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TECHNICAL NOTE

Spectroscopy

^{31}P-Nuclear Magnetic Resonance Spectroscopy of Blood: A Species Comparison

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ABSTRACT

^{31}P-nuclear magnetic resonance (NMR) spectroscopy is frequently used as a tool in the study of organs from various animal species and humans. Because signals arising from the presence of blood are visible in in vivo ^{31}P-NMR spectra of blood-filled organs, such as the heart, it is necessary to correct these spectra for the contribution of blood to the signal. It is unknown whether species differences in ^{31}P signals of blood exist. ^{31}P-containing metabolites of blood from various species were therefore quantified by means of ^{31}P-NMR spectroscopy. Signals of 2,3-bisphosphoglycerate (2,3-DPG); phosphodiesters (PDE); and γ-, α-, and β-ATP were detected in all ^{31}P-NMR spectra of blood. 2,3-DPG/ATP ratios were significantly higher in dogs, rats, and guinea pigs than in humans but lower in sheep. Pig and rabbit were the only animals with a 2,3-DPG/ATP ratio similar to that of humans. PDE levels varied among species but were significantly lower than in humans only in guinea pigs. The PDE/ATP ratio was relatively similar among all species compared with humans, except dog and guinea pig, where it was significantly higher and lower, respectively. We conclude that because of large species differences, species-specific ^{31}P metabolite ratios should be applied for the correction of in vivo ^{31}P-NMR spectra.

KEY WORDS: *ATP; Blood; Magnetic resonance spectroscopy; ^{31}P metabolites.*

INTRODUCTION

^{31}P-nuclear magnetic resonance (NMR) spectroscopy has been used to evaluate high-energy phosphate metabolism in organs from various animal species and humans. For some applications (e.g., spectra from skeletal muscle, liver, or brain), no distinct signals from blood can be resolved. This is due to the small relative amount of blood of these organ tissues. In the heart, a totally different situation arises. Although the amount of blood in the heart muscle itself is small (1), there are two large ventricular cavities filled with blood. Because of the use of rectangu-

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lar voxels on a spheric organ, most in vivo heart spectra are contaminated by substantial signals from blood. The resonances of ATP detected in such spectra represent the sum of ATP signals arising from the heart tissue and blood. Thus, phosphocreatine (PCr)/ATP ratios should be corrected for blood contamination when a significant amount of blood signal is present. Because the 2,3-bisphosphoglycerate (2,3-DPG)/ATP ratio depends on the metabolism of the species examined (e.g., the ratio of oxidative to anaerobic pathways for the synthesis of ATP), the 2,3-DPG/ATP values used for correction of blood contamination should be species specific. In this study, we characterize ^{31}P-NMR blood spectra from humans and from six animal species frequently used in laboratory research to arrive at species-specific correction factors.

METHODS

Blood was freshly drawn by venous puncture (human, dog, rat, guinea pig) or from the carotid artery (rabbit, sheep, pig). Coagulation was inhibited with Na_4EDTA. All blood was collected after a fasting period of at least 12 hr to ensure low activity of glucose metabolism in erythrocytes. Samples were placed on ice and transferred to the NMR unit within 30 min. Preliminary testing with human blood (2) showed no change of ^{31}P-NMR spectra within 12 hr at 0°C or within 4 hr at 37°C, whereas freezing and thawing led to a 20–30% reduction of ATP caused by destruction of erythrocytes. Blood stored at 0°C showed no changes in partial oxygen saturation for 5 hr. Additional experiments (data not shown) with blood from humans showed no significant variation in the 2,3-DPG/ATP ratio when comparing arterial (O_2 saturation, 92.3 ± 1.8%) and venous (O_2 saturation, 77.0 ± 3.3%) blood.

Blood was measured in a 10-mm NMR tube (outer diameter 10.00 mm, inner diameter 9.07 mm; Wilmad, Buena, NJ) equipped with a Wilmad special stem coaxial insert of 5 mm for the standard. Because sedimentation of erythrocytes could result in increased levels of ATP and 2,3-DPG by sedimentation of blood cells from the volume above the coil, or vice versa, in decreased levels by movement of erythrocytes below the sensitive volume, we prevented erythrocytes from drifting into or out of the sensitive probe volume by means of two plastic discs at the lower and upper end of the NMR-sensitive volume. An external O,O′-dimethyl methylphosphonate standard in D_2O was used for determination of chemical shift (δ = 38.5 ppm vs. 85% H_3PO_4) and B_0-field shim.

