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Concentrations of Free Mg^{2+}, pH and ^{31}P MR Metabolite Ratios in Calf Muscles of Healthy Controls and Patients with Primary Juvenile Hypertension

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Summary

^{31}P MR spectroscopy was used to measure the signal intensity ratios of high-energy metabolites for the calculation of free cytosolic magnesium concentration [fMg^{2+}] and pH in the calf muscles of patients with primary juvenile hypertension and of healthy controls. Surface coil and spectroscopic imaging techniques were used. In patients with hypertension, the concentrations of [fMg^{2+}] was 788±33 μmol/l and intracellular pH was 7.05±0.02; these values were not significantly different from the results obtained in healthy controls ([fMg^{2+}], 776±21 μmol/l and pH, 7.06±0.01). Biochemical assays of magnesium in the serum (S-Mg) and in urine (DU-Mg) confirmed this finding. Significant differences in the relative signal intensities of high-energy phosphates between patients with primary juvenile hypertension and healthy controls were observed: a) signal intensity ratios of P_{Cr}/P_i, $P_{Cr}/P_{\beta ATP}$, $P_{DE}/P_{\beta ATP}$ were increased, and b) P_i/P_{De}, P_i/P_{ATP} were decreased. The results were the same irrespective of whether the surface coil method or ^{31}P spectroscopic imaging were employed.

Key words

Concentration Mg^{2+} • pH • Muscles • ^{31}P MR spectroscopy • Juvenile hypertension

Introduction

Metabolism-related diseases (primary hypertension, insulin resistance, hyperinsulinemia, diabetes mellitus) can be considered as individual clinical components of a generalized cardiovascular-metabolic disease (Resnick 1993). Primary hypertension is a multifactorial condition manifesting itself primarily in adulthood. However, the last decade has furnished evidence suggesting that the critical period for the development of the disease is the intrauterine phase of development. Currently, the hypothesis of "chronic disease programming *in utero"*, including primary hypertension, is being tested (Barker 1992, 1998, Langley Evans *et al.* 1999). The existence of primary hypertension in childhood and adolescence has been confirmed by large multicentric studies (Report of the Second Task Force 1987, Update on the Task Force 1996). To date, no answer has been provided to the question whether or not the etiopathogenic mechanisms of hypertension in adulthood and in childhood or adolescence are identical. An option for evaluating the differences between

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hypertension in various age groups is to monitor the role of intracellular magnesium in energy metabolism.

The available data suggest that intracellular magnesium deficiency may play a role in a number of pathological states including hypertension, diabetes mellitus, perinatal morbidity in diabetic pregnancies, arrhythmia, congestive heart failure, early atherogenesis, etc. A possible pathophysiological concept for diabetes mellitus, obesity and cardiovascular diseases including primary hypertension has been described (Resnick *et al.* 1990, 1992a,b). Increased intracellular [fCa^{2+}] concentrations, lower magnesium ion [fMg^{2+}] concentrations, and decreased pH have been demonstrated in smooth muscle cells in adult patients with hypertensive disease (Touyz *et al.* 1992, Barbagallo *et al.* 1993, Resnik *et al.* 1993). Possible changes may result in increased sensitivity of smooth muscle cells, higher concentrations of free radicals, platelet aggregation, reduction of myocardial bioenergetics, etc. A partial explanation of the mechanism behind the above changes in patients with hypertension can be found in decreased membrane Na^+-K^+-ATPase and Ca^{2+}-ATPase activity (Touyz *et al.* 1992, Fu *et al.* 1998). The concentration of Mg is thus an important parameter, which can help to understand the pathophysiology of the above diseases. Routinely available methods for the determination of Mg concern serum and urine analysis. However, neither method provides information about the cellular Mg concentration, where magnesium plays its most important biochemical role. When referring to the concentration of Mg in cells, it is necessary to differentiate between the concentration of [fMg^{2+}] and the total concentration of magnesium [Mg_{tot}]. Using phosphorus NMR spectroscopy, we are able to determine the concentration of [fMg^{2+}] which, from the physiological point of view, is more important than the total concentration. The introduction of NMR markedly enhanced the accuracy of measuring intracellular [fMg^{2+}] concentrations (Gupta *et al.* 1978, reviewed by London 1991). The feasibility of ^{31}P MR spectroscopy in skeletal muscles, especially in calf muscles, is of interest for examination of patients. First, skeletal muscles contain about one half of the body's stores of available Mg; second, skeletal muscle is bioenergetically more similar to smooth muscles than red blood cells, where a relationship between low concentrations of [fMg^{2+}] and hypertension was initially established (Resnik *et al.* 1994).

