perfused for 10 min, then submitted to an ischemia for 30 min and, finally, reperfused for 45 min. The livers of the third group (DFO(+), n = 4) were submitted to the latter protocol, but were perfused with 0.165 mM of HES-DFO in K-H solution 5 min before the end of the ischemic period. For the last two groups, two samples of 0.1 ml of perfusate were collected at the onset and at the end of the reperfusion to measure the activity of ALT released in the perfusion fluid by the liver. ALT activity was measured by using a commercial kit (ALT 10 59–10, Sigma, St. Louis, MO) on a Hewlett-Packard 8452A Diode Array Spectrophotometer. The measurement was performed at 340 nm and 37°C.

Sequence of the Experiments

The various phases of the experiments are presented in Fig. 1. The isolated liver was first perfused in normoxic conditions for 10 min in a recirculating mode. The right lobe was then submitted to a total ischemia during 30 min as described above. Finally, the liver was normally reperfused. The temperatures of the liver and of the perfusion medium were maintained at 37°C throughout the experiment except during the administration of MSM, which was done at 4°C to avoid the phagocytosis of the microspheres by Kupffer cells. At the end of the contrast

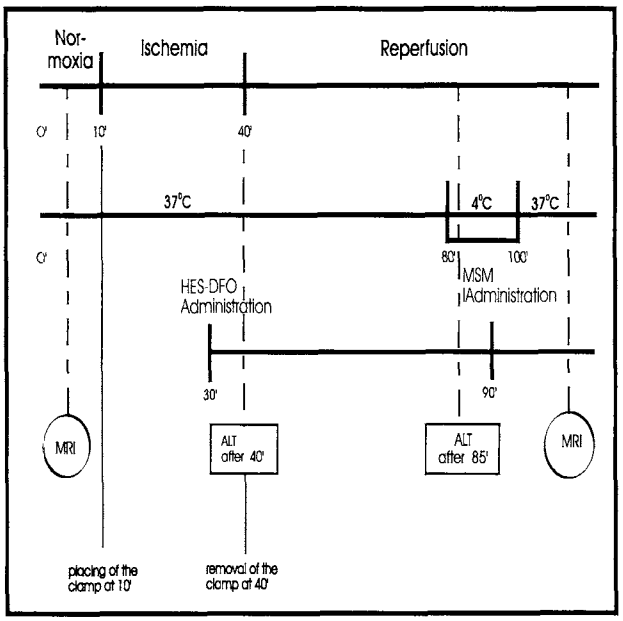

FIG. 1. Sequence of the experiments.

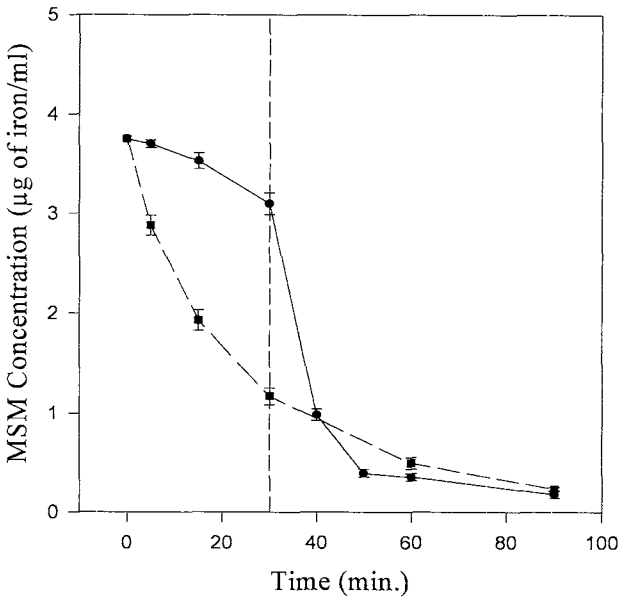

FIG. 2. Influence of temperature on the endocytosis of magnetic starch microspheres by the perfused rat liver. ■■■ = perfusion at 37°C (n = 10) and ●●● = perfusion at 4°C during the first 30 min and then at 37°C (n = 8). The slight uptake at 4°C is due to the fixation of MSM particles to cell receptors, a process still active even at this low temperature .

agent administration period, the washout of the liver was achieved in a single pass perfusion mode to avoid the recycling of the contrast agent.

