Kinetics of creatine uptake in the perfused mouse liver: a ^{31}P -n.m.r. study of transgenic mice expressing creatine kinase (CK_{BB}) in the liver

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Transport of creatine in the mouse liver has been investigated *in vivo* and in the perfused organ. Experiments were carried out with transgenic mice expressing creatine kinase in the liver (brain isoenzyme CK_{BB} ; EC 7.2.3.2.) [Koretsky, Brosnan, Chen, Chen and Van Dyke (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3112–3116] and in the corresponding control mice. The animals were fed a regular chow with or without the addition of 10% creatine (w/w) for 5 days. The kinetics of creatine uptake was measured in the perfused liver by ^{31}P -n.m.r. spectroscopy and biochemical analysis following infusion of creatine at concentrations ranging over 0–15 mM and at an extracellular pH of either 7.40 or 6.40. The results suggest that creatine is actively trans-

ported by a pH-dependent mechanism obeying a saturable Michaelis–Menten type of kinetics ($K_{\text{m}} = 0.80 \pm 0.18$ and 5.12 ± 2.40 mM; $V_{\text{max}} = 0.57 \pm 0.04$ and 1.72 ± 0.32 $\mu\text{mol} \cdot \text{g}$ of liver $^{-1} \cdot \text{min}^{-1}$ at pH 7.40 and 6.40 respectively). Creatine export was evaluated in the perfused liver preloaded with creatine and the results show that less than 2.5 and 5% of the total creatine pool is exported to the perfusate during 80 min of perfusion at pH 7.40 and 6.40 respectively. Taken together, these results seem to explain the observation that creatine accumulates in the mouse liver only when blood creatine is raised by creatine feeding.

INTRODUCTION

There is a growing interest in the biological and clinical relevance of creatine feeding both in experimental models and in humans. Thus various tumour cell lines implanted in rodents show a significantly decreased growth rate upon feeding the animals with creatine or creatine analogues (Miller et al., 1993). Extracellular creatine regulates the expression of a creatine transporter in L6 rat muscle cells (Loike et al., 1988). In humans, Osbakken et al. (1992) recently observed that creatine, but not cyclocreatine, feeding exerted a protective effect on the heart during a subsequent ischaemia. Furthermore, short duration high-intensity athletic performance may be increased by supplementing the diet of human subjects with 5 g of creatine per day, an effect which was tentatively ascribed to an increased intracellular phosphocreatine (PCr) concentration in the skeletal muscle of these individuals (Harris et al., 1992). The latter findings, however, seem to contrast with earlier observations on the rat where creatine administration led to a decreased level of creatine in skeletal muscle (Agostini et al., 1981); also performance of endurance exercise was not improved by creatine supplementation in trained subjects (Balsom et al., 1993).

PCr is synthesized *in vivo* exclusively through the reversible reaction catalysed by creatine kinase, from creatine and ATP (Bessman and Carpenter, 1985), whereas creatine itself originates either from the diet or via a two-step *de novo* biosynthesis pathway present in various organs (Walker, 1979). There are marked inter-organ and inter-species variations in the enzymic distribution of this biosynthesis (Van Pilsum et al., 1972). In particular, rodent liver (unlike human liver) lacks the first enzyme involved in creatine synthesis (glycine amidinotransferase), leaving the pancreas and kidneys as major sources for endogenous creatine (Walker, 1963; Koszalka, 1963; Koszalka, 1967).

There are few data concerning the hepatic transport of creatine, either *in vitro* (hepatocytes, perfused liver) or *in vivo* (Henderson

et al., 1983; Yanokura et al., 1984; Meert et al., 1991). In perfused liver from transgenic mice (expressing the gene of creatine kinase CK_{BB} isoenzyme in the liver), it has been shown recently that creatine uptake was dependent on the presence of P_i in the perfusate buffer (Koretsky et al., 1993). In the present work, we have undertaken a series of experiments with the same model, investigating the kinetics and quantitative aspects of creatine transport in the liver *in vivo* and in the perfused liver. A preliminary account of this study has been presented (Masson and Quistorff, 1993).

EXPERIMENTAL

Animals

Transgenic mice expressing the CK_{BB} isoenzyme in the liver (Koretsky et al., 1990) were kindly provided by Dr. Alan Koretsky, University of Pittsburgh, U.S.A. The animals have subsequently been inbred at the Animal Facility of the Panum Institute in the context of an ongoing study of zonation of the mouse liver creatine kinase (Quistorff et al., 1993). Male and female mice, weighing 18–28 g, were fed a regular chow diet or the same diet supplemented with 10% (w/w) creatine for 5 days. They had free access to water. Hepatic creatine kinase activity was 5.28 ± 0.66 and 4.99 ± 0.14 units $\cdot \text{mg}$ of total protein $^{-1}$ in the male and female mice respectively (Quistorff et al., 1993). Male hybrid mice (strain $\text{B}_6\text{D}_2\text{F}_1$), which were originally used for the construction of the transgenic mice (Koretsky et al., 1990), were used as controls in this study, and were obtained from Møllegaard, Denmark.

