Dynamic Evaluation of the Hepatic Uptake and Clearance of Manganese-Based MRI Contrast Agents: A ^{31}P NMR Study on the Isolated and Perfused Rat Liver

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This spectroscopic study compares the mechanisms of the hepatic uptake of manganese chloride ($MnCl_2$) and manganese dipyridoxyl diphosphate (MnDPDP). Alterations of the phosphorus-31 (^{31}P)-NMR spectrum of the intracellular adenosine 5'-triphosphate (ATP) are used to monitor the internalization of manganese by the isolated and perfused rat liver. Mn^{2+} delivered as $MnCl_2$ in the perfusate rapidly enters the hepatocytes, where it strongly interacts with ATP, inducing a broadening of the ^{31}P lines. The inhibition of the process by nifedipine confirms that manganese ions cross the cellular membrane at least partly through Ca^{2+} channels. MnDPDP induces weaker but still significant changes of the ATP spectrum. The inability of pyridoxine to compete for the uptake of manganese confirms that the vitamin B_6 carrier is not involved in the internalization process of the paramagnetic complex. Finally, preincubation of MnDPDP with blood does not increase the extent of the dissociation. The alterations of the ^{31}P spectrum of the liver subsequent to the administration of MnDPDP are attributable to a fraction of free Mn^{2+} released by the chelate and delivered to the hepatocytes.

Index terms: MRI contrast agent • Liver • MnDPDP • $MnCl_2$ • ^{31}P spectroscopy • Perfused organ

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Abbreviations: ATP = adenosine 5'-triphosphate, DPDP = dipyridoxyl diphosphate, DPE = phosphodiesters, EDTA = ethylenediaminetetraacetic acid, K-H = Krebs-Henseleit buffer, LB = line broadening, $MnCl_2$ = manganese chloride, MnDPDP = manganese dipyridoxyl diphosphate, Pi = inorganic phosphate, PME = phosphomonoesters, ROCC = receptor-operated calcium channels, ^{31}P = phosphorus-31.

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MANGANESE, AN ESSENTIAL nutrient metal, is an attractive paramagnetic ion for contrast enhancement in MRI because of its favorable magnetic properties (S=5/2 and long electronic relaxation time) and its specific metabolic pathway. In 1978, manganese chloride ($MnCl_2$) was tested as the first in vivo contrast agent for MRI, exhibiting relaxation enhancement in myocardium and liver (1). As a free ion or bound to proteins, manganese is efficiently taken up by the liver and any excess is excreted into the bile (2). It can also be eliminated by the gastrointestinal mucosa (3). In the portal blood flow, the manganese ion that is mainly bound to a α_2-macroglobulin is subsequently cleared up by the hepatocytes, a mechanism likely to involve the Ca^{2+} channels (4). In the systemic circulation, Mn^{2+} is carried by red blood cells, partially as Mn-porphyrin. Unfortunately, beyond a concentration of 10 μmol/kg, this metal is harmful, with an acute cardiac toxicity originating from the exchange of Ca^{2+} by Mn^{2+} (5). Manganese also induces late hepatotoxicity and neurologic alterations. Chelation is a classical way to lower the toxicity of an ion. Manganese dipyridoxyl diphosphate (MnDPDP) is an anionic chelate of Mn^{2+} associated with four meglumine molecules (6) that has been described as an efficient agent for the detection of focal hepatic lesions (7). The LD_{50} of MnDPDP in the mouse is 5 mmol/kg, compared to .3 mmol/kg for $MnCl_2$ (8). No serious adverse effect was demonstrated on patients during clinical phases II and III (9). The most common but reversible side effect is flushing. Surprisingly, it has also been shown that MnDPDP enhances the signal of the pancreas (10–12). The similarity of the kinetics of the signal enhancement arising from both liver and pancreas suggests a common mechanism of uptake. Based on the structural analogies between vitamin B_6 and dipyridoxyl diphosphate (DPDP) (6), early works have involved the carrier of the latter to explain the internalization. Kinetics studies have, however, revealed a dissociation of MnDPDP leading to a release of free Mn^{2+} in a time shorter than the plasmatic clearance (13). The biodistribution of MnDPDP is nevertheless clearly different from that of $MnCl_2$. In the rat, $^{54}MnDPDP$ showed 25% of fecal excretion of the paramagnetic ion within 5 days (8), whereas in the same conditions, $^{54}MnCl_2$ is characterized

