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In Vivo Metabolic Imaging of Cardiac Bioenergetics in Transgenic Mice

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Recent advances in transgenic technology have made the mouse a particularly interesting small animal in cardiovascular research. Increasingly sophisticated experimental methods and tools are needed for detailed characterization of cardiovascular physiology and biochemistry in the mice. The objective of this study was to develop a method for noninvasive evaluation of cardiac energy metabolism in the mouse. Cardiac gated ^{31}P magnetic resonance spectroscopy using Image Selected *in Vivo* Spectroscopy (ISIS) method was applied in old mice overexpressing bovine growth hormone (bGH) (n = 5) and control mice (n = 5). The localized volumes of interest were 128 and 112 μL, respectively. Phosphocreatine-to-ATP ratio was 1.5 ± 0.13 in the bGH mice and 2.1 ± 0.04 in the control group (P < 0.01). The study demonstrates the feasibility of application of volume-selective ^{31}P MRS for evaluation of cardiac energy metabolism in the mouse under maintained physiological conditions. © 2000 Academic Press

Key Words: **volume-selective ^{31}P MRS; ISIS; transgenic mice; myocardial energy metabolism; growth hormone; magnetic resonance spectroscopy; cardiovascular diseases; *in vivo* animal models.**

Recent advances in transgenic technology have made the mouse a particularly interesting small animal in cardiovascular research (1, 2). By manipulation of mouse genome it is now possible to study the consequences of altered gene expression (overexpression, absence, or suppression) of different proteins (e.g., receptors, enzymes) for normal physiology and pathophysiology of the heart. Although the importance of the mouse as experimental animal is continuously increasing, in comparison with other experimental animals, cardiovascular physiology and pathophysiology in the mouse is relatively scarcely described in the literature (3). Therefore, there is a clear need for further and detailed characterization of cardiovascular characteristics in the mouse. To achieve this objective one need to apply increasingly sophisticated experimental methods and tools. The use of magnetic resonance (MR) methods is growing in animal experimentation and MR is probably the most promising technique for characterization of different transgenic phenotypes. Thanks to the inherent properties, MR technique offers unique possibility for integrated and simultaneous study of organ anatomy, function, and biochemistry—a feature that no other technique offers at the present time (4). Myocardial energy metabolism has gained considerable attention in recent years since accumulating evidence indicates that disturbances in myocardial energy metabolism are associated with different diseases of the heart (5). Although mice and other small animals are most frequently used in cardiovascular research only few small-animal models for evaluation of myocardial bioenergetics with ^{31}P MR spectroscopy are described in the literature. Recently, we have described a new model for noninvasive evaluation of cardiac bioenergetics in the rat after induction of myocardial infarction (6). The purpose of this study was to extend the implementation of the method to the mouse and to evaluate its usefulness for detection of hypothetically disturbed myocardial energy metabolism in previously described transgenic mice with overexpression of bovine growth hormone (bGH) (7).

METHODS

Animals. The study protocol was approved by ethical committee of Gothenburg's University and was in agreement with the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animals were maintained on standard pellets and tap water *ad libitum*. Ten month old normal mice (BW = 25–35 g, n = 5) and transgenic mice overexpressing bovine growth hormone (bGH) (BW = 40–50 g; n = 5) were used. The animals were kept on 12-h light–dark cycle. The detailed description of experimental procedure for production of the bGH mouse strain has been reported previously (7).

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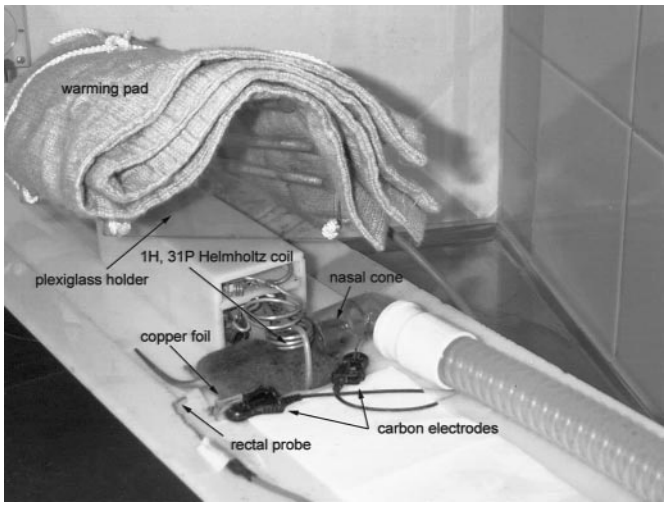

