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NMR detection of creatine kinase expressed in liver of transgenic mice: Determination of free ADP levels

(³¹P NMR/hepatic energy metabolism/phosphocreatine/transthyretin promoter)

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ABSTRACT **To use the equilibrium established by creatine kinase (CK) to determine hepatic free ADP levels, the transcriptional control elements of the transthyretin gene were used to direct expression of the CK B isozyme to the livers of transgenic mice. Activities of CK ranging from 80–250 μmol per min per g (wet weight) were detected in liver extracts from five founder mice. The CK activity was stably transmitted to subsequent generations. Isozyme gels and immunoblots confirmed that the activity detected in extracts was due to the B isozyme of CK. Immunohistology indicated that the protein was expressed uniformly throughout the liver and was localized primarily to the cytoplasm. ³¹P NMR spectroscopy was used to detect the metabolic product of the CK reaction, phosphocreatine, demonstrating that the enzyme was active *in vivo*. The phosphocreatine level fell rapidly during anoxia ($t_{1/2}$ = 1 min), indicating that the CK reaction was integrated into hepatic energy metabolism. The equilibrium established by CK was used to calculate a hepatic free ADP level of 0.059 ± 0.004 μmol/g (wet weight). *In vivo* NMR studies of these mice will be valuable for studying the role of free ADP in regulating liver metabolism.**

Adenosine diphosphate (ADP) is an important regulator of many metabolic pathways *in vitro*, including oxidative phosphorylation (1), glycolysis (2), gluconeogenesis (2), and ion transport (3). Due to difficulties in measuring free ADP levels in intact liver it has been impossible to assess the role of this key metabolite in regulating hepatic metabolism *in vivo* (4). To study changes that may occur during perturbations of liver metabolism, ADP is usually assayed from whole cell extracts or from liver cells after subcellular fractionation into cytoplasmic and mitochondrial compartments (4–6). However, it is generally acknowledged that for metabolites such as ADP, the concentrations of which are low and comparable to the number of intracellular binding sites, measurements from extracts lead to an overestimation of free metabolite levels (4).

Hans Krebs introduced the idea of using cellular reactions near equilibrium to determine the levels of free metabolites at low concentrations (7). This technique relies on measuring compounds present in relatively high concentrations that are in chemical equilibrium with the metabolite of interest. Ideally, a single equilibrium using metabolites that can be monitored *in vivo* should be used. The reaction catalyzed by creatine kinase (CK), phosphocreatine (PCr) + MgADP + H^+ = MgATP + creatine, is the method of choice for the determination of ADP (4). The value of the equilibrium constant is known for a variety of conditions, and free ADP levels can be calculated from *in vivo* nuclear magnetic resonance (NMR) measurements of PCr, ATP, creatine, Mg^{2+}, and pH. This approach has been successfully used to study the role of ADP in metabolic regulation in heart (8), muscle (9), and brain metabolism (10). However, it has not been useful for liver due to the lack of significant levels of CK.

In recent years transgenic animal technology has evolved into a powerful approach to studying and manipulating gene expression (11). DNA can be microinjected into the pronuclei of fertilized eggs enabling it to integrate into the genome and be present in every cell of the mature adult. A number of control elements have been mapped that define the specific cell type in which a transgene will express. Furthermore, it has been shown that these control elements can direct the expression of heterologous genes to the proper tissue. The transgenic mouse technique has been very useful for studies of gene expression and for producing animal models of disease (11).

To overcome the lack of CK activity in liver, we have used the transgenic mouse technique to direct high-level expression of CK to the liver. ³¹P NMR was used to detect CK *in vivo* by measurement of the product of the reaction, PCr. A combination of NMR and HPLC data was used to calculate a free ADP level of 0.059 ± 0.004 μmol/g (wet weight) in transgenic livers. This work illustrates that the combination of transgenic technology with *in vivo* NMR will provide a useful technique for understanding metabolic control in the intact animal.