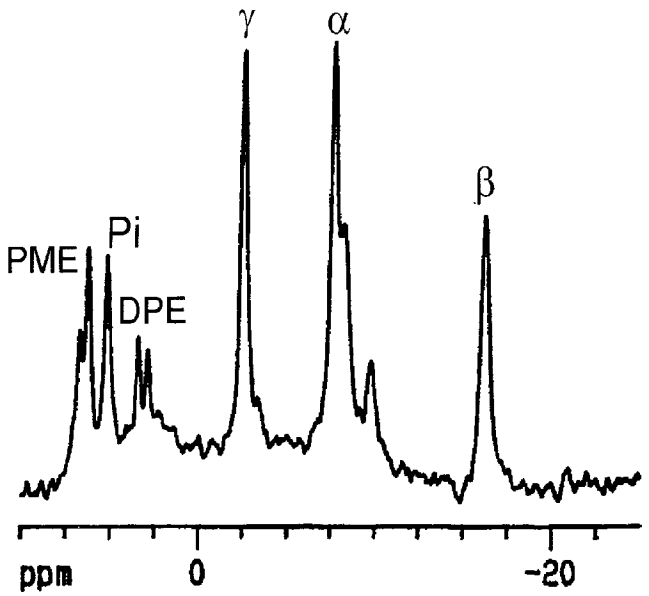

Figure 1. ^{31}P spectrum of isolated rat liver perfused with Krebs-Henseleit buffer. α = α−ATP, β = β-ATP, γ = γ-ATP.

by 50% of fecal excretion and 50% of body retention but no renal excretion. As shown by this pharmacokinetics study by Grant et al (8), clearly confirming earlier reports (14,15), MnDPDP dissociates in protein-containing media and is quickly degraded in blood (16). The remarkable MRI properties of the agent are thus likely to arise from the hepatic and pancreatic uptake of released Mn^{2+}. Despite its tendency to dissociation, DPDP is, however, able to detoxify Mn^{2+} as demonstrated by its lower LD_{50}. Among the suspected factors of degradation, the action of phosphatases located in the blood or inserted in the endothelium as well as the transmetallation with other ions like zinc can be proposed.

The influence of manganese ions on the ^{31}P NMR spectrum of the phosphate groups of acid adenosine 5'-triphosphate (ATP) was studied in solution by Sternlicht et al (17). At the physiologic temperature of 37°C, manganese induces a broadening and a subsequent lowering of the resonance peaks of ATP phosphates. Brown et al (18) have explained this evolution by a "slow exchange" model in which the paramagnetic enhancement of the transverse relaxation is essentially governed by a scalar interaction controlled by the exchange rate.

In the present work, ^{31}P-NMR spectroscopy was therefore used to monitor the hepatocytic uptake of manganese administered either as $MnCl_2$ or as MnDPDP to the isolated and perfused rat liver.

• MATERIALS AND METHODS

Liver Perfusion

Forty-five male Wistar rats weighing 150 g each were used for these experiments, fulfilling the requirements of the Committee of Ethics of our institution. The rats were anesthetized with .2 g of urethane (U-2500, Sigma, St. Louis, MO) and injected with 500 IU of heparin (Novo Nordisk, Bagsvaerd, Denmark) into the vena cava. The livers were isolated as described by Delmas-Beauvieux et al (19) and perfused at a constant flow rate (3–4 ml/g^{-1} of liver/min^{-1}) through the portal vein with the Krebs-Henseleit buffer (K-H) kept at 37°C. This aqueous solution contains (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (3), $MgSO_4$ (1.2), $NaHCO_3$ (25), D-glucose (5.5), and pyruvic acid (5.5). A one-through perfusion was performed to avoid the accumulation of bile products in the fluid.

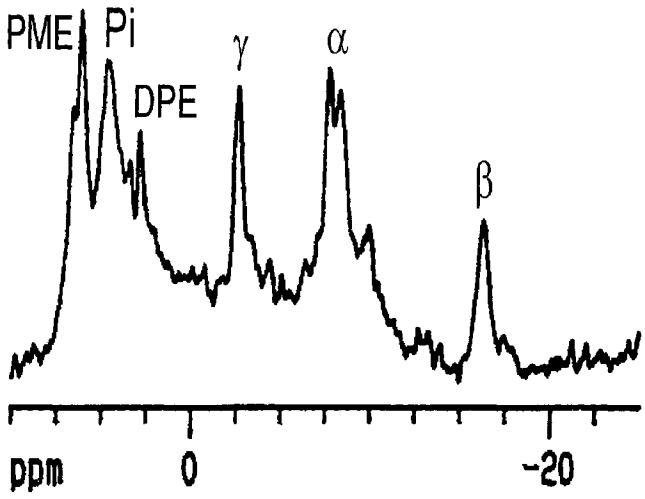

Figure 2. ^{31}P spectrum of isolated rat liver perfused with 10 μM of $MnCl_2$ according to protocol 1.