After rewarming to 37°C, one ^{31}P-NMR spectrum was obtained within 42 min using a 45-degree block pulse followed by sampling of 1032 acquisitions (4 k data points) with an interpulse delay of 2.1 sec. We used a Bruker AM 300 SWB system (7.05-T vertical magnetic system, Oxford Instruments, Bruker Aspect 3000 computer) with a standard 10-mm multinuclear probe. Homogeneity of the B_0-field was optimized by shimming on maximum lock level of ^{2}D-signal.

For the calculation of absolute metabolite concentrations, the area under the signal of each metabolite was calculated by comparison with the area of the standard solution. The ratio of signal areas would be calculated as the ratio of volumes in the glass tubes. However, because the length of the tubes and the constant π are given in the denominator and the numerator of the formula, the ratio of the sample volume reduces to the square of the ratio of the radius of the inner tube divided by the difference of the squares of dimensions of the inner and the outer tube.

Data were corrected for partial saturation (1.03 for 2,3-DPG, 1.00 for PDE and ATP) by comparison with fully relaxed spectra, obtained using a TR of 15 sec. Free inductions decays were processed with exponential multiplication (line broadening, 20 Hz) and Fourier transformation. Integration of peak areas was performed using the Bruker standard integration routine DISNMR89 program.

The signal of the outmost ^{31}P-atom of ADP and ATP overlapped in ^{31}P-NMR spectra. ADP concentration was calculated as the difference of the γ- and β-ATP signal.

Animal Species and Humans

Seven groups were examined. One group included 23 human volunteers with a mean age of 25 ± 1 yr. Sixteen volunteers were men and 7 were women. We used male Wistar rats with a weight of 350 g aged 12 weeks. Rabbits were all male aged of 9–12 months and weighing 6–8 kg. Pig blood was collected at the slaughterhouse from pigs with a weight of about 150 kg and an age of 9 months (females or castrated males). Dogs included three males and two females (German shepherd) with a weight of approximately 25 kg. Guinea pigs weighed 500–600 g, and two were female and three were male. Sheep (four male, one female) were 12–14 months old and weighed 35–45 kg (from the slaughterhouse). For each group, five animals were investigated.

Statistical Analysis

For each group, data for each parameter obtained were compared with those from humans using factorial ANOVA. Calculations were aided by the StatView SE+Graphics, Statistics Utility (Abacus Concepts, Berkeley, CA). Data are means ± SE, if not stated otherwise. Scheffe's F-test was used to test for significance (3).

For the comparison of γ- and β-ATP signals, we used the paired, two-sided, Student's *t*-test (3).

RESULTS

In all species, ^{31}P-NMR blood spectra showed resonances for 2,3-DPG; phosphodiesters (PDE); and γ-, α-, and β-ATP. Typical spectra are shown in Fig. 1. Absolute and relative amounts (ratios) of ^{31}P-compounds are shown in Table 1 and in Figs. 2 and 3.

The 2,3-DPG/ATP (Fig. 2) ratio showed a wide variation among species. Compared with 2,3-DPG/ATP ratios from humans (3.66 ± 0.10), only the pig had a similar 2,3-DPG ratio (3.96 ± 0.48), whereas the other species showed lower (sheep, 1.50 ± 0.36) or higher values (guinea pig, 11.60 ± 0.79; dog, 10.31 ± 0.91; rat, 7.07 ± 0.52; rabbit, 5.32 ± 0.19).

In blood spectra, a difference between the absolute concentration of γ- and β-ATP is measured due to overlap with ADP signal. Absolute ADP concentrations (Table 1) can be calculated from the difference of the γ- and β-ATP integrals. A significant difference between the γ- and β-signal was found in humans ($p = 0.0001$), guinea pigs ($p = 0.0082$), and pigs ($p = 0.005$), whereas the difference of the peak areas was not significant in other species, attesting the low ADP levels in these species.