MATERIALS AND METHODS

Production and Screening of Transgenic Mice. An *Eco*RI–*Acc* I fragment containing the liver-specific promoter and enhancer regions from the mouse transthyretin gene (12) was removed from plasmid preAlb/PC_2AT (gift from R. Herbst and J. Darnell, Rockefeller University, New York) and cloned into the corresponding sites in pGEMBlue3 (Promega). The vector *Pst* I site was changed to a *Kpn* I site by using *Pst* I linkers (Boehringer Mannheim), and an internal *Bam*HI site was removed by cutting with *Bam*HI, blunting the ends, and ligating. The *Eco*RI–*Kpn* I fragment was then isolated and cloned into corresponding sites of the IBI30 vector (IBI) to provide appropriate downstream cloning sites. A *Bam*HI–*Apa* I fragment containing the coding region from the rat brain CK gene (13) was removed from the plasmid pAM170 (gift from P. Benfield, DuPont) and was inserted downstream of the transthyretin regulatory elements, creating the plasmid pTTRCKB. The *Eco*RV–*Sph* I DNA fragment from pTTRCKB was used for generating transgenic mice after dialysis against injection buffer (5 mM Tris·HCl/5 mM NaCl/0.1 mM EDTA, pH 7.4) using a Millipore VM 0.05-μm filter. Standard recombinant DNA techniques were used in the above constructions (14), and all DNA fragments

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Abbreviations: CK, creatine kinase; CK_{BB}, the B isozyme of CK; PCr, phosphocreatine.

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were isolated by electrophoresis in low-melting-point agarose followed by NACS (BRL) chromatography, as described (15).

Transgenic mice were generated by microinjection of ≈2 pl of injection buffer containing DNA fragment at 2 ng/μl into one of the two pronuclei of fertilized mouse eggs as described (15). Four hundred and fifty embryos were injected, which produced 98 offspring. Offspring that had incorporated the injected DNA were identified by Southern hybridization (16) of digested tail DNA by using a portion of pTTRCKB as probe. In subsequent generations the mice were screened by the polymerase chain reaction assay (17) on tail DNA with oligonucleotides specific for the transgene. Isolation of tail DNA, hybridization, and labeling of probes were done as described (15).

Analysis of Tissue Extracts. Extracts for gels were made by homogenizing freshly excised tissue with a Polytron homogenizer (Brinkmann) at a setting of 6 for 15 sec in 1:10 dilution of buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM cysteine, 0.1 mM EGTA, and 10 mM Tris·HCl at pH 7.4. The extract was centrifuged at 14,000 × *g* for the immunoblot and 25,000 × *g* for the agarose gel. For the immunoblot, proteins were transferred electrophoretically from 10% SDS/polyacrylamide gels onto nitrocellulose filters. After being blocked for 30 min with 3% gelatin in Tris-buffered saline (pH 7.5), the filters were washed with Tris-buffered saline containing 0.05% Tween 20 for 10 min and then incubated with 1 μg of lyophilized anti-human B isozyme of CK (CK_{BB}) rabbit serum (Calbiochem) in 25 ml of Tris-buffered saline. After being washed for 10 min, the filters were incubated with horseradish peroxidase-conjugated anti-rabbit IgG and washed. The peroxidase reaction was initiated with horseradish peroxidase color development solution (Bio-Rad).

For the agarose gel electrophoresis, extracts were loaded onto 0.3-mm thick 1% agarose gels and run at 22 V/cm for 2 hr in 0.05 M barbitol buffer at pH 8.6. CK activity was visualized by coupling the production of ATP catalyzed by CK from PCr and ADP to the hexokinase and glucose-6-phosphate dehydrogenase reactions. Production of NADPH by glucose-6-phosphate dehydrogenase was visualized with a redox stain (Innovative Chemistry, Marshfield, MA). After electrophoresis, 0.5 ml of the CK visualization mixture was spread on the gel, incubated for 30 min at 37°C, washed in distilled water, and then dried at 55°C.

Quantitative CK assays were performed using a coupled enzyme assay (18). Tissue was homogenized for 10–15 sec in the Polytron at a setting of 6 in 1:5 dilution of extraction buffer containing 50 mM triethanolamine·HCl (pH 7.4), 20 mM 2-mercaptoethanol, 1 mM EDTA, and 2 mM $MgCl_2$. The homogenate was diluted 1:1000 in extraction buffer, and 40 μl of the diluted extract was added to 1 ml of assay buffer at 25°C containing 130 mM KCl, 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 2 mM AMP, 50 μM diadenosine pentaphosphate (to inhibit adenylate kinase activity), 5 mM glucose, 0.7 mM NADP, 1.5 mM ADP, and 2 units each of hexokinase and glucose-6-phosphate dehydrogenase (Boehringer Mannheim). The rate of increase in absorbance at 340 nm was used to determine the CK activity and was linear with the volume of extract added.