RESULTS

MSM Endocytosis Blockade

In a preliminary study (13), we investigated the influence of temperature on the endocytosis of MSM by the perfused rat liver and confirmed an earlier report of Fahlvik *et al.* (17). Figure 2 compares the kinetics of the clearance of MSM from the perfusate at 37°C and 4°C, as measured by NMR relaxometry of the perfusion fluid. As it is noticeable, the concentration of MSM rapidly decreases at 37°C because the particles are taken up by the liver, while at 4°C the evolution of the concentration is drastically reduced due to endocytosis impairment. The slight residual uptake is due to the attachment of the material to the membrane receptors (13, 15, 17). The phenomenon of blockade is reversible once the temperature of perfusion is brought back to 37°C. Thus, at 4°C MSM can be considered a vascular contrast agent.

Assessment of Microcirculation by MRI

Figure 3 shows the MR image of a transverse slice of the isolated perfused rat liver collected at 37°C, 45 min after the reperfusion of the ischemic lobe, thus after that MSM has crossed the sinusoids perfused at 4°C. On this transverse slice, the right ischemic lobe has markedly darkened due to the retention of the MSM in the sinusoids. On the contrary, the non-ischemic lobes do not darken since the superparamagnetic particles have been washed out of the sinusoids. The experiment has been successfully reproduced on a series of six animals. Table 1 illustrates the relative intensities measured as described above after the administration of the contrast agent.

Hydroxyethylstarch Deferoxamine Treatment

To evaluate the protective effect of HES-DFO against microvascular injury during the initial phase of reperfusion, the chelator was added to the perfusion medium 5 min before the end of the ischemia. Table 1 shows that no change of the signal intensity is noticeable for the liver treated with HES-DFO, indicating that the magnetic particles have not been retained in the sinusoids in spite of the ischemia/reperfusion sequence. Figure 4 shows the MR image of a transverse slice of the isolated rat liver treated with HES-DFO, acquired after the reperfusion with MSM. This picture clearly rules out any preferential

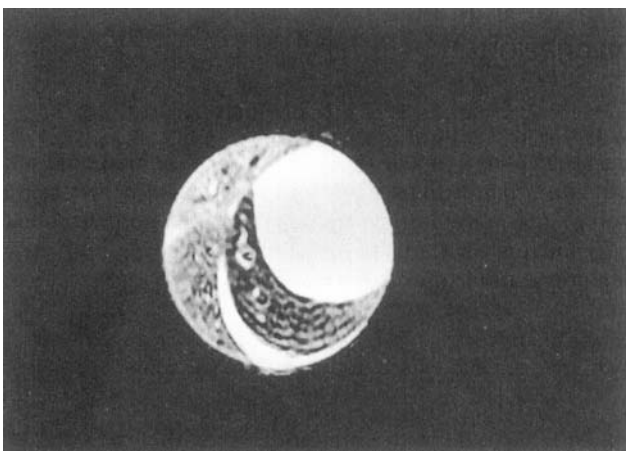

FIG. 3. Transverse MR slice of a perfused rat liver submitted to the ischemia/reperfusion protocol without protection by HES-DFO (TR = 1200 ms/TE = 32 ms). The lower intensity of the ischemic lobe is due to the strong T_2^* effect of the particles trapped in the damaged vessels.