NMR Measurements

The livers inserted in a 25-mm Pyrex tube were placed in the 7.05-T magnet of an AMX-300 spectrometer (Bruker, Karlsruhe, Germany). The ^{31}P signal was detected at 121.5 MHz, and 100 free induction decays were acquired with a 60° pulse angle and a repetition delay of 3 sec, allowing the total recovery of the excited spins to their thermodynamic equilibrium. In these conditions, each spectrum is collected in 5 minutes and only the spectral modifications due to transverse relaxation or metabolite concentration are detectable. A line broadening (LB) of 20 Hz was applied before Fourier transform. This value was subsequently subtracted from the linewidth measured on the spectrum. To evaluate the interaction of manganese with ATP, the peak of β-phosphate was characterized by the three following parameters: the height (H), the width at half-height ($\Delta\nu_{1/2}$) and the surface area (SA), using the standard Bruker integration routine.

Experimental Protocols

Protocol 1.

The livers were perfused with K-H for 15 minutes and three control spectra were recorded. After this initial stabilization period, the perfusate was delivered for 20 minutes (four spectra) by a second reservoir containing 1 liter of K-H and 10 μmol of either $MnCl_2$ (Riedel-de Haën, 5051, Seelze, Germany) (n = 8) or MnDPDP (Salutar, #BCO 3068.1, Sunnyvale, CA; 125 mM stock solution analytically checked for absence of free Mn^{2+} ions immediately before use) (n = 5). Finally, the livers were rinsed with fresh K-H for 145 minutes (29 spectra). In the control group (n = 5), the livers were continuously perfused with K-H. The three characteristic parameters H, $\Delta\nu_{1/2}$, and SA were measured for each spectrum.

Protocol 2.

Antagonists were used to inhibit the hepatic uptake of $MnCl_2$ and MnDPDP. Nifedipine (N-7634, SIGMA, St. Louis, MO), a calcium-channel blocker, was tested on 11

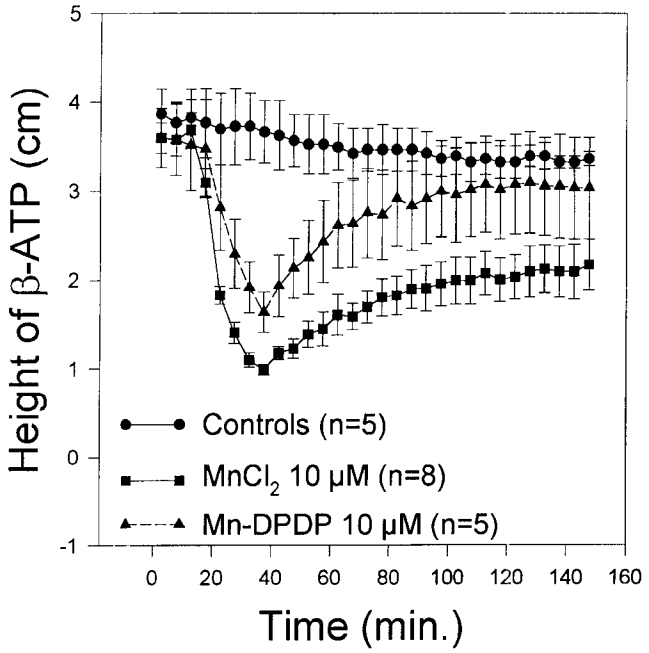

Figure 3. Evolution of the height (H) of the β-ATP peak of isolated livers perfused with K-H (control group), 10 μM of $MnCl_2$, or 10 μM of MnDPDP according to protocol 1.