Immunohistology. Liver was fixed with 95% ethanol/5% acetic acid and then embedded in paraffin. Sections (6 μm) were deparaffinized with xylene, rehydrated, and washed for 5 min with methanol containing 0.5% H_2O_2 to inhibit endogenous peroxidase. After being rinsed with 50 mM Tris·HCl (pH 7.6)/0.8% NaCl, the sections were blocked with blocker reagent (Biostain super ABC kit, Biomedia, Foster City, CA) and incubated with rabbit anti-human CK_{BB} antibody (1:500 dilution, Ventrex Laboratories, Portland, ME) for 1 hr. The slides were washed for 20 min with Tris-buffered saline/0.3% Triton X-100 and then incubated for 30 min with biotinylated anti-rabbit IgG antibody (Biostain super ABC kit, Biomedia). Sections were washed with Tris-buffered saline/0.3% Triton X-100 for 10 min and then with Tris-buffered saline alone for 5 min. Slides were then incubated with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 40 min, washed with Tris-buffered saline/0.5% Tween 20 for 10 min, and then washed with Tris-buffered saline alone for 10 min. Color was developed by incubating the slide for 5 min in 10 ml of 50 mM Tris·HCl (pH 7.6) containing 5 μl of 30% H_2O_2 and 5 mg of diaminobenzidine. Finally the sections were washed with H_2O and photographed through a Nikon Diaphot microscope. Slides were made containing both a section of nontransgenic tissue alongside a section of transgenic tissue so that both tissues were processed and photographed identically.

NMR. ^{31}P NMR spectra were acquired at 121.5 MHz in a Bruker 7.0-Tesla, 15-cm bore, Biospec II imaging spectrometer. A 0.8-cm surface coil tuned to 121.5 MHz was positioned just above the surgically exposed liver. For quantitating metabolite ratios and chemical shifts, 600 acquisitions were averaged by using a 90° pulse at the surface coil with a 7-sec delay between pulses. Increasing the delay time to 15 sec had no effect on metabolite ratios, indicating that 7 sec was long enough for full relaxation to occur. For anoxia experiments, 1-min time resolution was achieved by summing 300 acquisitions by using a 90° pulse at the surface coil and 200-msec recycle time. Thirty-hertz exponential line-broadening was applied to all spectra before Fourier transformation. Peak areas were determined by using the Bruker integration routine. pH was calculated based on the chemical shift of P_i with respect to PCr (19), and the free Mg^{2+} was calculated from the chemical shift difference between the β and α phosphate peaks of ATP (20).

Liver normally contains too low a level of creatine to easily quantitate on HPLC or to produce enough PCr to be easily detected by NMR. It has been shown that dietary supplementation with creatine leads to an accumulation of creatine in liver (21). Therefore, before experiments all mice were fed a diet supplemented with 2% creatine for 5 days. Immediately before experiments, mice were anesthetized with pentobarbital at 60 mg/kg. The liver was exposed, and the animal was placed on an NMR holder with a heated water pad that maintained temperature at 37°C. Anesthesia was maintained with 2% halothane in a 66% N_2O/33% O_2 mixture. Anoxia was induced by switching the gas flow to 100% nitrogen.

HPLC Analysis of Tissue Extracts. Liver was rapidly frozen either directly after NMR experiments or just after surgically exposing the liver by clamping between aluminum plates cooled with liquid nitrogen. Frozen tissue was pulverized in 5 vol of 4% (vol/vol) perchloric acid, warmed to 4°C, and centrifuged at 14,000 × *g* for 10 min. The supernatant was neutralized with 5 M KOH in 0.5 M triethanolamine·HCl and spun for 10 min at 14,000 × *g*. HPLC was used to quantitate levels of creatine, PCr, and ATP using a Partisil 10 Sax ion-exchange column (Whatman) according to the procedure of Harmsen *et al.* (22).

