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Effect of Cyclosporine on Hepatic Energy Status and on Fructose Metabolism after Portacaval Shunt in Dog as Monitored by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy *in Vivo*

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The effect of cyclosporin A on the hepatic energy status and intracellular pH of the liver and its response to a fructose challenge has been investigated using *in vivo* phosphorus-31 nuclear magnetic resonance spectroscopy in dogs. Three experimental groups were studied: (a) control dogs (n = 5), (b) dogs 4 days after the creation of an end-to-side portacaval shunt (n = 5), and (c) dogs 4 days after portacaval shunt and continuous infusion of cyclosporin A (4 mg/kg/day) by way of the left portal vein (portacaval shunt plus cyclosporin A, n = 5). The phosphorus-31 nuclear magnetic resonance spectra were obtained at 81 MHz using a Bruker BIOSPEC II 4.7-tesla nuclear magnetic resonance system equipped with a 40-cm horizontal bore superconducting solenoid. The phosphomonoesters ($p < 0.01$), inorganic phosphate and ATP levels ($p < 0.05$) were decreased significantly in portacaval shunt–treated and in portacaval shunt-plus-cyclosporin A–treated dogs compared with unshunted control dogs. After a fructose challenge (750 mg/kg body wt, intravenously), fructose-1-phosphate metabolism was reduced in portacaval shunt–treated dogs compared with either the normal or portacaval shunt-plus-cyclosporin A–treated dogs ($p < 0.05$). Both portacaval shunt– and portacaval shunt-plus-cyclosporin A–treated dogs demonstrated a reduced decline in ATP levels after fructose infusion when compared with the controls ($p < 0.05$). Immediately after the fructose challenge, the intracellular pH decreased from 7.30 ± 0.03 to 7.00 ± 0.05 in all animals ($p < 0.01$) and then gradually returned to normal over 60 min. These data, obtained *in vivo* using phosphorus-31 nuclear magnetic resonance spectroscopy of the liver after a portacaval shunt, suggest that: (a) the energy status of the liver is reduced in dogs with a portacaval shunt compared with that of normal controls and (b) cyclosporin A treatment ameliorates the reduction in hepatic metabolism normally observed after a fructose challenge to the liver with a portacaval shunt. (HEPATOLOGY 1991;13:780-785.)

The intrinsic hepatotoxicity of cyclosporin A (CsA) is well documented in the literature (1-10). Clinically, it can be manifested either as an increase in one or more liver injury parameters or as a cholestatic syndrome. Hepatic regeneration may be crucial during and immediately after transplantation, when the liver recovers from the injury experienced during the period of ischemic cold preservation and reperfusion. The acute effects of CsA on liver regeneration have been investigated recently by several different groups (11-16). These studies have shown that CsA may actually enhance the hepatocyte's regenerative response after partial hepatectomy.

In this study, the dog with portacaval shunt (PCS) (Eck's fistula) was used. PCS usually is associated with hepatic atrophy and low-grade hepatocyte hyperplasia (16-21). We have previously shown in this model that CsA prevents the atrophy and augments the hyperplasia (21). In this report, the effect of CsA treatment on the metabolic events caused by PCS is examined *in vivo*

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using phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) spectroscopy. Various *in vivo* studies using ^{31}P-NMR spectroscopy (22-24) have reported on the intracellular pH (pH_i), the hepatic content of ATP, sugar phosphates and inorganic phosphate (Pi) under basal conditions and after a physiological challenge with fructose. The only other NMR study relevant to hepatic regeneration or hepatocyte growth control was performed *in vitro* and used perchloric-acid extracts of tissue samples to calculate phosphate metabolites (25).