Table 1
CNR Measured at the End of the Reperfusion, after the Administration of the Particulate Contrast Agent at 4°C

Animals	CNR for untreated livers (n = 6)	CNR for HES-DFO-treated livers (n = 6)
1	−7.4	0.6
2	−10.4	0.3
3	−11.2	1.6
4	−10.5	−0.1
5	−10.6	−1.3
6	−11.7	−0.6
M	−10.3	0.1
S.E.M.	0.6	0.4

M, mean; S.E.M., standard error of the mean.

retention of the particulate contrast agent by the ischemic lobe.

Alanine Aminotransferase Activity Measurement

Figure 5 compares the ALT activities of normally perfused livers (SHAM) and ischemic reperfused livers, in the absence or in the presence of HES-DFO. As expected, the control livers show low levels of ALT release. In contrast, these levels rapidly increase in case of ischemia. Although high, the ALT activity in the presence of HES-DFO shows a 40% reduction as compared with the non-treated livers.

DISCUSSION

We hypothesized that MSM particles, the dimension of which is about 200 nm, would be retained in the microvascular circulation occluded after the ischemia/reperfusion sequence. Due to their behavior as a T_2 contrast agent (12), the MSM particles attenuate the MRI signal in the damaged regions. Unfortunately, this type of contrast agent is rapidly taken up by Kupffer cells at 37°C (18) so

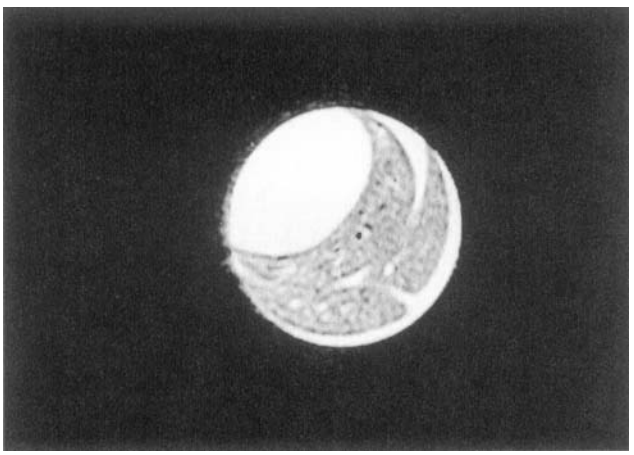

FIG. 4. Transverse MR slice of a perfused rat liver submitted to the ischemia/reperfusion protocol and protected by HES-DFO (TR = 1200 ms/TE = 32 ms). The absence of darkening of the ischemic lobe the intensity of which is similar to the non-ischemic lobe shows that the superparamagnetic particles are not retained in the vessels.

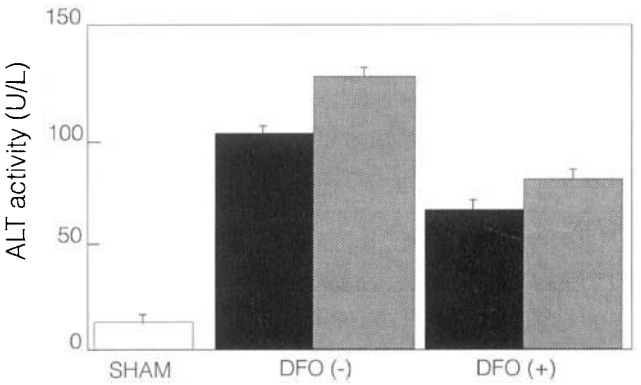

FIG. 5. Alanine aminotransferase (ALT) levels in the perfusion fluid at the onset (■) and at the end (▩) of reperfusion. Values are mean ± SEM for each group. SHAM = normally perfused livers after 2 h; DFO(+) = HES-DFO-treated livers; DFO(−) = untreated livers, $P < 0.05$ at the end of the ischemia, $P < 0.01$ at the end of the reperfusion.

that it would be difficult to determine whether they are retained in the liver because of the endocytosis or because of some mechanical trapping due to a restriction of sinusoids subsequent to cellular damage. However, from previous studies (13, 17), we know that at 4°C MSM can be considered as a vascular contrast agent. If during hypothermic conditions, the MSM particles are retained in the liver, only a mechanical trapping in the microcirculation caused by the ischemia/reperfusion sequence can be implicated. A possible uptake of those particles by the endothelial cells has been ruled out by electron microscopy in a previous work (15).