RESULTS AND DISCUSSION

To direct expression of CK to the mammalian liver, we generated a DNA construct in which the gene for the coding region of the mouse CK brain isozyme (CK_{BB}) was driven by the liver-specific transcriptional regulatory signals of the mouse transthyretin gene. The transthyretin elements have been shown to direct high-level expression to the liver of transgenic mice (23). Eight founder mice carrying the transgene were identified by Southern analysis. Five of the founder mice had CK activity in extracts from liver that ranged from 80 to 250 μmol/min/g (wet weight) at 25°C. This compares to <1 μmol/min/g (wet weight) in control liver and

250 μmol/min/g (wet weight) in mouse heart. The CK_{BB} gene and CK activity were transmitted to offspring in three mouse lines that have been established.

To determine that the CK activity that was detected in transgenic liver extract was due to CK_{BB}, immunoblotting analysis was performed (Fig. 1*A*). A single band was detected in extracts from mouse brain (lane 1) and transgenic mouse liver (lane 3) but was absent from the livers of nontransgenic littermates (lane 2). Agarose gel electrophoresis, which separates CK isozymes, confirmed that the protein detected in transgenic liver extract was CK_{BB} (Fig. 1*B*). Transgenic liver extracts (lanes 5 and 6) contained activity absent from nontransgenic littermates (lanes 3 and 4) and indistinguishable from purified rabbit CK_{BB} (lane 2) or from activity detected in mouse brain extracts (data not shown). Therefore, the transthyretin promoter was successful in directing expression of high levels of CK_{BB} to liver. CK has thus been expressed in an organ that normally does not contain it. Recently, CK has been expressed in *Escherichia coli* (24) and *Saccharomyces cerevisiae* (25).

Immunoblot analysis of subcellular fractions indicated that >80% of the CK was cytosolic with a small amount detected in the post 14,000 × *g* pellet. This could be due to nonspecific binding or to specific localization of CK. It is known that CK localizes to myofibrils, plasma membrane, and sarcoplasmic reticulum in muscle and heart (26). Immunohistology showed that transgenic liver (Fig. 2*A*, *Right*) stained darkly compared with nontransgenic liver (Fig. 2*A*, *Left*). The staining was diffuse throughout different regions of the liver, indicating that CK was expressed in periportal, as well as pericentral regions. This is consistent with the normal cell specificity of transthyretin in mouse as determined by *in situ* hybridization (C. Yan and T.V.D., unpublished observation). A higher magnification showed staining throughout the cytoplasm of hepatocytes with little staining in nuclei (Fig. 2*B*).

^{31}P NMR spectra were acquired from the liver *in situ* to demonstrate that the CK_{BB} detected in extracts was active *in vivo* (Fig. 3). To increase the liver content of creatine all mice were fed a diet supplemented with 2% creatine for 5 days.

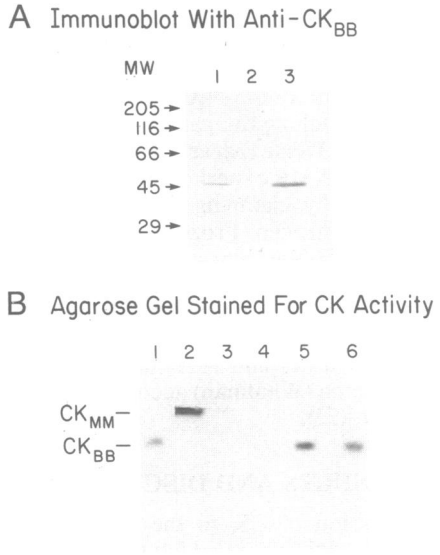

FIG. 1. (*A*) Immunoblot of tissue extracts using antibody against human brain CK (Calbiochem). Lanes: 1, mouse brain; 2, control mouse liver; 3, CK-positive liver. A single band of the appropriate molecular weight (MW) ($\times 10^{-3}$) for CK is detected in brain and CK-positive liver. (*B*) Agarose gel stained for CK activity with a histochemical stain. Lanes: 1, purified rabbit brain CK (Sigma); 2, purified rabbit muscle CK (CK_{mm}) (Sigma); 3 and 4, control mouse liver extract; and 5 and 6, CK-positive transgenic mouse liver extract.