Liver perfusion

The mice were anaesthetized by intraperitoneal injection of a mixture of sodium pentobarbital (100 mg $\cdot \text{kg}$ of body weight $^{-1}$) and propanidide (sombrevin, 4 ml $\cdot \text{kg}$ of body weight $^{-1}$). The

Table 1 The effect of feeding control and transgenic mice with a diet supplemented with creatine

Transgenic (CK_{BB}) and control mice (B₆D₂F₁) were fed a regular diet or the same diet supplemented with 10% (w/w) creatine for 5 days. After anaesthesia and laparotomy of the animals, a blood sample (100 µl) was withdrawn from the portal vein and a liver biopsy immediately freeze-clamped for biochemical analyses of creatine (Cr), PCr and ATP. Values are expressed in mM and represent means ± S.E.M. for three experiments in each group.

Metabolite (mM)		Regular diet		Creatine diet	
		Control	Transfected	Control	Transfected
Liver	PCr	0.16 ± 0.01	0.07 ± 0.03†	1.59 ± 0.18*	3.26 ± 0.78*
	Creatine	0.48 ± 0.08	0.51 ± 0.05	6.94 ± 2.66*	17.59 ± 2.09*†
	PCr + Cr	0.64 ± 0.09	0.58 ± 0.08	8.35 ± 2.83*	20.85 ± 1.84*†
	ATP	2.35 ± 0.15	2.96 ± 0.18	2.56 ± 0.08	3.15 ± 0.16†
Portal blood	PCr	0.011 ± 0.002	0.020 ± 0.004	0.022 ± 0.004	0.019 ± 0.009
	Creatine	0.06 ± 0.01	0.05 ± 0.01	0.31 ± 0.03*	0.76 ± 0.23*
	PCr + Cr	0.08 ± 0.01	0.07 ± 0.01	0.34 ± 0.03*	0.78 ± 0.22*
	ATP	0.63 ± 0.02	0.66 ± 0.02	0.59 ± 0.01	0.67 ± 0.06
Liver/blood	Cr/Cr	7.48 ± 0.57	11.89 ± 3.32	21.14 ± 7.56	25.45 ± 3.87*
	(PCr + Cr)/(PCr + Cr)	8.53 ± 0.38	9.15 ± 1.93	22.61 ± 6.51	29.55 ± 5.10

* $P < 0.05$; regular versus creatine diet (effect of creatine supplementation).

† $P < 0.05$; control (B₆D₂F₁) versus CK_{BB} transgenic mice (species effect).

excised livers (mean weight after dry-blotting: 1.37 ± 0.03 g) were perfused via the portal vein in a non-recirculating set-up, at a constant flow rate of $8.0 \text{ ml} \cdot \text{min}^{-1}$. The perfusion medium was a Krebs–Henseleit buffer equilibrated with O₂/CO₂ (19:1) passing through a silastic-tubing gas exchanger. The pH of the medium, (pH_i) was 7.40 or adjusted to 6.40 by decreasing the amount of NaHCO₃ to 2.2 mM. The temperature of the perfusate was 37 °C. After an initial equilibration (approx. 20 min), creatine was added to the medium in concentrations ranging over 0.5–15 mM. The perfusions were concluded after 80 min and the livers were rapidly freeze-clamped (Quistorff and Poulsen, 1980) and stored at –80 °C until analysed. Removal of the liver from the perfusion set-up, dry-blotting and freeze-clamping was accomplished in 5–10 s.

³¹P-n.m.r. spectroscopy

Experiments were carried out in an Otsuka Vivospec spectrometer interfaced to a 31-cm-bore Magnex 4.7 T superconducting horizontal magnet. Perfused livers were set in a 14-mm (i.d.) n.m.r. tube inside the detection volume of a three-turn, double-tuned (³¹P/¹H) and inductively driven solenoid coil. Loaded Q of the coil was > 100 for ³¹P. The magnetic field was shimmed on the water signal. ³¹P-n.m.r. spectra were recorded at 81.06 MHz without proton decoupling. They consisted of the accumulation of 80 free induction decays (f.i.d.) with 4K data points, using a nutation angle of 70°, a spectral width of 10 kHz and a repetition delay of 1.5 s. The total time of observation for each spectrum was 2 min. F.i.d.s were processed with an exponential filter corresponding to a 15 Hz line broadening. Methylene diphosphonic acid (28 µmol in a sealed glass capillary inside the detection volume of the coil) was used as an external quantification standard. Chemical shifts are expressed relative to an 85% H₃PO₄ (w/v) solution at 0.0 p.p.m. The resonance areas were corrected for signal saturation by comparing the signal acquired under the regular conditions described above and spectra recorded under fully relaxed conditions (repetition time of 7 s). Cytosolic pH was determined from the chemical shift of the P_i resonance (Desmoulin et al., 1987). The concentration of creatine was determined at the end of the experiments in freeze-clamped biopsies by biochemical analysis and expressed as µmol · g wet wt