FIG. 1. The image depicts the mouse on the examination board prepared for the placement in the magnetic bore for localized cardiac gated ^{31}P MRS experiment. The mouse is anesthetized with isoflurane through the nasal cone and lies with the heart region inside the double tuned (^{1}H and ^{31}P) Helmholtz coil. The paws are wrapped in thin copper foil and connected via carbon electrodes to the monitoring system for control of heart rate. Constant body temperature was maintained by homeothermic blanket system consisting of the warming pad and rectal probe for regulation of the heat output. A homemade holder for warming pad was used to keep the pad at 5 cm distance from the coil as it interfered with homogeneity of the B_1 magnetic filed.

^{31}P MRS of mouse heart. MR imaging and spectroscopy experiments were performed on a 2.35 Tesla (T) horizontal magnet with a 20 cm bore (Bruker Biospec 24/30) interfaced with an X-32 acquisition system giving operating frequencies for ^{1}H and ^{31}P MR 100 and 40.5 MHz respectively. The homogeneity of the global B_1 magnetic field was optimized by adjustment of the current in the shim coils while the free induction decay of the water ^{1}H-signal was observed. Magnetic field homogeneity was accepted when the line width at half height was $<$0.5 ppm. A gradient system with maximum gradient strength of 125 mT/m equipped with digital preemphasis was used for gradients switching. A double tuned (^{1}H and ^{31}P) Helmholtz coil of 2 cm diameter was used for transmission and reception of RF. Mice were anesthetized with fentanyl/fluanizon (Hypnorm, Janssen) 0.5 mg/kg and diazepam (Stesolid, Dumex-Alpharma) 2.5 mg/kg. Constant body temperature (37 $\pm$ 0.5°C) was maintained by specially adapted homeothermic blanket system (Harvard Apparatus) consisting of the warming pad and rectal probe for regulation of the heat output (Fig. 1). Maintenance of constant body temperature in the mouse is particularly problematic since mice have very low body weight/body surface ratio which makes them prone to rapid loss of body temperature in cool environment. A homemade plexiglass holder for warming pad (Fig. 1) was used to keep the pad at 5 cm distance from the coil as it interfered with homogeneity of the B_1 magnetic field. The animals were placed with heart region lying within the Helmholtz coil in prone position to minimize respiratory movements of the chest wall. The paws were wrapped in thin copper foil and connected via carbon electrodes to the Physiogard SM 785 MR monitoring system (Bruker, Karlsruhe, Germany). Continuous ECG signal was acquired and used for synchronization of RF pulses and monitoring of HR. The animals were maintained anaesthetized by continuous gas anesthesia through nasal cone. Isoflurane was delivered in the mixture of oxygen and nitrous oxide (1:1) in the concentration 0.4–1% and at flow rate 0.4 L/min. A gradient echo imaging method was used for visualization of the heart and selection of volume of interest (VOI). All images were obtained with synchronized RF pulses to the cardiac rhythm with triggering delay of 1 ms after the R wave. Five images (1 mm slice thickness and field of view of 4 cm) were acquired in the coronar plane in order to visualize the thoraco-abdominal region and the heart. Starting from the scout image in the coronar plane 4 additional images (1 mm slice thickness) were taken perpendicular on the LV long axis. The size of the VOI was $8 \times 4 \times 4$ mm^3 (128 μL) and $7 \times 4 \times 4$ mm^3 (112 μL) which included as much of the LV as possible. Selection of the VOI was made first on the images in the coronar plane and then the position was crosschecked on the images in the transverse plane (Figs. 2A, 2B, and 2C). If the VOI included parts of chest muscles, diaphragm or liver it was repositioned. Thereafter, localized shimming was performed on the VOI using cardiac gated STEAM localization pulse sequence observing the proton signal. Localized shimming improved the linewidth at half height about 12–15 and 30–40 Hz at the base. Cardiac gated image selected *in vivo* spectroscopy (ISIS) method (8) was employed for volume-selective ^{31}P spectroscopy. Acquisition parameters were 4096 scans with 2.5 s repetition time, 4k data points and 2500 Hz sweep width giving total scan time 2 h and 57 min. A 4 ms adiabatic amplitude modulated hyperbolic secant shaped pulse was used for inversion and a 3.5 ms half since cosine pulse was used for excitation in the ISIS method. Spectroscopic processing consisted of exponential multiplication (lb = 6 Hz) by first order phase correction. Phosphorous metabolites were calculated by computer integration of the areas under the respective peaks. Myocardial energy reserve was evaluated by means of PCr/ATP ratio calculated by dividing PCr with β-ATP resonance area. This ratio is proportional to the levels of intracellular ADP and therefore is an indicator of cellular phosphorylation potential (9). PCr/ATP ratio was corrected for partial saturation for each animal separately by use of the correction factor calculated from PCr/ATP measured from unlocalized spectra of the chest region acquired at 2.5 s and 15 s repetition time (Figs. 5C and 5D) (10).