In vivo ^{31}P MR spectroscopy is routinely used in determining the concentrations of metabolites, which play an important role in energy metabolism (Rudin *et al.* 1992). The signal intensities of phosphomonoesters (P_{Me}), inorganic phosphates (P_i), phosphodiesters (P_{De}), phosphocreatine (P_{Cr}) and three signals of adenosine triphosphates (P_{ATP}) can be obtained during *in vivo* ^{31}P MR measurement of muscle spectra (see Figure 1). The signal intensities are proportional to the concentrations of metabolites. The other parameters measured using the spectrum are chemical shifts, which describe the chemical structure and, in some circumstances, are sensitive to chemical exchanges. In the case of muscular tissues, the chemical shifts of inorganic phosphates and ATP are primarily controlled by the equilibrium between ATP, Mg^{2+} and H^+ ions described by equilibrium constants K_D, and K_H, and K_P (Equations 1- 3):

$$[ATP]^{4-} + [fMg^{2+}] \leftrightarrow [ATPMg^{2-}] \quad K_D \quad (1)$$

$$[ATP^{4-}] + [H^+] \leftrightarrow [ATPH^{3-}] \quad K_H \quad (2)$$

$$[HPO_4^{3-}] + [H^+] \leftrightarrow [H_2PO_4^{2-}] \quad K_P \quad (3)$$

Under conditions of fast exchange, the observed chemical shifts are given by the sums of individual limiting chemical shifts and molar fractions of pure complexes. Gupta (1978) proposed a method for measuring the concentrations of [fMg^{2+}] based on the difference of chemical shifts between α and β signals of ATP. The influence of pH was taken into account by Bock (1985) who developed the following equation for the calculation of free magnesium:

$$[fMg^{2+}] = K_D\{ (\delta_{\alpha\beta obs}-\delta_{\alpha\beta ATP}) (1+[H^+]/K_H)+[H^+]/K_H (\delta_{ATP}^{4-} - \delta_{\alpha\beta ATPH}^{3-})\}/ (\delta_{ATPMg}^{2-} - \delta_{\alpha\beta obs}) \quad (4)$$

where $\delta_{\alpha\beta obs}$ is the observed difference of chemical shifts of α and β signals of ATP in the tissue, K_D and K_H are equilibrium constants from equations (1-3), δ_{ATP}^{4-}, δ_{ATPMg}^{2-} are the limiting shifts of pure complexes derived experimentally from titration experiments, $\delta_{\alpha\beta ATP}^{4-}$, $\delta_{\alpha\beta ATPH}^{3-}$ are the differences of limiting shifts of α and β signals in pure complexes, and the intracellular concentration of [H^+] is calculated from equation (5):

$$pH = pK_P + \log[(\delta_{Pi} - \delta_{HP})/(\delta_P- \delta_{Pi})] \quad (5)$$

where δ_{Pi} is the chemical shift difference of P_i and P_{Cr} signals (with PCr serving as internal standard) and δ_{HP}, δ_P are the limiting shifts of protonized and nonprotonized phosphates. Several sets of equilibrium constants were used for the calculation of [fMg^{2+}] concentration by different authors and their application is discussed below.

Our previous studies have repeatedly confirmed significant hyperinsulinemia in pediatric and adolescent hypertensive patients (Palyzová *et al.* 1996, 2001). The aim of the present study was to evaluate calf muscle

[fMg^{2+}], intracellular pH and ^{31}P MR signal intensity ratios in primary juvenile hypertensive patients (PJH), and to compare their values with those of healthy controls along with the results of biochemical assays.