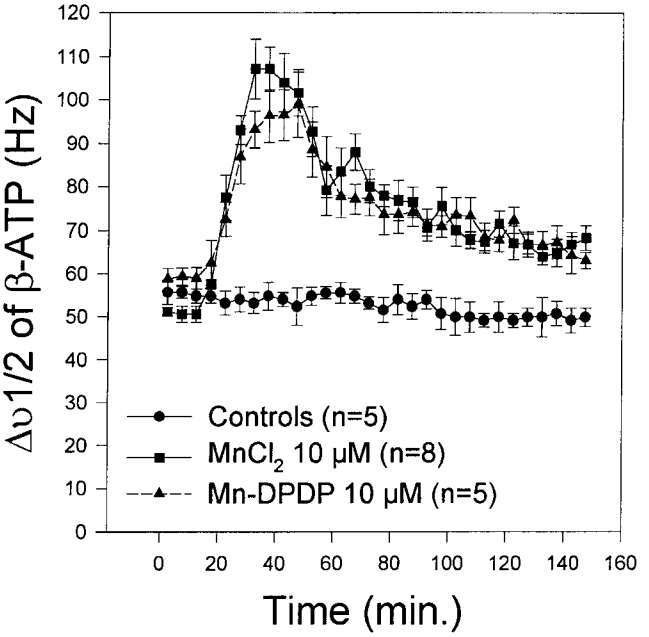

Figure 4. Evolution of the width at half-height ($\Delta\nu_{1/2}$) of the β-ATP peak of isolated livers perfused with K-H (control group), 10 μM of $MnCl_2$, or 10 μM of MnDPDP according to protocol 1.

livers as antagonist of the entry of free Mn^{2+} in the hepatocytes. The uptake of MnDPDP was competed by either DPDP alone (n = 4) or vitamin B_6 (P-5669, SIGMA, St. Louis, MO) (n = 4). The livers were perfused for 20 minutes with K-H before the passage of a solution containing nifedipine (10, 100, or 300 μM solubilized in dimethylsulfoxide), DPDP (100 μM), or vitamin B_6 (100 μM). To allow for the saturation of the suspected channels or carriers, the perfusion with the competitors was started 5 minutes before the administration of $MnCl_2$ or MnDPDP (10 μM), which, as in protocol 1, lasted 20 minutes (four spectra). Finally, the livers were rinsed for 115 minutes (23 spectra).

Protocol 3.

This set of experiments was run to evaluate the influence of blood on the dechelation of MnDPDP; 100 μmol of MnDPDP was thus added to the heparinized blood collected from the liver donor and maintained at 37°C for 20 minutes. The livers (n = 4) were perfused for 15 minutes (three spectra) with K-H and then with K-H supplemented with the MnDPDP blood mixture (final MnDPDP concentration = 10 μM) for 20 minutes (four spectra). The experiment was completed by a 115-minute washout period (23 spectra).

Protocol 4.

To evaluate the contribution of the free manganese released from the solution of MnDPDP, four livers were perfused with a solution of K-H containing 2 μM of ethylenediaminetetraacetic acid (EDTA; Aldrich, 25,235-2, Steinheim, Germany) and 10 μM od MnDPDP. This strong chelator is expected to reduce the availability of free manganese ion.

Statistical Evaluation

The results were expressed as means ± SEM. The variations between groups were compared using the bilateral Student t test and the Fisher test for variances. Significance was assumed when P was found to be less than .05 (significant for $P \leq .05$ or highly significant for $P \leq .01$).

• RESULTS

Protocol 1.

Figures 1 and 2 show the ^{31}P NMR spectra of the isolated rat liver before and after the perfusion of 10 μM of $MnCl_2$, respectively. The major peaks are assigned to the α-, β- and γ-phosphates of ATP, inorganic phosphate (Pi), phosphodiesters (DPE), and phosphomonoesters (PME).

The presence of $MnCl_2$ (10 μM) in the perfusion fluid induced a fast and significant alteration of the peaks corresponding to the three phosphates of ATP. The decreased height (H) and the strong broadening ($\Delta\nu_{1/2}$) are evidence of a close interaction between ATP and Mn^{2+} after Mn^{2+}'s incorporation in the intracellular compartment. The decreased area (SA) is concomitant with an increase of the lines corresponding to Pi and DPE. The time evolutions of the β-ATP peak parameters observed after perfusion with $MnCl_2$ and MnDPDP are shown in Figures 3, 4 and 5.

The influence of manganese on the ^{31}P spectrum significantly differed when the ion was administered as MnDPDP. Although the time course of the linewidth evolution is comparable to the situation encountered with $MnCl_2$, the transient decrease of H and SA was lower and the control levels are almost completely recovered after the washout period. This was clearly not observed with $MnCl_2$, the biliary excretion of which was not complete, as shown by the partial recovery of H, $\Delta\nu_{1/2}$, and SA.

Protocol 2.