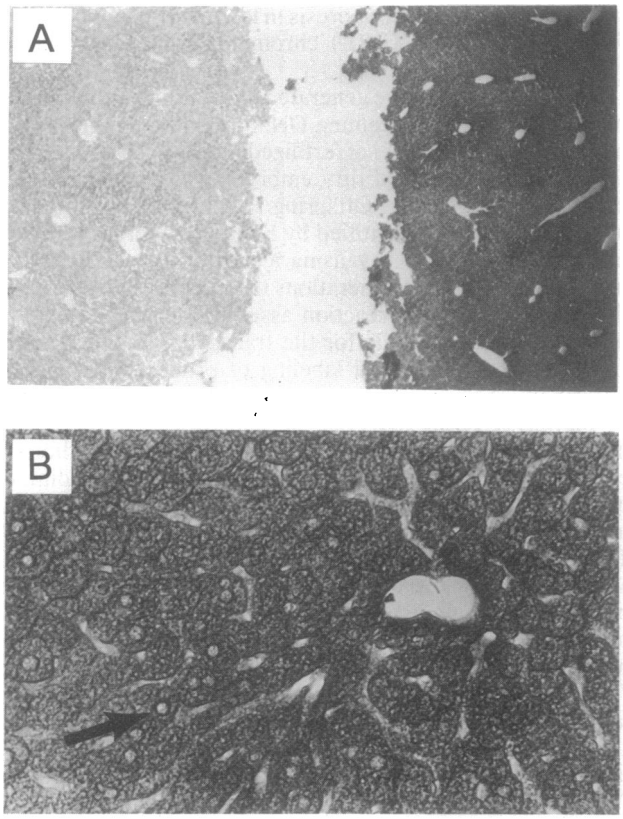

FIG. 2. Immunolocalization of CK in liver of transgenic mice. (*A*) Low-magnification view (×36) showing nontransgenic liver (left) without CK and transgenic liver (right) with CK. Staining is seen in the liver containing CK (right) and is diffuse throughout all regions of the liver. (*B*) High-magnification view of transgenic liver containing CK (×180) showing that the staining is cytoplasmic with little staining of nuclei (arrow).

Peaks due to phosphomonoesters (PME), inorganic phosphate (P_i), and the three phosphates of ATP were detected in both

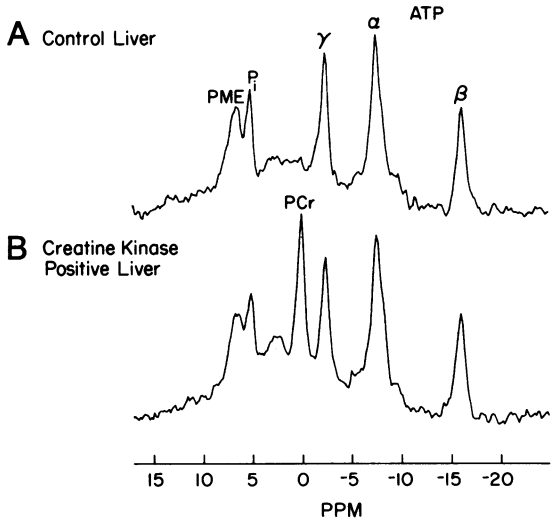

FIG. 3. ^{31}P NMR spectra obtained from control (*A*) and CK-positive liver (*B*). Peaks are as labeled with PME, phosphomonoester; P_i, inorganic phosphate; PCr, phosphocreatine; γ, α, and β, the three phosphates of ATP. The PCr peak resonates 2.47 ppm downfield from the γ phosphate of ATP peak. Spectra represent the accumulation of 600 free induction decays using a 90° pulse at the surface coil and 7-sec recycle time. Thirty-hertz linebroadening was applied before Fourier transformation.

control and CK-positive liver. A new peak resonating between the P_i peak and the γ phosphate of ATP peak was clearly detected in the spectrum from the transgenic liver (Fig. 3*B*); this peak was absent from control liver (Fig. 3*A*). The peak resonated 2.47 ppm downfield from the γ phosphate of ATP peak, which is at the proper chemical shift to represent PCr (27). In addition, HPLC analysis of extracts from CK-positive liver showed a peak with the same retention time as pure PCr, the area of which increased when PCr was added to the extract. This peak was absent from control liver extract. Thus, the new peak detected in the NMR spectrum of transgenic liver was PCr formed from the reaction catalyzed by CK_{BB}. The fact that the targeted expression of a transgene leads to a detectable NMR peak opens the possibility of using NMR as a noninvasive assay for gene expression.