PDE levels (Table 1) were similar in all species (2.24 ± 0.16) except guinea pigs, which had extremely low PDE levels (0.19 ± 0.03). With little variation in PDE concentrations in humans, dogs, rats, rabbits, pigs, and sheep, the PDE/ATP ratio is dominated by the ATP levels as denominator of the ratio.

Because blood from rats and guinea pigs was taken under anesthesia (pentobarbital sodium), the effects of treatment with anesthetics was tested using an additional group of three nonanesthetized rats killed by a blow on the head (data not shown) and by comparing the results within the dogs (three were anesthetized with thiabutabarbital, two were untreated). We found no effect of anesthesia on ratios of ^{31}P-metabolites and on ^{31}P-NMR spectra.

DISCUSSION

In this study, we report on relative amounts of ^{31}P-compounds in blood. In all species the ^{31}P metabolites 2,3-DPG, PDE, and ATP were found. There is a wide variation of absolute and relative concentrations of the ^{31}P metabolites among species. The aim of this study was the correction of integral values for signal contribution of blood metabolites. We focus on the most important parameter: the 2,3-DPG/ATP ratio.

Our data show that 2,3-DPG/ATP ratios are substantially different among various species. Thus, blood correction should be applied to organ spectra with values typical for each respective species. Because the regional blood volume differs from organ to organ (1) (Table 2) and the amount of blood passing through the organ (blood weight/organ weight, Table 2, adapted from ref. 4) does not necessarily reflect the extent of blood contamination, the appearance of signals of 2,3-DPG is the best guide to the decision whether blood correction of organ spectra is required or not.

Despite technical development for the use of curved voxels in spectroscopy (5,6), today commercial scanners allow only the use of rectangular voxels. Signals of 2,3-DPG, visible in ^{31}P-NMR spectra indicate the need for blood correction of data to get true PCr/ATP ratios.

The contribution of ATP signal arising from blood is calculated as previously reported (2). Briefly, the integral value for ATP arising from blood is calculated by the 2,3-DPG integral multiplied with the 2,3-DPG/ATP ratio and subtracted from the total ATP integral before the calculation of PCr/ATP ratios.

ADP levels (Table 1) can be detected indirectly by comparing the peak areas of β- and γ-ATP, as the resonance signals of the outmost ^{31}P atoms of ADP and ATP coincide. Briefly, the outmost P atom in ATP (γ-ATP) is in a chemical environment, which is nearly identical to the environment of the P-atom in β position in ADP. The same is true for the P atom closest to the adenosine structure. The signal of the P atom in the α position in ATP is overlapping with the P atom in the position of the ADP molecule. Thus, the concentrations for α- and γ-ATP show values from overlap with ADP, which cannot be separated in in vivo measurements. Although we are aware of this overlap of ADP and ATP signal, we used γ-ATP values for calculation of metabolite ratios. As the