The evolution of H for β-ATP of livers perfused with nifedipine (10 and 100 μM) and $MnCl_2$ (10 μM) is shown in Figure 6. The results of the statistical analysis indicate that a nifedipine dose of 10 μM does not significantly reduce the hepatic uptake of $MnCl_2$, whereas an important inhibition appears at a dose of 100 μM. To demonstrate this antagonistic effect more clearly, the perfusion was

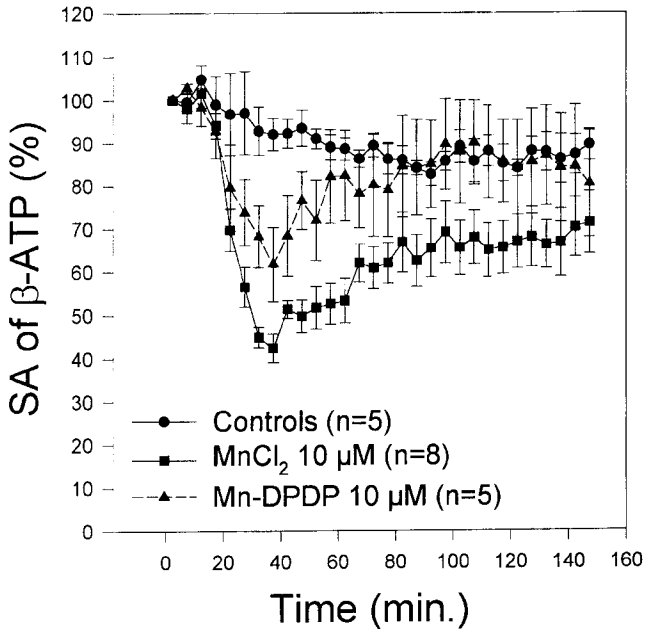

Figure 5. Evolution of the surface area (SA) of the β-ATP peak of isolated livers perfused with K-H (control group), 10 µM of $MnCl_2$, or 10 µM of MnDPDP according to protocol 1.

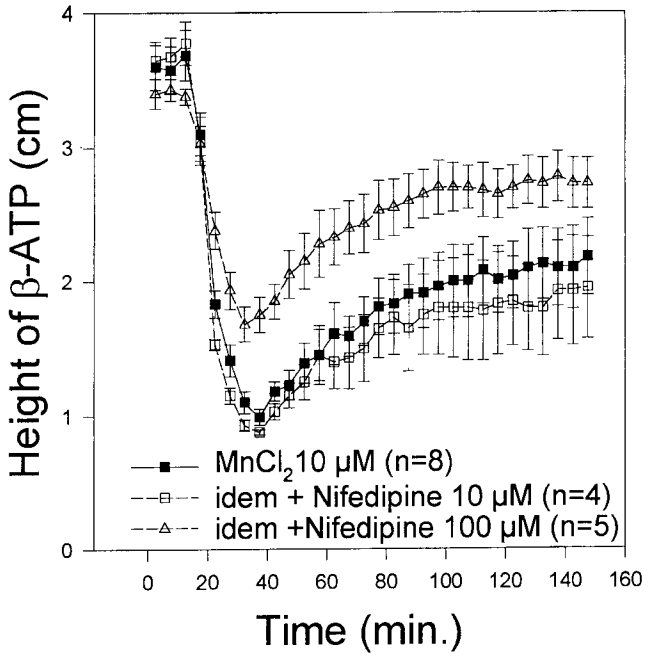

Figure 6. Influence of nifedipine on the hepatic uptake of $MnCl_2$ according to protocol 2. Evolution of the height (H) of β-ATP.

tentatively run with nifedipine at a dose of 300 µM. Unfortunately, nifedipine that is not completely solubilized at this high concentration induced a cellular ischemia consecutive to sinusoidal microthrombi, as demonstrated by a large increase of the perfusion pressure and the subsequent alteration of the cellular physiology, resulting in a quasi total loss of the intracellular ATP. These perturbations were not noticed at the lower doses of 10 and 100 µM, for which the compound is completely solubilized.

The effects of DPDP and vitamin B_6 on the hepatic uptake are illustrated in Figures 7 and 8. The preperfusion of the DPDP ligand completely suppressed the paramagnetic effect induced on the transverse relaxation of β-ATP. By comparison, no influence of vitamin B_6 on the uptake phase was observed. The slightly stronger decrease and the lower recovery levels of H (Fig. 7) and SA (data not shown) during the clearance phase could be related to a weak toxic effect of pyridoxine at this concentration.

Protocol 3.

The preincubation of MnDPDP for 20 minutes in the blood of the liver donor did not significantly modify the evolution of the β-ATP transverse relaxation. Although the peak height H seemed to be more affected by this incubation (Fig. 9), the two other parameters, $\Delta\nu_{1/2}$ (Fig. 10) and SA (data not shown), followed the evolutions observed without this operation.

Protocol 4.

Finally, the addition of a small amount of EDTA (20% of the MnDPDP concentration) to the perfusion fluid drastically reduced the spectral alterations (Figs. 9 and 10).