MATERIALS AND METHODS

Experimental Design. Fifteen female purebred Beagle dogs weighing between 8.5 kg and 15 kg were studied after care and conditioning at the University of Pittsburgh Animal Research Facility. Three groups of animals were studied: (a) normal dogs (control, n = 5); (b) animals subjected to PCS (n = 5); and (c) animals subjected to PCS and treated with a continuous infusion of CsA (4 mg/kg/day) into the left lobes of the liver through the left portal vein that had been cannulated at the time of the PCS procedure (PCS + CsA, n = 5). The basic design of the experiment using other test substances has been described previously (19, 20). Four days after the procedure and after general anesthesia induced with 20 mg/kg sodium pentobarbitone intravenously and ventilation with 2% halothane in 50% O_2/50% N_2O, femoral arterial and venous catheters were inserted for blood sampling. The proximal airway pressure, arterial pressure and blood gases were monitored continuously during the experiments using a Gould RS3600 recorder and a Radiometer ABL2 instrument (Radiometer America, Westlake, OH), respectively. Body temperature was maintained at 37° C with a heated water pad. Laparotomy was performed, and a surface coil was placed over the liver for NMR measurements. The left and right hepatic lobes were studied separately.

NMR Measurements. ^{31}P-NMR spectra were acquired on a Bruker BIOSPEC II 4.7-tesla NMR system equipped with a 40-cm horizontal bore superconducting solenoid. A 3.5-cm surface coil tuned to the resonance frequency of ^{31}P nucleus (81 MHz at 4.7-tesla) was used both for ^{31}P-NMR spectroscopy and for shimming on the water signal. The typical line width of the water resonance was 100 Hz. Generally, ^{31}P-NMR spectra were acquired in 4-min blocks of 64 transients with an approximate 3-sec pulse delay (respiratory cycle) and 90 degree pulses. Gating was accomplished with a gating device that was armed by the respirator and triggered before each respiratory cycle. A 5-mm spherical bulb containing a 0.15 mol/L solution of methylenediphosphonic acid (MDPA) (23 ppm downfield from phosphocreatine [PCr] at 37° C) in D_2O at pH 9 was placed in the center of the surface coil to serve as chemical shift and signal strength references for pH_i and relative concentration measurements.

A calibration curve for the determination of pH from the ^{31}P chemical shifts of Pi and fructose-1-phosphate (F-1-P) was obtained as follows. Liver specimens (4 gm wet wt) obtained from a normal dog were homogenized and diluted 1:4 (wt/vol) in physiological saline. The homogenate was centrifuged (2,500 rpm) at 4° C for 15 min, and the supernatant (12 ml) was transferred to a 30-mm NMR sample tube. A spherical glass bulb containing MDPA (0.15 mol/L in D_2O, pH 9.0) was centered in the sample as an external standard. After the addition of 10 mmol/L F-1-P (Sigma Chemical Co., St. Louis, MO) and 10 mmol/L PCr (Sigma), a titration curve (from pH 4.24 to pH 9.45) was generated at 37° C. For each pH studied, the solution was positioned in the magnet, and 10-min acquisitions were accumulated using a 3.5-cm diameter solenoid coil, 90-degree pulses and a 5-sec interpulse delay. The ^{31}P chemical shifts of Pi and F-1-P in the ^{31}P--NMR spectra were referenced to the internal signal of PCr. Titration curves were obtained by plotting the ^{31}P chemical shifts of Pi and F-1-P as a function of pH. A least-squares fit was performed to establish the titration parameters for each curve. The pH_i was determined using the following equations (26):

$$pH_i = pK_{Pi} + \log ([\sigma_{Pi} - 3.34]/[5.81 - \sigma_{Pi}])$$

for Pi, and

$$pH_i = pK_{F\text{-}1\text{-}P} + \log (\sigma_{F\text{-}1\text{-}P} - 3.68)/(7.48 - \sigma_{F\text{-}1\text{-}P})$$

for F-1-P, where pK_{Pi} and $pK_{F\text{-}1\text{-}P}$ are 6.76 and 6.03 and σ_{Pi} and $\sigma_{F\text{-}1\text{-}P}$ are the observed values of the chemical shift in ppm of Pi and F-1-P, respectively, error ± 0.03 pH unit.