To evaluate the amount of possible contaminating signal from the regions outside the VOI, experiments were performed on phantom consisting of two separate compartments filled with two different buffers: Na_2HPO_4 and NaH_2PO_4, respectively. Localized spectroscopy using ISIS method was performed following the same protocol as described above. When VOI was localized in one of the compartments no external signal from the other compartment was observed indicating no contamination (Figs. 5A and 5B).

^{31}P spectroscopy of the blood. When ventricular chamber is included in the VOI, blood signal from the LV chamber can result in false myocardial PCr/ATP and PDE/ATP ratio. This occurs because blood contains ATP and PDE but not PCr. Therefore, to calculate the real myocardial PCr/ATP and PDE/ATP ratio, correction for blood contribution to β-ATP and PDE resonance areas is necessary (11, 12). In order to calculate appropriate correction factors one needs to know ATP/2,3-DPG and PDE/2,3-DPG ratio of the blood since 2,3-DPG signal in cardiac spectrum originates from blood. Furthermore, concentration of 2,3-DPG in the blood may change during cardiac diseases (13). Therefore, ^{31}P MRS of blood was performed in separate experiments according to the protocol previously described (14). The blood was drawn from the right ventricle and pooled (because of limited blood volume) from both, bGH mice and controls at the end of the study. The ^{31}P MRS was performed within 15 min after collection of the blood during which time it was placed on ice (14). The experiments were performed on a BRUKER DMX 500 NMR Spectrometer at 11.75 T. Blood samples of 1 ml were placed in 10 mm standard MR tubes and spun at 18 Hz for optimization of shimming. WALTZ-16 proton broadband decoupling was applied to eliminate ^{1}H–^{31}P MR multiples. ^{31}P MR spectra were obtained at 310 K, by accumulating 1032 free induction decays (FIDs) with a repetition time of 1 s. After exponential multiplication (lb = 4 Hz) data was Fourier transformed and first order phase corrected. Resonance areas for ATP, PDE,