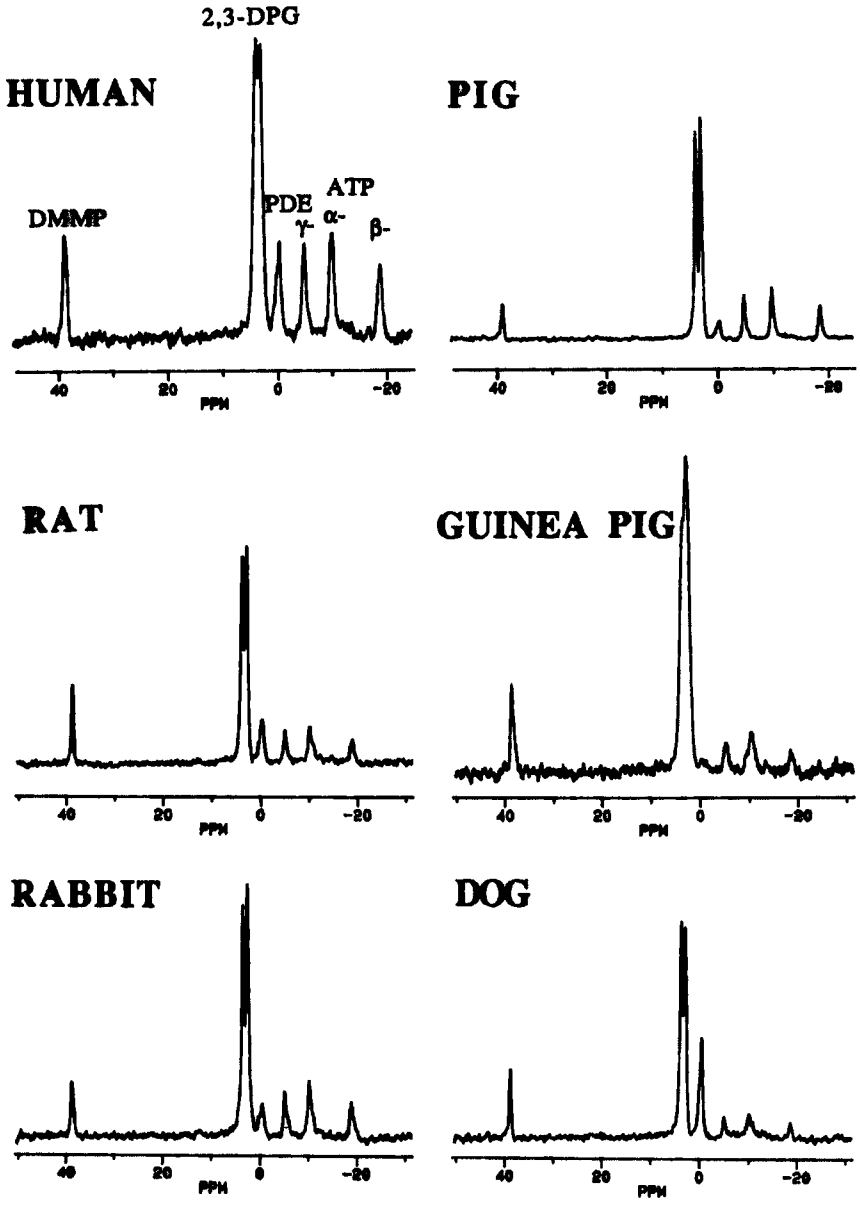

Figure 1. Examples of ^{31}P-NMR spectra of blood of human, pig, rat, guinea pig, rabbit, and dog. Values are as follows: 1032 acquisitions, 42 min, interpulse delay 2.1 sec, pulse angle 45 degrees. DMMP, O,O'-dimethylmethylphosphonate as standard.

situation of signal coincidence is the same in in vivo measurements as in the examination of blood presented here, there is a difference in the excitation profile of surface vs. Helmholtz coils.

A profound off-resonance effect can be observed when using surface coils in in vivo studies, which leads to a reduced signal intensity from the β-ATP resonance. In our experiments, no off-resonance effects were observed in the in vitro measurements of blood. In some species (dog, pig, guinea pig), significant differences (Table 3) between the two peak areas were found. This might be explained by differences in energetic pathways. In human erythrocytes (7), only 11% of all substrate flows through the oxidative phosphogluconic pathway, whereas 89% undergo the anaerobic Embden-Meyerhoff pathway. Variation in the ratio of glucose utilization by the

Table 1

^{31}P-NMR Spectroscopic Data for Humans and Animal Species

	Human	Dog	Rat	Rabbit	Pig	Guinea Pig	Sheep
n	23	5	5	5	5	5	5
2,3-DPG, mM	6.67 ± 0.55	6.49 ± 0.96	6.47 ± 0.49	8.57 ± 0.54	11.25 ± 0.83*	7.16 ± 0.15	0.91 ± 0.08*
PDE, mM	2.58 ± 0.23	3.74 ± 0.48	1.93 ± 0.20	1.68 ± 0.14	1.07 ± 0.16	0.19 ± 0.03*	1.21 ± 0.10
γ-ATP, mM	1.86 ± 0.18	0.94 ± 0.10	1.62 ± 0.13	0.63 ± 0.09+	0.63 ± 0.04+	2.91 ± 0.18	0.68 ± 0.09
α-ATP, mM	2.56 ± 0.27	1.12 ± 0.11	1.67 ± 0.12	2.53 ± 0.20	3.63 ± 0.25	1.28 ± 0.09	1.40 ± 0.08
β-ATP, mM	1.53 ± 0.16	0.59 ± 0.09	0.76 ± 0.09	1.49 ± 0.13	2.36 ± 0.16	0.40 ± 0.05*	0.52 ± 0.07
ADP, mM	0.33 ± 0.06	0.04 ± 0.02*	0.18 ± 0.07	0.13 ± 0.15	0.56 ± 0.05	0.23 ± 0.05	0.15 ± 0.07
PDE/γ-ATP,	1.44 ± 0.09	6.04 ± 0.52*	2.19 ± 0.41*	1.08 ± 0.15	0.38 ± 0.05*	0.30 ± 0.03*	1.92 ± 0.31