The content of phosphate metabolites (ATP, F-1-P, and Pi) in the liver was calculated by cutting out the corresponding peaks in the ^{31}P-NMR spectra from the recording paper, weighing each peak and normalizing the data obtained using the MDPA peak area as a reference. This agreed satisfactorily with computer integration after baseline correction. After an injection of fructose (750 mg/kg body wt in 30% saline administered intravenously over 2 min), hepatic metabolism was monitored sequentially with a time resolution of 4 min.

Statistical Analysis. The statistical analysis of the experimental data for intergroup variations was performed using a one-way ANOVA combined with the Fisher test. Student's *t* test for paired data was used to compare the change in parameters after fructose within each group. A p value < 0.05 was considered significant. All results are expressed as mean value ± S.E.M.

RESULTS

Three typical 81-MHz ^{31}P-NMR spectra obtained *in vivo* from normal control, PCS-treated and PCS-plus-CsA–treated dogs are shown in Figure 1. The phosphomonoester (PM) peak (peak 2) represents the sum of signals from various sugar phosphates, glycolytic intermediates and AMP. The chemical shift of the Pi peak (peak 3) relative to the external standard (peak 1) permitted us to determine the pH_i of the liver. This value was 7.30 ± 0.05 for all three groups of animals studied. The phosphodiester (PD) resonance (peak 4) is the sum of glycero-3-phosphocholine, glycerol phosphoethanolamine and other related compounds. The γ-ATP-phosphate and β-ADP-phosphate resonances (peak 5) occur at about −2.4 ppm upfield from PCr; the α-phosphates of both ATP and ADP (peak 6) occur at −7.5 ppm upfield from PCr, and the β-ATP resonance (peak 7) has a single peak at −16 ppm upfield from PCr. Because the right and left hepatic lobes yielded identical ^{31}P-NMR spectra, only the left lobes were studied after the first two experiments.

The relative concentrations of the various phosphate metabolites in the liver as determined by ^{31}P-NMR spectroscopy *in vivo* are shown for the three groups of animals studied in Figure 2. The PM ($p < 0.01$), Pi ($p < 0.05$) and ATP ($p < 0.05$) decreased in the presence of PCS-treated and in the PCS-plus-CsA–treated dogs

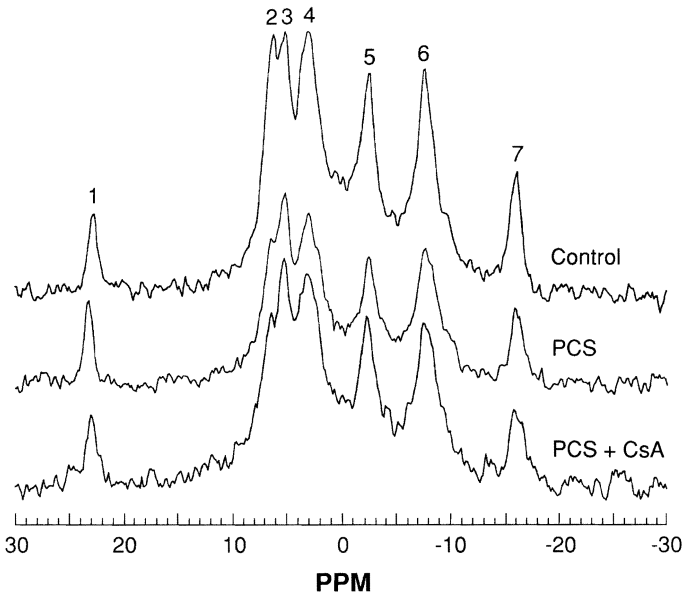

FIG. 1. *In vivo* ^{31}P-NMR spectra of dog liver in normal dog liver (control) 4 days after PCS and 4 days after PCS plus continuous infusion of CsA in the left portal vein branch (PCS + CsA). Peak assignments: *(1)* MDPA; *(2)* PM; *(3)* Pi; *(4)* PD; *(5)* γ-ATP plus β-ADP; *(6)* α-ATP plus α-ADP; and *(7)* β-ATP. These spectra consisting of 64 acquisitions were accumulated in 4 min with a ~3 sec pulse delay (respiratory cycle) and 90-degree pulse. MDPA was used as the external reference standard for chemical shift and for intergroup and intragroup relative concentration measurements.