Experiments conducted under hypothermic conditions demonstrated a significant decrease of the MRI signal intensity of the ischemic part indicating the retention of the MSM in the sinusoids of the lobe exposed to ischemia/reperfusion. This impairment effect is caused by a restriction of the sinusoids causing trapping of the MSM. *In vivo*, three different cellular types, hepatocytes, non-parenchymal cells (Kupffer cells and endothelial cells), and neutrophils (19), can be suspected to be involved in the observed microvascular constriction. In the isolated rat liver perfused with a neutrophil-free solution, the neutrophils can obviously be excluded. Furthermore, since this phenomenon occurs in the initial injury phase of reperfusion as described by Jaeschke *et al.* (7), it is reasonable to think that Kupffer cells are responsible for the observed changes. The involvement of hepatocytes and endothelial cells is less probable since these cells do not elaborate superoxide radicals even in the presence of neutrophils *in vitro* (20).

Transition metals in general, and iron in particular, are known to play an important and toxic role during reoxygenation of an ischemic tissue because of their catalytic activity in the reactions of Fenton and Haber-Weiss leading to the formation of a hydroxyl radical. The hydroxyl radical is extremely harmful because it causes peroxidation of cellular membrane lipids. Iron chelators are thus expected to attenuate injury caused by the hydroxyl radical by suppressing the catalytic potential of trace quantities of iron. In this context, deferoxamine is a remarkable agent with extremely high affinity for iron ($K_d \approx 10^{-30}$) (10). Unfortu-

nately, it has been reported to induce severe hypotension when administered to many animals and humans (21). The coupling of DFO to biocompatible polymers such as hydroxyethylstarch (HES-DFO) has considerably lowered the toxicity while leaving the iron chelating properties unchanged (10). Our results demonstrate that the addition of HES-DFO to the reperfusion fluid delivered to the livers submitted to the ischemia/reperfusion sequence attenuates the retention of the contrast particles in the sinusoids. The protective role of HES-DFO against cellular damages during the reperfusion of the tissue was confirmed by the ALT activity in the perfusion fluid. The levels of this enzyme released by the damaged liver cells were lower in our experimental conditions than those reported by Drugas *et al.* (11) for the liver *in situ*. This difference can be explained by a larger volume of distribution and by a less severe ischemia used in our protocol. From these results, it is clear that hydroxyethylstarch-deferoxamine provides a very efficient protection against cellular damage during the post-ischemic reperfusion period.

CONCLUSIONS

Contrast-enhanced MRI is well suited to evaluate the status of the microcirculation of the isolated perfused rat liver submitted to an ischemia/reperfusion sequence. Since endocytosis is interrupted at 4°C, the magnetic microspheres are retained in damaged sinusoids after the ischemia/reperfusion sequence, leading to a decrease in the MRI signal. The restriction of the sinusoids is likely to result from the deleterious effects of the highly reactive free radicals formed through iron-catalyzed reactions. As shown by changes in the MRI signal and by the release of lower specific enzymes, HES-DFO, a powerful iron chelator, provides a protective effect against the reperfusion damage of the microcirculation during reperfusion subsequent to an hepatic ischemia. The imaging protocol as well as the therapeutic properties of HES-DFO exemplified in this work could be useful in the context of organ transplantation and other situations in which assessment of microcirculatory patency is important.

ACKNOWLEDGMENTS

The authors thank Dr. A.K. Fahlvik of NYCOMED Imaging, Oslo, Norway, for helpful discussions and for providing the MSM; Professor Canioni of the University of Bordeaux, France, and to his co-workers for precious advice regarding the liver perfusion protocol; Professor Y. Van Haverbeke for constant interest and support; and Mrs. Patricia de Francisco for help in preparing the manuscript.

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