*$p < 0.05$ for dog, rat, rabbit, pig, guinea pig, sheep vs. human.

Embden-Meyerhoff pathway and phosphogluconic pathway among species are most likely and should result in variations of 2,3-DPG and ATP levels. Although we investigated mammals only, it is known that erythrocyte ATP levels vary among animal classes (8). ATP levels are lowest in mammalians, somewhat higher in amphibians, and highest of all in fish.

In general, species with a higher metabolic rate, estimated by heart rate (9–12) or basal heat production (13), showed high 2,3-DPG/ATP ratios (Fig. 4). In dog and guinea pig, we found no correlation of the 2,3-DPG/ATP ratio with metabolic parameters. Only the pig and the rabbit had 2,3-DPG/ATP levels similar to humans. However, a trend toward higher 2,3-DPG/ATP ratios was seen in the rabbit in accordance with the high metabolic rate of rabbits.

Preliminary reports on phosphorus-containing metabolites in human blood or erythrocytes using enzymatic methods (14), in vivo labeling with radioactive ^{32}P-phosphate (15), or ^{31}P-NMR of perchloric acid extracts (14) show ATP values close to those obtained in our study, whereas 2,3-DPG levels calculated based on literature values (14) are lower than the values reported here (Table 3). Overlap of 2,3-DPG with 1,3-bisphosphoglycerate leading to increased integration areas can be excluded, because the concentration of 2,3-DPG levels in human blood cells is four orders of magnitude greater than levels of 1,3-DPG (8). The discrepancy of previously reported 2,3-DPG levels and our results remains unexplained.

The nature of the PDE signal is still incompletely un-

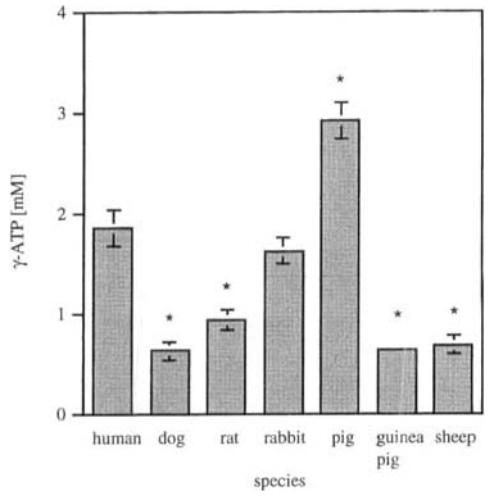

Figure 2. Concentration (mM) of blood γ-ATP for various species. *$p < 0.05$ vs. human.

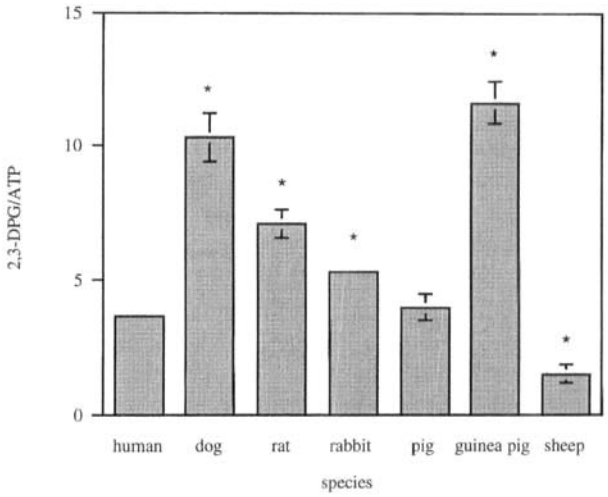

Figure 3. Blood 2,3-DPG/γ-ATP ratios of various species. *$p < 0.05$ vs. human. The 2,3-DPG/γ-ATP ratio in pigs and rabbits is close to the ratio in humans, whereas different ratios are found in other animals.