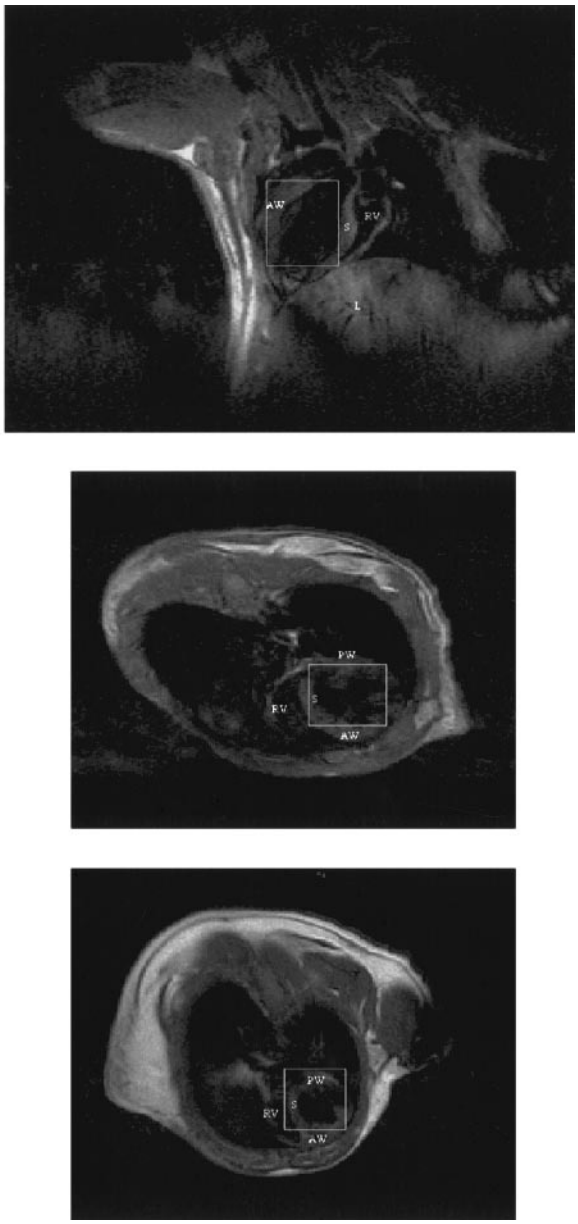

FIG. 2. (A) ^{1}H gradient-echo scout image from thoracoabdominal region of the bGH mouse in the coronar plane. The delineated volume of interest (VOI) includes parts of the left ventricular myocardium and blood in the chamber. Care was taken to not include chest skeletal muscle and liver in the VOI. AW, left ventricular anterior wall; PW, left ventricular posterior wall; RV, right ventricle; S, septum; L, lever. (B) ^{1}H gradient-echo scout image of the bGH mouse thoracic region in the transversal plane. Observe cardiac enlargement and presence of thoracal deformity. The size of the delineated VOI is $8 \times 4 \times 4$ mm^3 (128 μL). AW, left ventricular anterior wall; PW, left ventricular posterior wall; RV, right ventricle; S, septum. (C) ^{1}H gradient-echo scout image of the control mouse thoracic region in the transversal plane. The size of the delineated VOI is $7 \times 4 \times 4$ mm^3 (112 μL). AW, left ventricular anterior wall; PW, left ventricular posterior wall; RV, right ventricle; S, septum.

2,3-DPG peaks were identified and integrated. The correction of myocardial β-ATP and PDE resonance areas were then calculated according to the formulae:

$$\text{ATP} = \text{contaminated ATP} - 0.5 \times (\text{ATP}/2{,}3\text{-DPG}_{\text{blood}}) \times 2.3\text{-DPG}_{\text{total}} \quad (4)$$

$$\text{PDE} = \text{contaminated PDE} - 0.5 \times (\text{PDE}/2{,}3\text{DPG}_{\text{blood}}) \times 2{,}3\text{-DPG}_{\text{total}} \quad (12).$$

Statistics. Computer software (Statview 5.01) was used to perform standard statistical procedure with calculation of mean value and standard deviation (SD) in the different groups. Unpaired Student *t* test was used to determine if changes in parameters between the studied groups were statistically significant. Normal distribution of the data was assessed with Kolmogorov–Smirnov test. When data were not normally distributed, nonparametric test was applied (Mann–Whitney). The value $P < 0.05$ was considered as statistically significant. All data are presented as means ± SEM, unless otherwise indicated.