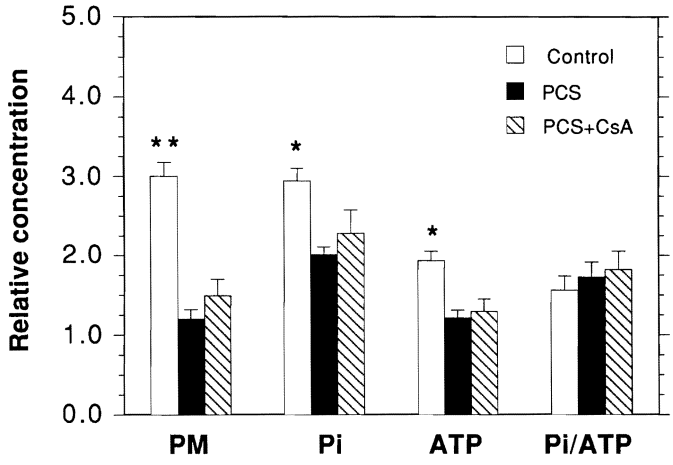

FIG. 2. Relative phosphate metabolite concentrations in dog liver. The relative concentrations of phosphate metabolites in the liver were measured by dividing the integrated area under each peak by the MDPA area used as an external reference in each experiment. The n value for each group was 5. The values of the columns marked with asterisks are significantly different from the values of the other columns (*$p < 0.05$, **$p < 0.01$).

with respect to the control. The (Pi/ATP) ratio that correlates inversely with the energy state of the liver did not significantly change in the three groups of dogs studied (Fig. 2).

After the administration of a fructose challenge (Fig. 3), major changes were observed in the ^{31}P-NMR spectra of all three groups. An increase in the sugar phosphates, caused by the accumulation of F-1-P, and a reduction in the Pi and ATP content of the liver was evident immediately.

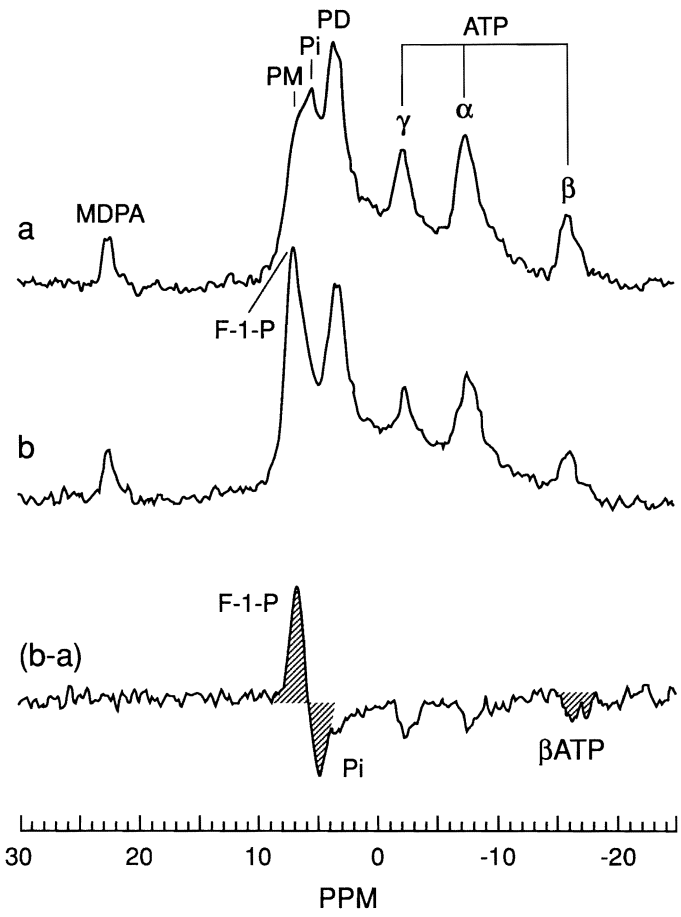

FIG. 3. Effect of a fructose challenge on the *in vivo* ^{31}P-NMR spectrum of normal dog liver. Spectra obtained before fructose *(a)* and 8 min after fructose administration *(b)* administered at a dose of 750 mg/kg body wt intravenously as a bolus. The difference spectrum is labeled *(b-a)*.