of liver⁻¹. When relevant these data were corrected for the contribution of the extracellular space, multiplying by 1/0.83, i.e. µmol · ml of liver cells⁻¹ (Weibel et al., 1969).

Determination of the intrahepatic PCr/creatinine ratio during creatine perfusion

Another series of experiments was devised to monitor the evolution of the intrahepatic PCr/creatinine ratio during infusion of creatine at different concentrations (up to 15 mM) and at pH_o values of 7.40 or 6.40. After an initial interval of equilibration (approx. 20 min), livers from transgenic mice fed *ad libitum* without creatine supplementation were infused with creatine and rapidly freeze-clamped after 3, 5, 10, 15, 30 or 45 min respectively ($n = 2$ or 3 for each time-point). The biopsies were thereafter analysed for their content of PCr and creatine, and the ratio $([\text{PCr} + \text{Cr}]/[\text{PCr}])_{\text{BIO}}$ (where Cr is creatine) calculated for each time-point of the experiments. This ratio was subsequently used in the n.m.r. experiments described above to compute the intrahepatic total creatine content, i.e. $[\text{PCr} + \text{Cr}]$, using the formula:

$$[\text{PCr} + \text{Cr}]_t = [\text{PCr}]_{\text{NMR},t} \times ([\text{PCr} + \text{Cr}]/[\text{PCr}])_{\text{BIO},t}$$

where $[\text{PCr}]_{\text{NMR},t}$ is the concentration of PCr at a particular time, t , of perfusion determined from ³¹P-n.m.r. experiments, and $([\text{PCr} + \text{Cr}]/[\text{PCr}])_{\text{BIO},t}$ is the term calculated from biopsy experiments.

In vivo experiments

Male control and transgenic mice (body weights: 25–30 g) were fed *ad libitum* either a regular chow diet or the same diet supplemented with 10% creatine for 5 days. After anaesthesia and incision of the abdominal wall, 100 µl of blood was withdrawn from the portal vein and immediately extracted with 200 µl of 1 M ice-cold perchloric acid. A liver biopsy was rapidly freeze-clamped and the samples were kept at –80 °C following centrifugation and neutralization with 1.5 M K₂CO₃. Concentrations of creatine and PCr, intracellular hepatic creatine, PCr and ATP were thereafter assayed in the extracts.

Biochemical analyses

Hepatic metabolites were extracted with 2 M perchloric acid as previously described (Masson and Quistorff, 1992). Standard enzymic assays were used to measure ATP, PCr and creatine (Lowry and Passonneau, 1972), and a colorimetric assay for P_i (Penney, 1976). Hepatic creatine kinase activity was measured as described by Bergmeyer (1970).

Statistics

Data are presented as means $\pm$ S.E.M. for the number of observations stated. Statistical analysis was performed using a two-tailed Student's *t* test, and the level of significance was set at $P < 0.05$ (C-Stat software package, Cherwell Scientific Publishing, Oxford, UK.). The kinetics were analysed with a curve-fitting program (GraphPad InPlot software, GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Effect of creatine feeding *in vivo*

Table 1 shows the concentrations of creatine, PCr and ATP in liver and in portal blood sampled from the anaesthetized mouse *in vivo*. The animals had been fed either regular chow or the same diet supplemented with 10% (w/w) creatine for 5 days. The creatine feeding resulted in a 5–14-fold increase in the creatine concentration measured in the portal blood, significantly higher in the transgenic than in the control mice. Portal blood and a liver biopsy were sampled from the same animal. In the practical experiment, however, it was necessary to withdraw the blood before sampling the liver biopsy, introducing a brief ischaemia during blood sampling, which caused some hydrolysis of PCr in the liver. For this reason, only total creatine [PCr + Cr] was taken into consideration in the quantitative evaluation of these data. In the liver biopsies of regularly fed animals, the total creatine concentration was of the order of 0.6 mM with no difference between control and transgenic mice. However, upon creatine feeding, the total creatine content increased more than 10 times in the controls and 36 times in the transgenic mice. ATP concentration in the biopsy samples was about 2.5 mM in controls and 3 mM in transgenic mice, with no significant effect of creatine feeding. Taken together, these results suggest that an active uptake mechanism for creatine in the mouse liver is in operation.