RESULTS

Total experimental time was 3.5 h including preparation of animal, shimming and acquisition of spectra. All ten animals tolerated well the examination procedure. After acquisition of spectra and discontinuation of anesthesia the mice recovered spontaneously within minutes. There were no signs of adverse effect of anesthesia or experimental protocol on ECG pattern and body temperature. The mean heart rate was not significantly different between the groups i.e. 570 ± 22 in the control group and 559 ± 18 in the bGH group. The mice remained healthy after the examination and were sacrificed 1 week later. After induction of anesthesia and placement of the mouse in the magnet, another 10–15 min were required to establish normal body temperature because of initial drop caused by anesthesia and cold environment in the magnetic bore. Flow rate and isoflurane concentration in the anesthetic gas needed to be adjusted when HR was in decrease or increase. Heart rate fluctuations were easy to control and occurred only in the beginning of the experiment. When steady state condition was established no further adjustment of anesthesia was needed. During whole examination procedure the animals were maintained normothermic between 37–38°C. The PCr/ATP values corrected for partial saturation are summarized in the Table 1. Significantly lower corrected PCr/ATP ratio was observed in the transgenic mice (1.5 ± 0.13 versus 2.1 ± 0.04, $P < 0.01$) (Fig. 3). There was a prominent phosphodiester (PDE) signal in the cardiac spectra from bGH mice. However, when corrected for blood contamination, myocardial PDE/ATP ratio was not significantly different from the control group indicating that PDE signal originated mainly from the blood. Increased myocardial PDE/ATP ratio is proposed to be an indicator of cellular destruction (15). Blood ATP/2,3-DPG was 1.3 lower while PDE/2,3-DPG ratio was 3.8 times higher in the bGH mice compared to the controls (Fig. 4) resulting in different corrections of myocardial β-ATP and PDE signals. Blood PDE/ATP

TABLE 1

	Control	bGH
BW (g)	32.7 ± 0.8	45.2 ± 2.2*
HW (mg)	92 ± 2.3	242 ± 25.6*
HR beats/min	570 ± 22	559 ± 18
Myocardium		
PCr/ATP corrected	2.1 ± 0.04	1.5 ± 0.13*
PDE/ATP corrected	0.44 ± 0.12	0.58 ± 0.13
Blood		
PDE/ATP	6.1	28.9
ATP/2,3-DPG	0.053	0.041
PDE/2,3-DPG	0.29	1.1

Note. BW, body weight; HW, heart weight; HR, heart rate; PDE, phosphodiesters; PCr, phosphocreatine; ATP, adenosine-3-phosphate; 2,3-DPG, diphosphoglycerate.

* $P < 0.01$ versus control.

ratio was 4.7 times higher in the bGH mice indicating presence of hyperlipidemia.

DISCUSSION

The main aim of the present study was development of the method for *in vivo* noninvasive evaluation of cardiac energy metabolism in mice. To our knowledge, our group was first to report, in a preliminary form, feasibility of performing noninvasive cardiac ^{31}P MRS in the mouse (16).

Despite recent advances in the treatment, cardiovascular diseases (atherosclerosis, ischemic heart disease and heart failure) remain leading cause of morbidity and mortality in the industrialized countries. Understanding of pathophysiological mechanisms involved in development of heart disease as well as the search for new pharmacological treatments to improve patient symptoms and survival will continue to depend on relevant animal models. Although rats and mice are the two most frequently used experimental animals in cardiovascular research, only few models for cardiac ^{31}P MRS in small animals are described in the literature. Continuously increasing number and availability of different transgenic mice models related to myocardial diseases creates a demand for detailed characterization of cardiovascular physiology and biochemistry in mice. We have recently demonstrated the advantage of using volume-selective cardiac ^{31}P MRS for *in vivo* detection of disturbances in cardiac bioenergetics in rats with myocardial infarction (6). In this paper, the method has been further developed along with our previous strategy (17) and adjusted for *in vivo,* noninvasive application in the living mouse. The results demonstrate that generally recommended protocol for volume-selective cardiac ^{31}P MRS can be successively applied no only in humans and large animals but also in small rodents as well.

The importance of myocardial bioenergetics has been emphasized recently by the increasing body of evidence indicating that disturbances in myocardial energy metabolism are involved in the development of ventricular dysfunction and heart failure in different diseases of myocardium (18–20). Large proportion of this recent evidence were provided by use of ^{31}P MRS as experimental tool in experiments conducted both *in vivo* and *in vitro* (5, 18).