The time course of the F-1-P peak for the three groups of animals is shown in Figure 4. In the control dogs, the F-1-P resonance appeared within the first 4 min after the fructose infusion and reached a maximum value in the second 4 min. It then decreased rapidly as a result of the metabolism of the fructose load. This process was complete within 20 min, and the sugar phosphates region in the ^{31}P-NMR spectrum returned to pre-fructose levels. In PCS-treated dogs, the metabolism of F-1-P was greatly reduced, as shown by a larger increase and a slower decline of the F-1-P resonance in the sugar phosphate region ($p < 0.05$). In contrast, the metabolism of the fructose load by the PCS-plus-CsA–treated dogs was normal and equivalent to control.

The ATP level (Fig. 5) decreased significantly ($p < 0.01$) to about 50% of the initial value 8 min after the fructose challenge. It began to recover at 12 min and

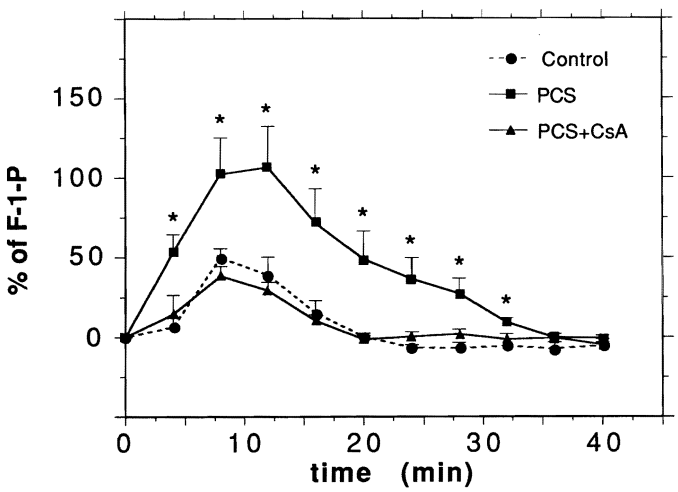

FIG. 4. F-1-P levels in dog liver after a fructose challenge. The time course of F-1-P levels in dog liver in the three groups of dogs (control, n = 5; PCS, n = 5; PCS + CsA, n = 5) after a fructose load (750 mg/kg body wt administered intravenously as a 30% solution in saline, over 2 min, beginning at 0 time). Values are shown as relative changes from basal value at 0 time for the PM area relative to the MDPA area, mean ± S.E.M. The asterisk denotes $p < 0.05$.

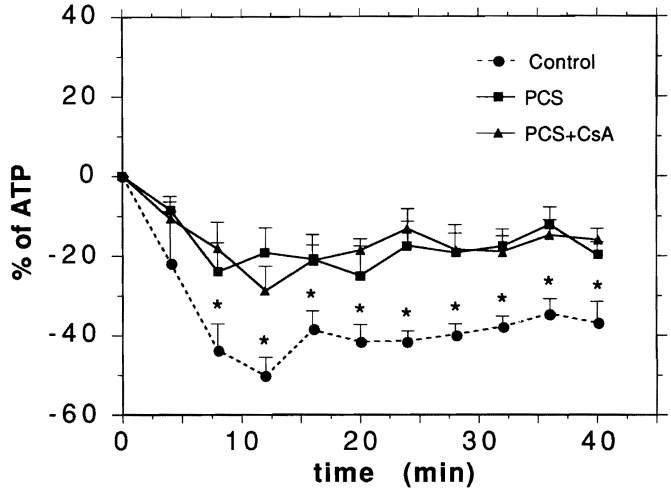

FIG. 5. ATP levels after a fructose challenge. The time course of intrahepatic ATP levels in three groups of dogs (control, n = 5; PCS, n = 5; PCS + CsA, n = 5) after a fructose load (750 mg/kg body wt administered intravenously as a 30% solution in saline, over 2 min, beginning at 0 time). Values are relative changes from the basal value at 0 time for the β-ATP area relative to the MDPA area, mean ± S.E.M. The asterisk (*) denotes $p < 0.05$.