Kinetics of creatine transport in the perfused liver

In order to describe creatine transport in quantitative terms, we therefore perfused mouse livers with different concentrations of creatine in the perfusate (ranging over 0–15 mM). Furthermore, these experiments were carried out at perfusate pH values of 7.40 and 6.40. The perfusions were performed with continuous recording of ^{31}P -n.m.r. spectra, allowing the direct measurement of PCr accumulation as well as changes of intracellular pH. A typical ^{31}P -n.m.r. spectrum of the liver of a transgenic mouse fed a regular diet is presented in Figure 1(a). Figure 1(b) displays the spectrum of the same liver after 80 min of perfusion with a Krebs–Henseleit medium (pH_0 7.40) supplemented with 5.0 mM creatine. There was a large accumulation of a compound with a chemical shift of -2.385 ± 0.009 p.p.m. ($n = 22$), which is assigned as PCr. No significant alteration of intracellular pH was observed.

At the completion of the perfusion (80 min), the liver was freeze-clamped and the intracellular concentrations of creatine and PCr were measured, assuming an extracellular space of 15% of the biopsy wet weight. These results are given in Table 2. Excellent correlation was observed between the n.m.r. and the biochemical determination of PCr, indicating 100% visibility for this compound in the liver, in agreement with earlier observations *in vivo* in the liver (Koretsky et al., 1990) and muscle (Bangsbo et al., 1993). The intracellular/extracellular concentration gradient of creatine diminished with increasing extracellular creatine concentration. Thus, at pH_0 7.40 the gradient decreased 2-fold when increasing the perfusate creatine concentration from 0.5 to 5.0 mM. At pH_0 6.40, the creatine uptake rate was lower than at pH_0 7.40 (see below) and an additional series of experiments with a perfusate creatine concentration of 15 mM was therefore

Figure 1 ^{31}P -n.m.r. spectra of a perfused liver from a transgenic mouse

The liver from a mouse transfected with the gene for creatine kinase BB was perfused and ^{31}P -n.m.r. spectra acquired as described in the Experimental section. The animal was fed a regular chow. Trace (a) corresponds to a control spectrum, recorded after 20 min of perfusion with a Krebs–Henseleit buffer. Trace (b) has been recorded on the same liver, after 80 min of infusion with 5.0 mM creatine at pH_0 7.40. Assignments of resonance: 1, phosphomonoesters (mainly phosphorylcholine and AMP); 2, inorganic phosphate (P_i); 3, phosphodiester (glycerol 3-phosphoethanolamine and glycerol 3-phosphocholine); 4, phosphocreatine; 5, γ -phosphorus of NTP and β -phosphorus of NDP; 6, α -phosphorus of NTP, α -phosphorus of NDP and NAD^+ ; 7, β -phosphorus of NTP.

Table 2 Dose- and pH-dependent transport of creatine and PCr accumulation in livers from transgenic mice

Livers from transgenic mice were perfused for 20 min with a Krebs–Henseleit buffer at pH₀ 7.40 or pH₀ 6.40, and then infused with various concentrations of creatine as reported in the Table. The intrahepatic concentration of PCr (as determined by ³¹P-n.m.r. spectroscopy and biochemical analysis of biopsies) and creatine (biochemical analysis) measured after 80 min of creatine infusion are reported. Data are means ± S.E.M. for three experiments in each group. Abbreviation: nd, not detectable.

pH ₀	Perfusate creatine concn. (mM)	PCR _{80min} (μmol/g of liver wet wt.)		Creatine _{80min} (μmol/g of liver wet wt.)	PCr/Cr
		N.m.r.	Biochemistry		
7.40	0.0	nd	< 0.01	0.19 ± 0.09	—
	0.5	1.58 ± 0.10	1.30 ± 0.17	1.70 ± 0.05	0.75 ± 0.08
	2.0	3.85 ± 0.07	3.19 ± 0.48	4.91 ± 0.54	0.65 ± 0.03
	5.0	5.32 ± 0.33	5.29 ± 0.60	8.71 ± 0.27	0.61 ± 0.07
6.40	0.5	nd	—	—	—
	2.0	1.23 ± 0.03	1.17 ± 0.12	4.94 ± 0.44	0.27 ± 0.02
	5.0	1.66 ± 0.01	1.70 ± 0.24	10.21 ± 0.23	0.17 ± 0.03
	15.0	2.95 ± 0.03	2.84 ± 0.09	20.43 ± 1.79	0.14 ± 0.02