^{31}P MRS evaluation of transgenic mouse hearts have been performed recently to address specific questions regarding the regulation of myocardial energy metabolism (21, 22). However, these experiments were conducted *in vitro* on perfused heart preparations. *In vitro* experimentation offers the advantage to alter only one variable at the time and to measure the effect of that particular perturbation (e.g., substrate availability and/or concentration, pO_2, pH, temperature, preload, afterload, pharmacologically active substances) on cellular bioenergetics. Even though important information has been gained from these experiments regarding regulation of cellular energy metabolism in myocardium there are several limitations inherent to this

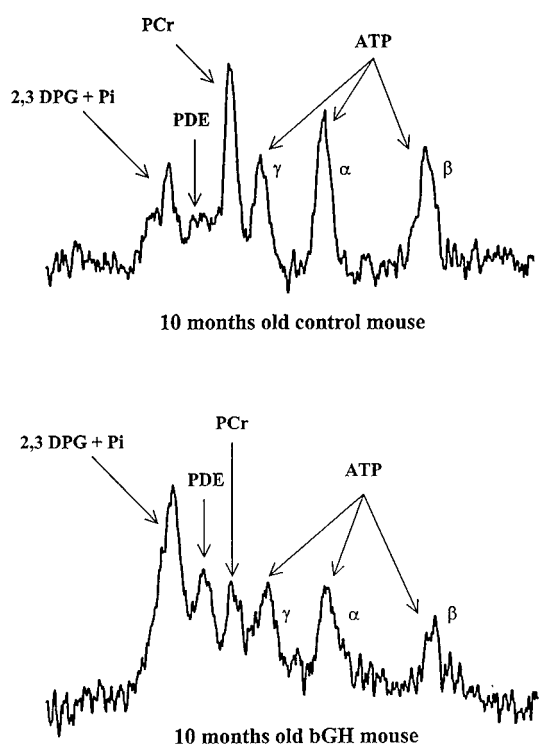

FIG. 3. Representative cardiac ^{31}P MR spectra from the control mouse (top) and bGH mouse (bottom). Observe marked difference in the intensity of PCr resonance area. Higher PDE signal was observed in the spectra from bGH mice; however, after correction there was no difference in the PDE/ATP ratio between the groups, indicating that a large proportion of PDE signal originated from the ventricular blood. PCr, phosphocreatine; α, β, γ-ATP, α, β, γ-phosphates of adenosine-3-phosphate; 2,3-DPG, diphosphoglycerate; Pi, inorganic phosphate; PDE, phosphodiesters.

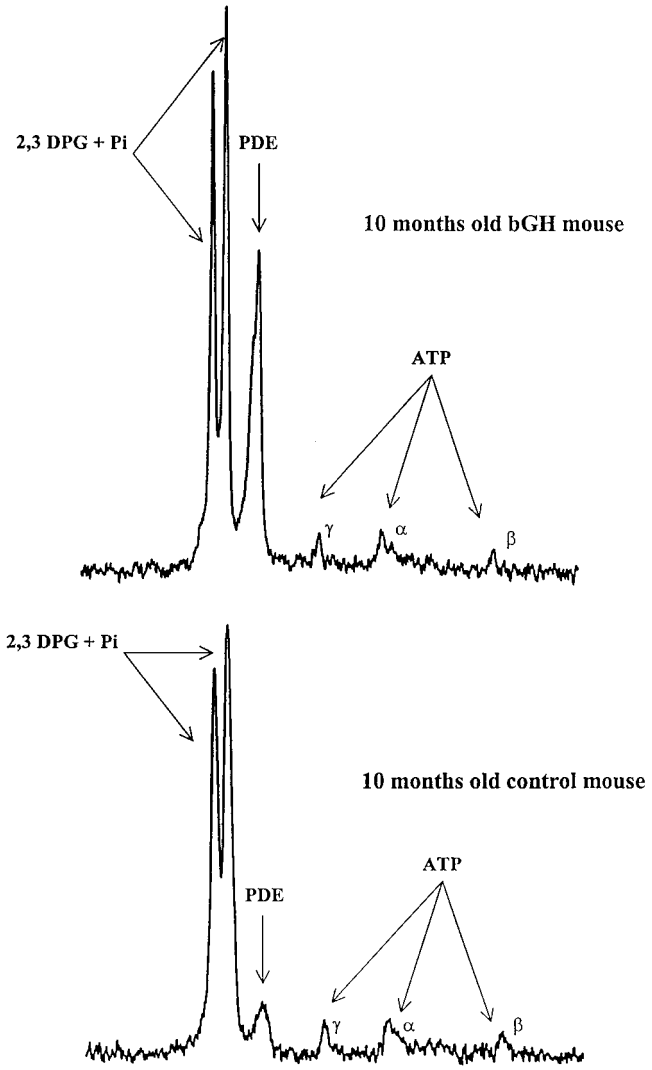

FIG. 4. Representative ^{31}P MR spectra of the pooled blood from bGH mice (top) and control mice (bottom). Observe higher PDE signal in the spectrum from the bGH mice. Also 2,3-DPG/ATP ratio was higher in bGH mice, indicating that different correction factors should be applied for calculation of myocardial β-ATP signal. α, β, γ-ATP, α, β, γ-phosphates of adenosine-3-phosphate; 2,3-DPG, diphosphoglycerate; Pi, inorganic phosphate; PDE, phosphodiesters.