Table 2

Blood Flow and Weight of Organs in Humans and Regional Blood Volume (RBV) in Rats (1)*

Organ	Blood Flow (ml/min)	Organ Weight (g)	Blood Flow/Organ Weight (ml/min/g)	RBV (%)
Kidney	1100	300	367	15.9 ± 0.9
Brain	750	1500	50	2.8 ± 0.1
Heart	250	300	83	7.7 ± 0.3
Skeletal muscle	1200	30,000	4	1.5 ± 0.4
Skin	500	5000	10	—

*Adapted from ref. 4, 70 kg body weight, 1.7 m^2 surface.

Table 3

Comparison of Concentration of 2,3-DPG and ATP in Human Blood and Erythrocyte Preparations from Various Studies

Reference	Sample	Technique	2,3-DPG (mM)	ATP (mM)	2,3-DPG/ATP
Bartlett (15)	Whole blood	^{32}P-labeled in vivo, acid extract	9.0	2.5	3.6
	Whole blood	^{14}C-labeled in vitro, acid extract	12.0	2.5	4.8
	Whole blood	^{32}P-labeled in vitro, acid extract	10.0	2.4	4.17
Labotka et al. (14)	Packed erythrocytes	Enzymatic assay	—	1.37 ± 0.12	—
	Packed erythrocytes	^{31}P-NMR	—	1.31 ± 0.08	—
	Model erythrocyte system	Enzymatic assay	4.64 ± 0.02	—	—
	Model erythrocyte system	^{31}P-NMR	4.53 ± 0.30	—	—
Sakuma et al. (3)		^{31}P-NMR	—	—	7.14
This study	Whole blood	^{31}P-NMR	6.67 ± 0.55	1.86 ± 0.18	3.59

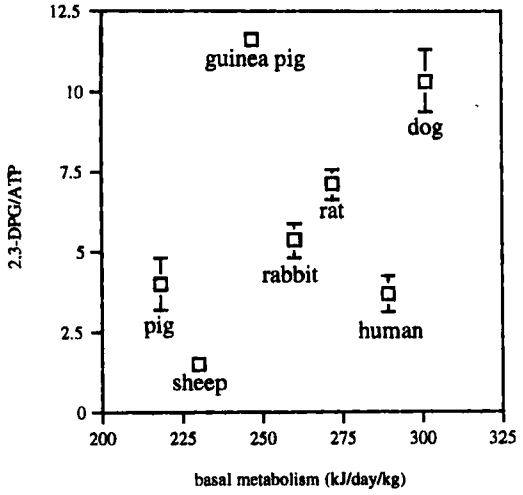

Figure 4. 2,3-DPG/γ-ATP ratios vs. metabolic rate. High metabolic rates of various species also show high 2,3-DPG/γ-ATP ratios.

derstood at present, although phosphatidylcholine and phosphatidylethanolamine are known to contribute to the PDE signal (4). Membrane-bound lipids contribute to the hump of the baseline. Thus, if lipids contribute to PDE signals, the signal is caused only by small amounts of free phospholipids. We could not find correlations of PDE levels with metabolic parameters in this study.

Limitations

Glucose utilization of blood during cooling and rewarming was not examined. In our study, blood was drawn after a fasting period of minimum 12 hr; thus, glucose levels were low and glucose metabolism should not be influenced by temperature.

Increased inorganic phosphate (P_i) levels stimulate glycolytic metabolism and yield an increase of the 2,3-DPG level (16). As the signals of P_i and 2,3-DPG overlap at physiologic pH values (16), P_i levels in blood cannot be determined by ^{31}P-NMR, and thus we are unable to evaluate the effects of P_i on the rates of glycolytic metab-

olism in the various species. As stated, the amounts of P_i are known to be not significantly different among the various species. Thus, it is likely that there is no effect on the glycolytic metabolism in blood collected after the fasting period among the species examined.

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