The transgenic mice show normal behavioral, reproductive, and aging patterns. Preliminary measurements (data not shown) indicate that there are no ultrastructural effects as visualized by electron microscopy and that the level of activity of lactate dehydrogenase (a cytosolic enzyme) and citrate synthase (a mitochondrial enzyme) are the same in normal and transgenic mice. In addition, pH, Mg^{2+}, P_i, and ATP levels are not different in normal and transgenic mice (Table 1). By these criteria, expression of CK in liver had no effects on metabolism.

In tissues that normally contain CK, such as brain and heart, inhibiting the oxidative production of ATP by removing oxygen leads to a rapid fall in PCr due to an increase in ADP. To test whether the PCr detected in mouse liver behaved similarly, the time course of changes in phosphate metabolites was followed after anoxia. Fig. 4 shows that anoxia led to a dramatic fall in both PCr ($t_{1/2}$ = 1 min) and ATP ($t_{1/2}$ = 7 min). There was a large increase in P_i associated with the fall in PCr and ATP (data not shown). This result indicates that the PCr level was sensitive to the energy status of the liver.

The PCr peak detected in the NMR spectrum (Fig. 3*B*) was in a steady state for at least 2 hr. The CK activity measured in extracts was >10 times the net ATP turnover rate as estimated from liver oxygen consumption data (28). The fact that the PCr fell much more rapidly than did the ATP during anoxia (Fig. 4) shows that the CK activity *in vivo* is greater than cellular ATPase activity. These data indicate that CK activity in transgenic liver was high enough to maintain the reaction at equilibrium. The free ADP level was calculated using the equilibrium expression, [ADP] = [ATP][creatine]/[PCr]K_{eq}, where K_{eq} is the equilibrium constant for the reaction and is equal to 158 at pH 7.2 and a free [Mg^{2+}] of 0.65 mM (29). HPLC was used to determine ATP and total creatine [creatine + PCr], and NMR was used to determine the PCr/ATP ratio, pH, and free Mg^{2+} concentration. Table 1 summarizes the data obtained from control and transgenic mice. By using these data a value of 0.059 ± 0.004 μmol/g (wet weight) for the free ADP level was calculated. This level of free ADP is in the range where changes can substantially modulate the activity of a number of important processes, such as oxidative phosphorylation (1). This is in contrast to

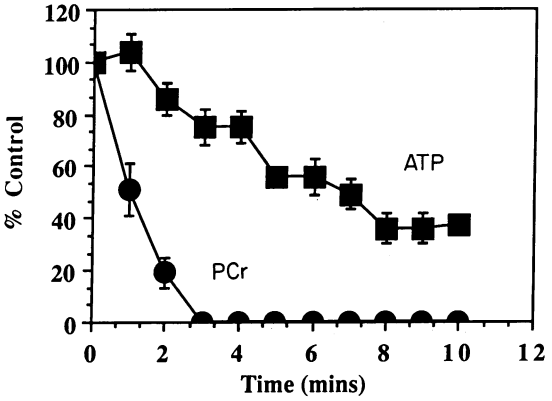

FIG. 4. Time course of changes in NMR detected levels of PCr (●) and ATP (■) after inducing anoxia. Peak areas for PCr and the β phosphate of ATP were integrated from spectra acquired in 1 min. Three spectra were acquired before inducing anoxia, and the areas from these spectra were averaged and assigned the value 100%. All other time points are reported as % of this control value. Each point represents the average of 5 animals, and the error bars correspond to ± SEM.

the value of ≈1 μmol/g (wet weight) measured in whole tissue extracts (4), which is far above the level of ADP expected to be regulatory.

Introduction of the CK reaction into a cell should have no direct effect on steady-state levels of ADP because PCr is not involved in any other reactions and the reaction is at equilibrium (30). Therefore, the value of ADP obtained in the transgenic livers probably reflects the level in nontransgenic liver as well. A potential problem with using the CK reaction to determine ADP levels would arise if the measured metabolite concentrations did not reflect the levels accessible to CK. This is clearly not a problem for PCr because anoxia leads to a rapid utilization of all the PCr. However, 12% of total liver ATP may be mitochondrial (5), and some portion of the creatine may not be available to CK (31). An overestimation of ATP or creatine concentrations accessible to CK would lead to an overestimation of the calculated free ADP level. The value determined here, therefore, represents an upper limit.