• DISCUSSION

Studies on the influence of manganese on the ^{31}P relaxation of ATP in solution have been reported in the literature (17,18). They have shown that for a low $[Mn^{2+}]$/[ATP] ratio, the transverse relaxation of the phosphate groups is essentially controlled by the scalar contribution of the interaction with manganese, whereas the longitudinal relaxation is directed by a dipolar contribution of lesser spectral consequence. In aqueous solution, the broadening of the α-, β- and γ-phosphate peaks is similar, demonstrating a simultaneous binding of Mn^{2+} to the three phosphates of ATP.

We have followed in quasi real time the hepatic internalization of the manganese ion delivered as $MnCl_2$ or MnDPDP through its paramagnetic broadening effect on the cellular ATP ^{31}P-NMR spectra. The addition of $MnCl_2$ to the perfusion fluid delivered to the isolated rat liver rapidly induced a broadening of the peaks. The effect, followed on the β-ATP resonance, indicates a close interaction between Mn^{2+} and ATP. In addition, the area of the peak decreased. This fact, which is obviously not observed in solution, can be attributed to a hepatotoxic effect of manganese because the decrease in ATP content is accompanied by a parallel increase of the Pi level. The hypothesis of longitudinal relaxation enhancement that could induce such an increase of the height of the Pi peak can be rejected because the spectra were acquired in fully relaxed conditions (θ = 60°, TR = 3 seconds, and T1 Pi = .6 second as measured under our experimental conditions). The origin of the toxicity of manganese on hepatocytes is unclear but could arise from the competition of Mn^{2+} for Ca^{2+} and/or Mg^{2+} in the context of the respective ion-dependent functions.

The extent — but not the time course — of the spectral changes was smaller when manganese was delivered as MnDPDP. A comparison between the maximal $\Delta\nu_{1/2}$ measured on the organs might be speculative because the measurement of linewidths larger than 120 Hz (LB = 20 Hz included) becomes uncertain. Nevertheless, this value of 120 Hz, which has therefore to be considered as a threshold, was always reached (and probably exceeded) in the case of the livers perfused with $MnCl_2$ but was

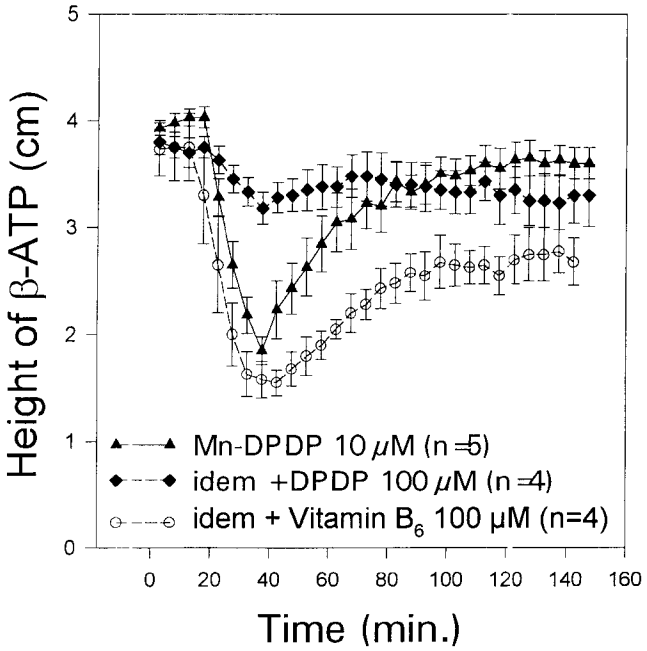

Figure 7. Influence of DPDP and vitamin B_6 on the hepatic uptake of MnDPDP according to protocol 2. Evolution of the height (H) of the β-ATP peak.

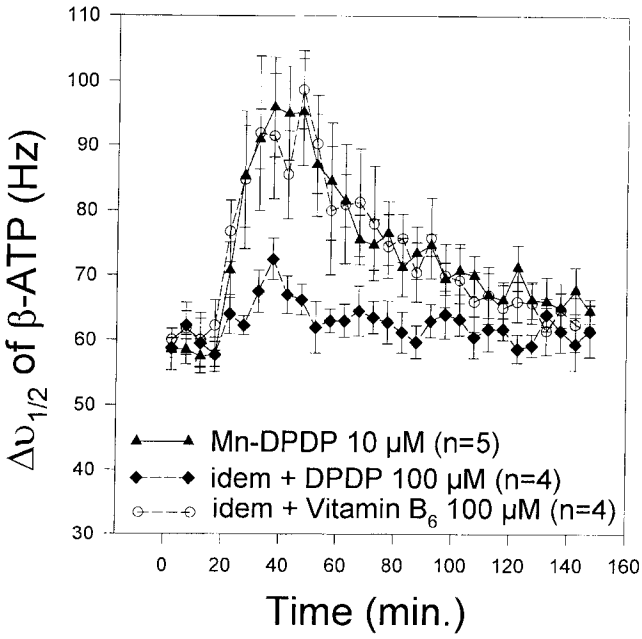

Figure 8. Influence of DPDP and vitamin B_6 on the hepatic uptake of MnDPDP according to protocol 2. Evolution of the width at half height ($\Delta\nu_{1/2}$) of the β-ATP peak.