undertaken. In analogy with the data at pH₀ 7.40, the intracellular/extracellular gradient was decreased from 2.2 to 1.36 when creatine concentration in the medium was increased from 2.0 mM to 15 mM. The intracellular PCr/creatinine ratio determined after 80 min was about 0.7 at pH₀ 7.40, decreasing only slightly with increasing creatine concentration. However, at pH₀ 6.40, the PCr/creatinine ratio decreased very significantly as expected from the decreased intracellular pH. In fact, taking into account the effect of lowered pH on the equilibrium of the creatine kinase reaction (Lawson and Veech, 1979), and the observed decrease in intracellular pH (from 7.20 ± 0.05 to 6.70 ± 0.05), the expected change in the intracellular PCr/creatinine ratio may be calculated to be 3.2-fold, which is in good agreement with the observed value (see Table 2). During creatine infusion ATP levels remained constant within 5%, except at 15 mM creatine where a decrease of 15–20% was observed (results not shown).

Figures 2(a) and 2(b) display the ³¹P-n.m.r.-determined time course of hepatic PCr concentration at pH₀ 7.40 and 6.40 respectively. It is obvious that the rate of PCr accumulation was a function both of perfusate creatine and extracellular pH. The time course of total creatine ([PCr + Cr]) accumulation in the liver has been computed from the n.m.r. measurements of PCr accumulation, applying the conversion factor ([PCr + Cr]/[PCr])_{B10} to these data as described in the Experimental section. From these data the individual initial rates of creatine uptake were subsequently derived for each concentration of perfusate creatine and at both pH values. The initial rates of creatine uptake were fitted to a Michaelis–Menten expression as shown in Table 3, giving apparent K_m values for creatine uptake of 0.80 ± 0.18 mM and 5.12 ± 2.40 mM at perfusate pH values of 7.4 and 6.4 respectively. The corresponding values for V_{max} were 0.57 ± 0.04 and 1.72 ± 0.32 μmol · g of liver⁻¹ · min⁻¹.

Efflux of intracellular creatine during liver perfusion

In order to evaluate creatine efflux, livers from transgenic animals were preloaded with creatine by the standard creatine feeding schedule (see the Experimental section). The isolated liver was then perfused for 80 min with Krebs–Henseleit buffer devoid of creatine either at pH 7.40 or 6.40. Liver biopsies were freeze-clamped at the beginning (*t* = 0 min) and at the end of the perfusion (*t* = 80 min) and analysed for their content of creatine and PCr. Total creatine (PCr + Cr) amounted to

21.43 ± 2.88 μmol · g of liver⁻¹ in the start biopsies and was not significantly altered after 80 min of perfusion at pH₀ 7.40 (20.89 ± 3.22 μmol · g of liver⁻¹). At pH₀ 6.40, the corresponding values for total creatine at *t* = 0 and *t* = 80 min were 17.85 ± 3.97 and 15.50 ± 3.96 μmol · g of liver⁻¹ respectively (no significant difference).

Alternatively, creatine efflux was evaluated in perfused livers preloaded with creatine by imposing a rapid transient depletion of intracellular PCr and thereby a corresponding increase in the intracellular creatine concentration. This procedure was accomplished by adding fructose to the perfusate as suggested by Brosnan et al. (1991). Figure 3(a) shows the time course of PCr with a time resolution of 1 min during a 10 mM fructose infusion, as well as the recovery phase after discontinuation of the fructose infusion. The initial level of PCr was 12.06 μmol · g of liver⁻¹ (100%) and decreased to 18.5 ± 3.8% (*n* = 10) during the 15 min of fructose infusion. The rate constant of this decay was 0.27 ± 0.03 min⁻¹. Removal of fructose led to a full recovery of PCr (99.3 ± 5%) with a rate constant of 0.27 ± 0.04 min⁻¹. However, the PCr level decreased somewhat thereafter, reaching 94.11 ± 4.27%, 20 min after the withdrawal of fructose. In separate experiments with the same protocol the PCr/Cr ratio was determined biochemically in biopsies sampled in the beginning before fructose infusion (1.41 ± 0.32) and at the end of the experiment after recovery from fructose (1.38 ± 0.40; *n* = 3). These figures are not significantly different, and consequently the complete recovery of PCr in the n.m.r. experiment indicates that there is no significant loss of creatine during the 80 min perfusion interval.

In a second set of experiments, perfused livers underwent the same protocol as described above, except that 5 mM creatine was co-infused with fructose (10 mM). During recovery, both creatine and fructose were omitted from the buffer. Figure 3(b) compares the time course of PCr recorded with the latter protocol. PCr decreased with a rate constant of 0.24 ± 0.02 min⁻¹, a value similar to the experiment performed without exogenous creatine. A nadir (11.1 ± 1.3%, *n* = 3) was reached after 14 min of fructose infusion (difference not significant). Although the onset of the recovery phase of PCr was delayed by 2 min when compared with experiments without creatine, the accumulation rate was not significantly different. At the end of the recovery phase, PCr concentration represented 118 ± 6.2% of its initial level (Figure 3b), indicating a significant creatine uptake during the interval when both fructose and creatine were present in the perfusate.