model which deserve to be mentioned. It is not possible to study time-depended alterations in myocardial bioenergetics after specific manipulation of mouse genome or after induction of certain disease process in the same animals or to evaluate the effects of pharmacological intervention. One must not forget that *in vitro* models, although valuable, are oversimplification of normal physiological conditions. Conclusions drawn from these experiments should be interpreted with caution because the heart was extracted from its physiological milieu which is characterized by numerous neurohumoral counterbalancing systems and subsystems in dynamic interaction. Therefore, it is apparent that *in vivo* models should be used as an important complement and extension of *in vitro* models. When possible, the results from *in vitro* experiments should be confirmed *in vivo*.

Numerous studies designed to evaluate parameters of cardiac function and hemodynamics in mice were conducted at heart rate below 300 beats/min (23–25). This is rather unphysological condition for the mouse and the question arises regarding interpretations of the results from these studies. This problem has been recently addressed (26). Our study demonstrates that proper use of adequate anesthesia and active thermoregulation are the two most important measures for preservation of normal heart rate.

The PCr/ATP ratio reported in this study is in agreement with previously published *in vitro* data (27) although others has reported lower values (22). This ratio is an intrinsic index of myocardial energy reserve and has been shown to be a predictor of mortality in patients with congestive heart failure (28). Mice with overexpression of bGH have been described in literature previously as a model for studies of *in vivo* effects of excessive GH. Several different pathophysiological alterations in the body of these animals have been reported including liver and renal failure, skeletal defects, increase incidence of mammary cancer, cardiac hypertrophy and also changes in normal myocardial histology (29, 30). The old bGH mice were used in order to investigate if the method is sufficiently sensitive to detect possible disturbances in myocardial energy metabolism. Indeed, the apparent decrease in myocardial PCr/ATP ratio in the bGH mice demonstrates both, the usefulness of the method for detection of bioenergetic abnormalities in the mouse heart and possible negative effect of chronic exposure to high plasma GH levels on myocardial energy metabolism. The fact that the bGH group had higher blood 2,3-DPG/ATP ratio suggests that determination of correction factor for β-ATP resonance area should be performed for each experimental group separately when experimental groups differ for the presence of disease. Furthermore, high 2,3-DPG signal may indicate presence of cardiac dysfunction in bGH mice as previous study have reported increased blood concentrations of this metabolite in the patients with cardiac diseases (13, 31). Both decreased myocardial PCr/ATP and increased blood 2,3-DPG/ATP strongly suggests the presence of cardiac dysfunction in bGH mice. These intriguing findings warrant further studies in which assessment of functional, pathohistological and boenergetic properties of myocardium of these animals should be investigated in more detail. Previous studies have shown that species differences exist regarding blood 2,3-DPG/ATP ratio (14). It appears, however, that there is no difference in this ratio between normal rats and mice (6). Another