reached a level of about 65% of the prefructose value at 40 min. The recovery of the hepatic ATP content was never complete even 2 hr after the fructose challenge, when
the level is approximately 90% of the basal value (results not shown). The hepatic ATP level decreased less rapidly and to a smaller extent in the PCS-treated and PCS-plus-CsA–treated dogs ($p < 0.05$) after the fructose challenge. However, the subsequent recovery in ATP content paralleled that noted in the controls. The difference between shunted and control dogs was statistically significant ($p < 0.05$) (Fig. 5).

The Pi changes noted after fructose are shown in Table 1. The Pi was depressed early after the fructose challenge ($p < 0.01$) and returned toward normal values as the F-1-P peak disappeared. No statistically significant differences among groups were observed.

After the fructose challenge, the pH_i fell from a value of approximately 7.3 to 7.0 ($p < 0.01$) in all three animal groups within 12 min and returned to a value close to baseline values thereafter (results not shown), confirming the findings of an earlier study performed in rat liver (28). Forty minutes after the fructose challenge, the pH_i of the PCS-plus-CsA–treated dogs was not significantly different from that noted before the challenge. In contrast, the pH_i of control and PCS-treated dogs was still reduced ($p < 0.01$ and $p < 0.05$, respectively).

DISCUSSION

The advantages of using ^{31}P-NMR spectroscopy to study liver metabolism and function have been reviewed recently (23). *In vivo* ^{31}P-NMR spectroscopy allows a nondestructive monitoring of tissue pH and a study of the levels of hepatic phosphorus-containing compounds associated with energy metabolism (23, 24, 27). In this study, this method was used to investigate the consequences of PCS and PCS in conjunction with cyclosporine treatment on hepatic function and basal energy status. The response of the liver to a fructose challenge was monitored by examining the changes in ATP, Pi, and F-1-P levels within the liver as a function of time (28).

The dog with a portacaval shunt was chosen as a well-established model for studying hepatic atrophy and contemporaneous hyperplasia (17-20). The typical injury pattern after PCS consists of a severe reduction in hepatocyte cell size (atrophy) plus organelle disruption and a moderate but persistent stimulation of cell renewal (hyperplasia). Insulin and cytosolic extract of the regenerating liver are able to restore the hepatocyte to normal size and to further stimulate hyperplasia (19, 20). A disadvantage of earlier studies was that the animals had to be killed, and *in vitro* assays of the metabolites of interest could be obtained only at selected times.

Interestingly, despite its recognized potential for intrinsic hepatotoxicity (1-10), CsA has been shown to have "hepatotrophic" effects after partial hepatectomy (11-16) or a PCS (21). Furthermore, CsA does not appear to either prevent or limit the hepatic regenerative response observed when a small-for-size liver is transplanted into a larger recipient (29) or when a reduced-size liver is used in pediatric recipients (30).

This study provides new data obtained by *in vivo* examination of the energy status and the metabolic responsiveness of the liver in an experimental situation that could have clinical relevance. The decline in the hepatic-phosphate metabolites observed after partial