Determination of total ADP levels in perchloric acid extracts of liver gives a value of ≈1 μmol/g (wet weight) (4) or an ATP/ADP ratio of ≈3. This is significantly less than the value of 46 for the ATP/ADP ratio determined in the present study based on the ADP detected by the CK equilibrium. Our value of ATP agrees with previous results (4), indicating that the discrepancy in ATP/ADP ratio is due to the difference in ADP levels. A similar difference between extract determination of total ADP and equilibrium calculations of free ADP also occurred in muscle and brain (4). An overestimation of free ADP levels by extracts results because there are a number of enzymes that bind ADP as well as other cellular sites (e.g., mitochondria, actin) that can sequester ADP. Fractionation of

Table 1. NMR and HPLC data from control and CK-positive liver

	pH*	Mg^{2+},† mM	PCr/ATP	HPLC ATP	HPLC‡ total creatine, μmol/g (wet weight)	PCr	ADP§
Control	7.17 ± 0.03	0.58 ± 0.20	0.06 ± 0.03	2.70 ± 0.35	—	—	—
CK-positive	7.22 ± 0.02	0.65 ± 0.20	1.40 ± 0.09	2.64 ± 0.22	16.8 ± 01.9	3.77 ± 0.41	0.059 ± 0.004

All values are mean ± SEM with n = 6 − 13.

*pH was calculated using the relation, pH = 6.75 + log (δ − 3.27/5.69 − δ) with δ being the chemical shift between P_i and PCr in ppm (19).

†[Mg^{2+}] was calculated using the relation [Mg^{2+}] = 0.05 [10.81 − $\delta_{\alpha\beta}/\delta_{\alpha\beta}$ − 8.32], where $\delta_{\alpha\beta}$ is the chemical shift between the α and β phosphate peaks of ATP (20).

‡Total creatine = creatine + PCr.

§ADP was calculated from the equation: [ADP] = [ATP][creatine]/[PCr]K_{eq}, with K_{eq} = 158.

liver cells to determine the cytosolic ATP/ADP increases the ratio determined by extracts to between 6 and 8 (5, 6). This is still far below 46, implying there are cytosolic sites in liver that can sequester large amounts of ADP.

NMR spectroscopy has previously been used to determine ADP levels directly in normal liver by determining the difference in areas between the γ peak of ATP (which can contain contributions from the β phosphate of ADP) and the β ATP peak (which only comes from ATP). Values ranging from undetectable to 0.400 μmol/g (wet weight) have been reported by others (32–35). For low ADP levels this measurement is not accurate for two reasons. (*i*) NMR is not an inherently sensitive technique, making it difficult to detect molecules below 0.3 mM *in vivo*. (*ii*) The direct NMR measurement of ADP relies on determining a small difference between two large ATP peaks, making it difficult to quantitate ADP when it is low compared with ATP levels. In general, NMR peaks are detectable *in vivo* only when the metabolite is free to tumble. However, it is not clear whether ADP detected by this method represents free ADP because NMR signals from ADP bound to a number of enzymes have been detected *in vitro* (36) and may contribute to the direct NMR measurement of ADP *in vivo*. In addition, other nucleoside diphosphates, such as GDP or UDP, would contribute to the peaks. In the present study, we were not able to detect significant differences between the γ and β ATP peak areas (data not shown), consistent with the low value of free ADP calculated from the CK equilibrium.

A third method of determining liver ADP used a similar concept as that in the present study. However, rather than CK, the equilibrium established by the glycolytic enzymes, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, enolase, lactate dehydrogenase, and phosphoglyceromutase was used to calculate values ranging from 0.04 to 0.06 μmol/g (wet weight) for liver ADP levels (4, 37). This is in agreement with the value reported here. Use of the glycolytic enzymes has not found widespread application because of the large number of metabolites that must be measured and the number of equilibria involved. Using CK has major advantages because only one equilibrium is involved, and changes in ADP can be inferred easily and noninvasively by monitoring PCr, ATP, and creatine levels with NMR.

In conclusion, CK is an ideal marker enzyme for the measurement of free ADP levels in liver. This transgenic animal model will be extremely valuable for physiologic studies on the role of ADP in control of hepatic metabolic pathways *in vivo*. Many experiments can be envisioned using transgenic animals to vary enzyme levels or localization to study aspects of intermediary metabolism that are not yet fully elucidated. The combination of transgenic technology with NMR greatly expands our ability to study normal and abnormal physiology *in vivo*.

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