Figure 2 Progress curves of hepatic PCr accumulation during creatine infusion

Livers from transgenic mice (fed a regular diet) were infused for 80 min with creatine at various concentrations as indicated on the figure and pH₀ values of 7.40 (a) or 6.40 (b). ³¹P-n.m.r. spectra were continuously recorded covering a 2 min time interval. Data are means ± S.E.M. for three experiments in each group.

DISCUSSION

It has been shown that PCr accumulation in the transgenic mouse liver depended on prior supplementation of the food with high amounts of creatine [10 % (w/w) of the diet] for several days (Koretsky et al., 1990). In a recent study aimed at examining the possible zonation of the artificially induced creatine kinase (Quistorff et al., 1993), we observed that total creatine content ([PCr + Cr]) in the liver of both normal and transgenic mice was highly variable when the animals were on the creatine feeding schedule mentioned above. Prompted by this observation we decided to investigate the kinetics of creatine uptake in the perfused mouse liver.

Creatine synthesis in human and rodents

Creatine is either derived directly from the diet (e.g. in meat) or synthesized in several organs from amino acids in a two-step process. In the first step, guanidoacetate is formed from arginine and glycine in a reaction catalysed by an amidinotransferase (EC 2.1.4.1.; Walker, 1979). This enzyme is co-repressed by creatine and some of its synthetic analogues (Roberts and Walker, 1985), and displays vastly different distributions between species and organs. In human, the pancreas appears to be the most active organ for creatine biosynthesis, more so than liver and kidney (Walker, 1963; Van Pilsum et al., 1972). Muscle and brain tissues are not competent for creatine synthesis, but rely on PCr for their energy metabolism. The situation is different in rodents where the liver apparently lacks the amidinotransferase (Walker, 1963; Van Pilsum et al., 1972), which leave the pancreas and the kidneys as the main sources of guanidoacetate (Koszalka, 1967). In general, it seems that organs capable of *de novo* creatine synthesis do not express creatine kinase significantly and vice versa (Van Pilsum et al., 1972; Yanokura and Tsukada, 1982) although this appears not to be the case for the rodent liver.

Creatine concentration varies significantly between species and in different organs. Blood creatine is reported to be 0.14–0.16 mM in rats (Fitch and Shields, 1966; Meert et al., 1991), 0.24 ± 0.03 mM in mice (Kim et al., 1983) and as low as 40 ± 1 μM in the plasma of healthy human (Mussini et al., 1984). In the portal blood of normal and transgenic mice, we found creatine concentrations of 65 ± 9 and 53 ± 13 μM respectively (Table 1). Highly variable concentrations of creatine are similarly reported in rodent muscle tissues, ranging from 3 to 30 μmol · g wet weight⁻¹ (Haughland and Chang, 1975; Kim et al., 1983; Yanokura et al., 1984; Reddy et al., 1992; Brosnan et al., 1993). On the other hand, rodent liver contains much lower creatine,

Table 3 Kinetic parameters of creatine transport in perfused liver from transgenic mouse

The data are calculated from the progress curves of PCr accumulation (Figure 2) at different concentrations of creatine, after conversion into total creatine content (see the Experimental section). The initial rates of creatine uptake were calculated for each concentration of infused creatine and each perfusate pH. V_{max} and K_m were computed from these initial rates and the perfusate concentration of creatine assuming a Michaelis–Menten type of kinetics.

Kinetic parameter	Creatine (mM) ...	pH ₀ 7.40			pH ₀ 6.40		
		0.5	2.0	5.0	2.0	5.0	15.0
Initial rate (μmol · g of liver ⁻¹ · min ⁻¹)		0.21 ± 0.02	0.43 ± 0.03	0.48 ± 0.03	0.37 ± 0.06	0.97 ± 0.04	1.25 ± 0.02
K _m (mM)		—	0.80 ± 0.18	—	—	5.12 ± 2.40	—
V _{max} (μmol · g of liver ⁻¹ · min ⁻¹)		—	0.57 ± 0.04	—	—	1.72 ± 0.32	—

Figure 3 Evolution of hepatic PCr during fructose infusion to perfused livers from transgenic mice fed a diet supplemented with creatine

Transgenic mice were fed for 5 days with a diet supplemented with 10% (w/w) creatine. Livers were first perfused for 20 min with a Krebs–Henseleit buffer (pH_0 7.40). At time zero, either 10 mM fructose (a) or 10 mM fructose plus 5 mM creatine (b) were added to the medium. After 15 min, fructose or fructose + creatine infusion was discontinued. ^{31}P -n.m.r. spectra were recorded every 2 min and the evolution of PCr is presented. Data are means $\pm$ S.E.M. for ten experiments (fructose infusion alone) or three experiments (co-infusion of fructose and creatine).