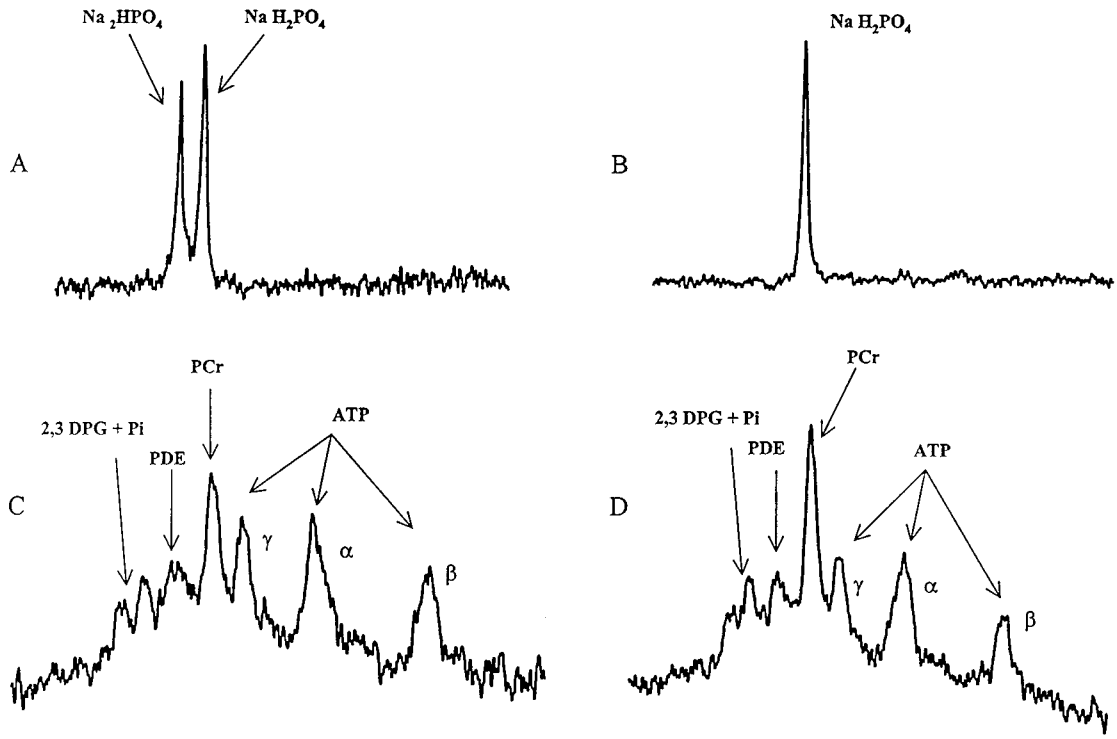

FIG. 5. (A) Unlocalized ^{31}P MR spectrum from the phantom consisting of two separate compartments filled with two different buffers: Na_2HPO_4 and NaH_2PO_4. Note two different signals from each buffer. (B) Localized ^{31}P MR spectrum from the phantom with VOI ($8 \times 4 \times 4$ mm) placed in the compartment filled with NaH_2PO_4. Absence of the Na_2HPO_4 signal in the localized spectrum indicates no contamination from the outer volume. (C) Unlocalized ^{31}P MR spectrum from the mouse (repetition time = 2.5 s). PCr, phosphocreatine; α, β, γ-ATP, α, β, γ-phosphates of adenosine-3-phosphate; 2,3-DPG, diphosphoglycerate; Pi, inorganic phosphate; PDE, phosphodiesters. (D) Unlocalized ^{31}P spectrum from the mouse (repetition time = 15 s). The PCr/ATP ratio in the fully saturated spectrum is 1.4 higher than in partially saturated (C). PCr, phosphocreatine; α, β, γ-ATP, α, β, γ-phosphates of adenosine-3-phosphate; 2,3-DPG, diphosphoglycerate; Pi, inorganic phosphate; PDE, phosphodiesters.

interesting finding was the presence of high PDE signal in the blood from bGH mice. The reason for this is unknown but it could be a result of known lipolytic effects of GH hormone and presence of hyperlipidemia in the bGH mice as blood PDE signal originates mainly from serum lipids (12, 31). Indeed, previous studies have reported the occurrence of hypercholesterolemia in the bGH mice (29). Although the examination time was relatively long, all animals recovered spontaneously without any sign of adverse effect of experimental procedure. The relatively long acquisition time could be significantly shortened with experimentation at stronger magnetic fields (MR systems designed for animal experimentation at 4.7 T and 7 T are available from several manufacturers) due to improved signal-to-noise ratio for the same amount of scans.

In summary, the study demonstrates the feasibility of performing noninvasive cardiac ^{31}P MRS in living mice and detection of disturbed myocardial bioenergetics under maintained physiological conditions. The method could be particularly useful for longitudinal studies and for evaluation of effects of different treatments on cardiac bioenergetics in transgenic and other experimental models in the mouse.

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