never observed with MnDPDP. The possible underestimation of $\Delta\nu_{1/2}$ for $MnCl_2$, therefore, biases the comparison with MnDPDP. Another major difference clearly lies in the preservation of a higher ATP content during the perfusion of the paramagnetic chelate as compared to the situation observed with $MnCl_2$. This suggests a less extensive interaction of manganese with cellular components, a phenomenon for which several hypotheses must be considered. First, MnDPDP could be incorporated as such in the intracellular compartment, where it would remain undissociated or partly dissociated. The presence of the ligand would, in this case, reduce the amount of Mn^{2-} binding to ATP. According to a second hypothesis, a fraction of the manganese would be released as a free ion from the complex either in the perfusion fluid or at the level of the plasma membrane and could enter the cytosol where the interaction with ATP would develop. In numerous nonexcitable cell types, Mn^{2+} indeed competes with Ca^{2-} for incorporation through the receptor-operated calcium channels (ROCCs) (20). Antagonists of voltage-dependent calcium channels are known to inhibit ROCCs: a 50% reduction of Ca^{2-} influx through the hepatocyte membranes has been shown for nifedipine at doses ranging from 50 to 100 μM (21). In the present work, a dose of 100 μM of nifedipine markedly reduced the effect of manganese on the relaxation of β-ATP, confirming the use of ROCCs by Mn^{2-}.

The inhibition of the hepatic uptake of MnDPDP by the preperfusion of the livers with the DPDP ligand alone demonstrates either that the mechanism of uptake is saturable or that free Mn^{2-} ion is trapped by the excess of DPDP. The structural analogy between DPDP and pyridoxine has indeed led to the early suggestion that MnDPDP could use the vitamin B_6 carrier to enter the hepatocytes. In our experimental conditions, however, the addition of pyridoxine to the perfusion fluid did not counteract the influence of MnDPDP on the broadening of the β-ATP peak. This could indicate a binding of MnDPDP to another carrier or, more likely, a dissociation of the complex before the ion uptake. The presence of 2 μM of EDTA, a strong manganese chelator able to trap any free ion, strongly reduced the effect of MnDPDP on the relaxation of β-ATP. This again confirms the release of free manganese ions from MnDPDP in the perfusion fluid. Like EDTA, the DPDP in excess reduced the amount of free manganese ion released by the original complex. At the pH of oxygenated K-H (7.4), the phosphate groups of DPDP are negatively charged and thus behave as powerful chelators of divalent cations.

Two recent studies have appeared on the metabolism of MnDPDP in humans (16,22). The first study demonstrated a dephosphorylation of the complex and a transmetallation with zinc 20 minutes after infusion or 2 minutes after bolus injection (16). The second study (22) revealed the presence of five metabolites in addition to the parent compound MnDPDP in plasma. At 1 hour after administration, zinc dipyridoxyl ethylenediamine was the most abundant metabolite, indicating a transmetallation and a dephosphorylation of MnDPDP. In our experiments, the incubation of MnDPDP in the blood of the liver donor did not significantly modify the behavior of MnDPDP, at least concerning its effect on the relaxation of ATP phosphates. This seems to preclude the sole intervention of phosphatases in the context of our experimental protocol. To definitively exclude the contribution of endothelial phosphatases, the same experiments were performed and the same results were obtained on isolated and perifused hepatocytes. In this protocol, the absence of endothelium did not prevent the release of Mn^{2-} from MnDPDP (23). Finally, we did not observe a major transmetallation with zinc during the preincubation of MnDPDP with blood. This difference with the in vivo situation (22), where the fraction of plasma zinc bound to dipyridoxyl ethylenediamine is rapidly restored from tissues, can be attributed to the large excess of complex relative to the minute amount of zinc available in the small volume of blood used in our protocol. Complementary experiments have been performed that indicate the involvement of magnesium and calcium in the decom-