ranging over $0.16\text{--}0.53\ \mu\text{mol}\cdot\text{g wet weight}^{-1}$ (Yanokura and Tsukuda, 1982; Yanokura et al., 1984; Meert et al., 1991; Reddy et al., 1992), in accordance with the present results (0.48 ± 0.08 and $0.50 \pm 0.05\ \mu\text{M}$ in control and transgenic mice respectively). It is therefore obvious that the gradient of total creatine across the plasma membrane is much higher in muscle than in liver tissues. For instance, this gradient amounts to 48-fold in the sheep *biceps femoris* but is only 5-fold in the liver of the same animal (Henderson et al., 1983). In the perfused rat heart the gradient is about 100-fold (Seppet et al., 1985) and 45-fold in fibroblasts (Daly and Seifter, 1980). Accordingly, we found a gradient of total creatine in the mouse liver *in vivo* of about 8-fold (Table 1).

In the present study, the portal concentration of creatine was increased 5–15 times by the creatine feeding protocol (see Table 1). However, the evaluation of the creatine concentration in the portal blood was based on only one time point, after approx. 6 h into the light period of a 12 h/12 h alternate dark/light circadian cycle. It is therefore possible that the blood creatine concentration could have been significantly higher at an earlier time point. In fact, this would seem to be the only explanation for the very high concentrations of total creatine reached in the transgenic animals subjected to creatine feeding (20.8 mM, see Table 1), since it appears from the perfusion experiments that the maximal attainable intracellular/extracellular creatine concentration gradient is of the order of 2–3-fold. Furthermore, as explained in the Results section, it is likely that the creatine concentration measured in the liver biopsies taken *in vivo* is too high due to unavoidable hydrolysis of PCr in the interval between blood sampling from the portal vein and freeze-clamping of the liver.

The fact that PCr is present in the liver of control mice fed with creatine in significant amounts (see Table 1), even though creatine kinase does not appear to be expressed in the hepatocytes (except perhaps for a low activity of the mitochondrial enzyme CK_m), can probably be accounted for by significant activity of CK_{BB} in sinusoidal and Kupffer cells (Vaubourdolle et al., 1993).

Transport of creatine into the perfused mouse liver

Transport of creatine across the plasma membrane has mainly been investigated in tissues relying on creatine uptake for their synthesis of PCr, such as skeletal muscle, heart and brain. In these organs, creatine is co-transported with sodium ions by a saturable process with a K_m of the order of $50\ \mu\text{M}$, matching the systemic creatine concentration in the rat (Seppet et al., 1985). Such transporters have been described in fibroblasts and smooth muscle cell lines (Daly and Seifter, 1980) or in the human muscle cell line L_6 (Loike et al., 1988). However, a much higher K_m of $0.5\text{--}0.6\ \text{mM}$ has been found in the rat extensor digitorum longus muscle (Fitch and Shields, 1966; Haughland and Chang, 1975). Recently, the cDNA for a creatine transporter has been cloned and shown to depend upon Na^+ and Cl^- ions for its activity. In the rabbit, this carrier was mainly expressed in kidney, brain, heart and skeletal muscle, but remained undetectable in the liver (Guimbal and Kilimann, 1993). Creatine transport is modulated by (i) the hormonal (Haughland and Chang, 1975) and feeding states of the animal (Kim et al., 1983); (ii) the presence of P_i in the perfusion medium as shown in rabbit heart (Gitomer et al., 1992), and (iii) is downregulated by a physiological concentration of creatine (Loike et al., 1988). In addition to the saturable creatine uptake process, a non-saturable mechanism has been described in red blood cells (Syllm-Rapoport et al., 1980), which, however, appears to account for only a small fraction of the total creatine uptake in skeletal muscle (Fitch and Shields, 1966; Haughland and Chang, 1975; Kim et al., 1983), heart (Seppet et al., 1985), smooth muscle cell lines (Daly and Seifter, 1980), monocytes and macrophages (Loike et al., 1986).