TABLE 1. Changes observed in hepatic Pi after a fructose challenge

Time (min)	Control (n = 5)	PCS (n = 5)	PCS + CsA (n = 5)
0	100	100	100
4	75.8 ± 10.2	78.6 ± 8.4	86.2 ± 3.9[a]
8	58.2 ± 5.7[b]	57.8 ± 2.7[b]	64.4 ± 6.0[b]
12	63.8 ± 4.6[b]	62.8 ± 3.3[b]	73.4 ± 8.1[a]
16	89.0 ± 3.7[a]	74.2 ± 4.9[b]	89.8 ± 8.2
20	109.0 ± 5.0	92.0 ± 7.4	101.2 ± 9.9
24	113.0 ± 8.3	105.4 ± 9.5	110.6 ± 8.2
28	116.8 ± 6.6	112.0 ± 8.8	116.8 ± 8.6
32	109.2 ± 9.7	116.4 ± 11.3	121.4 ± 7.3[a]
36	106.4 ± 10.4	118.2 ± 12.0	121.8 ± 8.0
40	105.0 ± 8.9	113.6 ± 10.6	117.4 ± 8.8

The time course of hepatic Pi changes in three groups of dogs (control, PCS and PCS + CsA) after a fructose load (750 mg/kg body wt administered intravenously as a 30% solution in saline, more than 2 min, beginning at 0 time). Values are mean ± S.E.M. Changes observed over time were analyzed statistically with Student's *t* test for paired data ([a]$p < 0.05$, [b]$p < 0.01$, vs. 0 time) and among groups by one-way ANOVA test and the Fisher test.

hepatectomy by *in vitro* studies (25) has been confirmed in the PCS model and *in vivo*. The effect of portal CsA infusion on these parameters was also determined. Using a fructose challenge as a test of metabolic responsiveness, the clearance of F-1-P in PCS dogs treated with CsA was found to be similar to that observed in the control and occurred considerably more rapidly than that observed in PCS dogs not receiving CsA treatment.

An apparent discrepancy arises when one considers the total balance of phosphates in the data. After fructose infusion, negative changes in ATP and Pi and positive changes in F-1-P did not seem to add up to zero in all single time points. In particular, when phosphorylation occurred (0 to 12 min after fructose), more phosphate is seen by ^{31}P-NMR spectra in the PM region of the PCS-treated animals. In the same time period, the concomitant drop of ATP is larger only in the control group, and the decrease in Pi is not significantly different among the three groups. One possible explanation could lie in the slightly greater decrease in Pi in the PCS-treated dogs at most time points. Another possibility is that the low signal/noise ratio of the *in vivo* experiments and the proximity of the PM and Pi peaks in the ^{31}P-NMR spectra limit an accurate measurement of relative changes in the two areas. Finally, the rapid changes in Pi could be underestimated in the 4-min time frame of our study. Under the present ^{31}P-NMR protocol (3-sec repetition), the phosphate resonances are not likely to be significantly saturated (31), allowing us to relatively quantify the changes of the various phosphate metabolite concentration over time. These findings suggest that CsA preserves the capacity of the liver to metabolize a fructose load after a PCS treatment. This may reflect the fact that intracellular levels of the Pi that inhibits AMP degradation (32) are better preserved. Also, it is interesting that the changes for pH_i noted in this study are consistent with data obtained using the perfused rat liver (28), suggesting that the fall in pH_i after fructose is caused mainly by production of hydrogen ions accompanying the formation of lactate from fructose.

How these biochemical changes relate to prevention of atrophy by CsA and the augmentation of hyperplasia (21) is not clear. A simple explanation would be that the better response to a fructose load simply reflects the increased function under CsA of more healthful and more plentiful hepatocytes. However, a more sophisticated hypothesis may be forthcoming now that information exists concerning the action of drugs like CsA and FK 506 on *cis-trans* peptidyl-prolyl isomerase, an enzyme at the cytosolic binding sites for these agents. Peptidyl-prolyl isomerase inhibition or activation seems to have a wide-ranging effect on cell physiology, including responsibility for the so-called hepatotrophic mechanisms and control of carbohydrate metabolism (33).

Meanwhile, it must be remembered that cyclosporine is not a panacea for the liver. The drug can also be hepatotoxic even at therapeutic doses (1-10), impair hepatocyte transport processes (34, 35) or have an adverse effect on intracellular membranes (36, 37) after having gained entrance to the cells by passive diffusion (38). It is probable that CsA can be either hepatotrophic or injurious to the liver, depending on the dose.

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