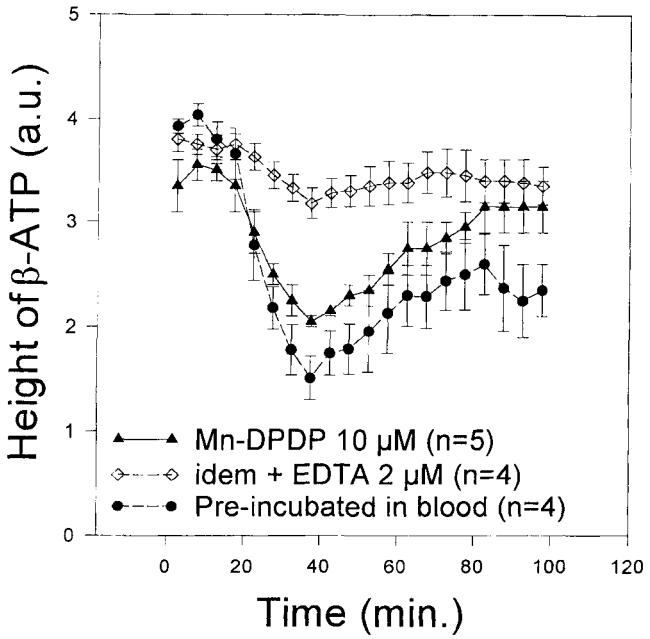

Figure 9. Influence of blood and EDTA on the hepatic uptake of MnDPDP according to protocols 3 and 4, respectively. Evolution of the height (H) of the β-ATP peak.

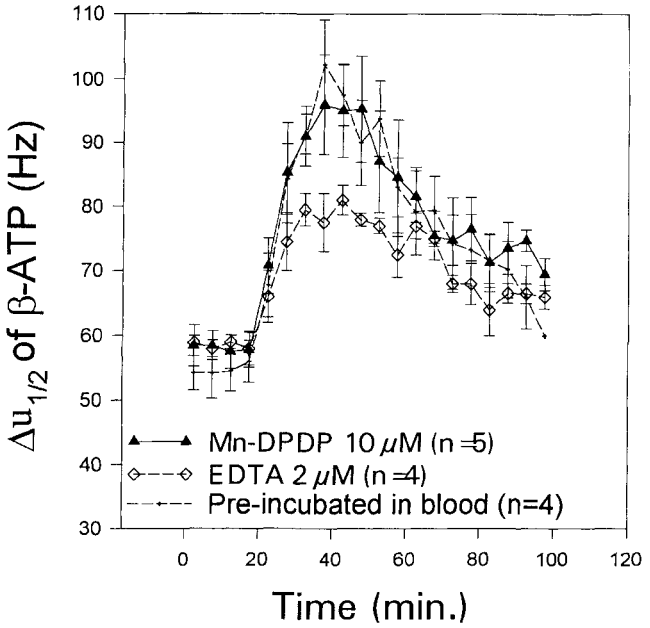

Figure 10. Influence of blood and EDTA on the hepatic uptake of MnDPDP according to protocols 3 and 4, respectively. Evolution of the width at half height ($\Delta\nu_{1/2}$) of the β-ATP peak.

plexation of MnDPDP in the perfusion fluid (Vander Elst et al, unpublished results), which does not contain zinc, another powerful partner for transmetallation.

• CONCLUSIONS

Contrast-enhanced MRI of the liver is expected to increase both sensitivity and specificity in the diagnostic effort to detect primary and secondary cancer of the liver. It might also contribute to the solution of the diagnostic challenges to detect and characterize lesions smaller than 1 cm and to differentiate between benign and malignant lesions. So far, the nonspecific gadolinium-based contrast agents of the first generation have only shown limited success as liver agents, mostly in dynamic studies enhancing highly vascularized lesions such as focal nodular hyperplasia.

In phase II and phase III clinical studies, MnDPDP, as a targeted agent, has shown its ability to considerably improve the image contrast in hepatic, pancreatic, and cardiac tissues due to an incorporation of paramagnetic manganese into the target cells. In addition to minor side effects such as flushing and warmth, apparently related to the rate of intravenous administration rather than to the product itself, no severe adverse reactions have been reported.

From our data, it is reasonable to attribute the potential of MnDPDP as a liver-specific agent to its efficiency in carrying and releasing manganese ions to the hepatocytes. The present results support those obtained in an analogous preclinical study performed on the heart (24). It is necessary, however, to point out that the extent of Mn^{2+} internalization is much greater in the liver than in the myocardium.

All the effects subsequent to the administration of MnDPDP and to the uptake of manganese ions through the calcium channels are transient and do not seem to affect the liver physiology as monitored with our protocol. However, the same does not hold when $MnCl_2$ is used in the perfusate at the same concentration, a difference that can be attributed to a larger availability and incorporation of free manganese ions.

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