Creatine transport has not previously been studied in the rodent liver and in the present study we have demonstrated that creatine is transported by an active, saturable mechanism strongly dependent on extracellular pH. At an external pH of 7.40, the K_m value of creatine transport was $0.80 \pm 0.18\ \text{mM}$ (Table 3), a value more than one order of magnitude higher than in muscle, brain and heart. Lowering pH_0 to 6.40 led to a K_m increase of more than 6-fold, as opposed to reported results from human erythrocytes, where an external pH of 6.9 did not change the creatine transport characteristics (Syllm-Rapoport et al., 1980). We do, however, have indications that the pH effect on K_m is not linear

and may be much more pronounced between pH_0 6.90 and 6.4 than between 7.4 and 6.9 (results not shown). The progress curves for creatine uptake are very different at the two pH values. While at pH_0 6.4 an apparent equilibrium was reached after only 15–25 min of perfusion, steady state was clearly still not reached after 80 min at pH_0 7.4, corresponding to the lower V_{max} measured at this pH (Figure 2 and Table 3). The value of creatine intracellular/extracellular concentration gradient at pH 7.4 calculated after 80 min is therefore an underestimation of the equilibrium value, which in the perfused organ at pH 7.40 may be much closer to the value observed *in vivo* of about 9. The pronounced decrease of the creatine concentration gradient across the plasma membrane by increasing external creatine seen at both pH values remains unexplained, although it may suggest that there is more than one creatine transport mechanism in operation. There are no reports about the pH effect of creatine transport in skeletal muscle. Under *in vivo* conditions the observed creatine transport kinetics would correspond to a net uptake of about $35 \text{ nmol} \cdot \text{g of liver}^{-1} \cdot \text{min}^{-1}$ at a portal creatine concentration ($53 \mu\text{M}$) characteristic of the non-supplemented animal.

P_i plays a role in creatine transport. Indeed, removing P_i from the perfusion buffer increased the intracellular content of creatine in rabbit heart, presumably through a decrease of creatine efflux mediated by an enhanced sodium electrochemical gradient across the plasma membrane (Gitomer et al., 1992). Similarly in the perfused liver from transgenic mice omission of P_i from the perfusate induced a very significant decrease of PCr accumulation (Koretsky et al., 1993). In the present study, we have found that decreasing the pH of the perfusion increased both K_m and V_{max} for creatine transport (Table 3). It is not clear whether these changes may be explained as secondary effects of pH on the electrochemical sodium gradient.

Efflux of creatine from the liver

The liver of higher mammals synthesizes creatine from amino acids and exports it to other organs dependent on PCr for their energy metabolism (Walker, 1979). This would seem to require an efficient process of creatine excretion. However, rodent liver lacks arginine:glycine amidinotransferase, the first of the two enzymes involved in *de novo* synthesis of creatine and in those animals creatine is mainly synthesized in the kidneys and the pancreas (Van Pilsum et al., 1972). Since the rodent liver does not appear to synthesize creatine, it was of interest to investigate the creatine efflux capacity from the perfused liver. Two series of experiments were conducted in order to evaluate creatine efflux. In the first series, the mice were fed for 5 days with a diet supplemented with creatine to induce an hepatic accumulation of PCr ($12.06 \pm 0.11 \mu\text{mol} \cdot \text{g of liver}^{-1}$). Since a non-recirculating perfusion set-up was used, the fact that no significant decrease of total creatine could be observed during 80 min of perfusion (see Figure 3), would seem to corroborate the notion that the mouse liver does not contribute significantly to body creatine synthesis. This conclusion was further confirmed with the second series of experiments where a rapid PCr depletion (and concomitant creatine increase) was elicited by fructose infusion, again with no significant export of creatine (Figure 3). These results are in agreement with other investigators who found a very limited efflux of creatine from human macrophage (Loike et al., 1986), isolated rat heart (O'Brien and Nutbeam, 1976) or atria (Savabi, 1988). Similarly, perfused rat hearts acutely preloaded with β -guanidinopropionic acid (an analogue of creatine and a competitive inhibitor of its transport) do not display a decrease of the corresponding intracellular phosphorylated metabolite when

infused without the guanidine compound, suggesting a very limited efflux in these conditions (Unitt et al., 1993). To the best of our knowledge, there have been no investigations of creatine efflux from the liver of higher animals, which is supposed to export creatine. Creatine efflux from skeletal muscle is also very limited (Fitch and Shields, 1966) although increased by fasting (Kim et al., 1983) or tri-iodothyronine-induced hyperthyroidism (Kurahashi and Kuroshima, 1977).

Conclusion

At physiological blood creatine concentrations creatine does not accumulate in the normal or transgenic mouse liver. However, when supplemented with creatine either *in vivo* in the diet or in the medium to the perfused organ, creatine accumulates with an intracellular/extracellular concentration gradient which depends on the external creatine concentration and pH, but which is much lower than in organs like skeletal muscle or heart. The creatine uptake of the mouse liver appears to follow Michaelis–Menten-type kinetics where both K_m and V_{max} depend strongly on external pH. Creatine export from the perfused mouse liver is slow and undetectable within 80 min of perfusion, suggesting that in the mouse the liver does not play a significant role in the supply of creatine to other organs.

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