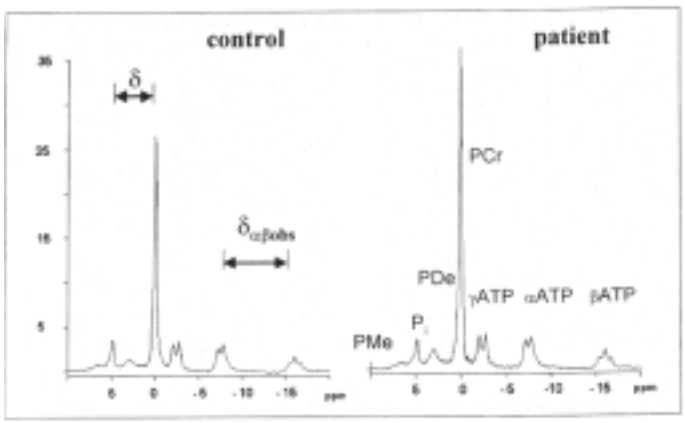

Fig. 1. *^{31}P MR spectrum of calf muscles of healthy volunteers and patients, as measured by the surface coil (Method I). The signal-to-noise ratio calculated for the signal of PCr is approx. 150.*

Methods

Localized phosphorus MR spectroscopy

Localized ^{31}P spectra and MR images were obtained on a Siemens 1.5 Tesla Magnetom "Vision" imager (Siemens Erlangen, Germany), equipped with actively shielded gradients, standard head coil and a dual $^{1}H/^{31}P$ surface coil.

The MR protocol consisted of multislice sagittal, coronal and transversal turbo spin echo T2 weighted MR images (TR (repetition time) = 5400 ms, TE (echo time) = 99 ms, FA (flip angle) 180^0, 5 mm slice thickness, 260 mm field-of-view, 192x256 matrix, acquisition time 1.21 min) to determine the exact position of the coil.

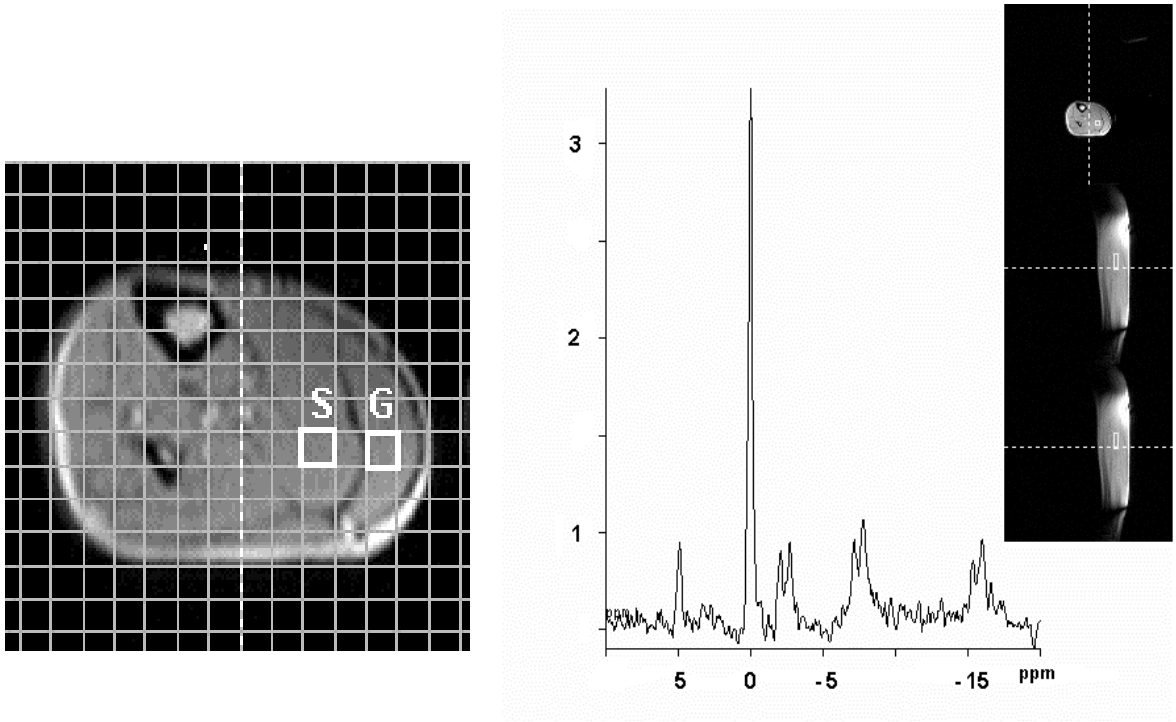

Fig. 2. *Localization of VOIs (4 cm x 1 cm x 1 cm) from the soleus (S) and gastrocnemius muscle (G) and a typical spectrum measured by the ^{31}P CSI technique (Method II) from the calf muscle. The spectra were measured with an approx. signal-to-noise ratio of 30.*

The spectra were obtained using two different techniques:

Method I.

Mean values of chemical shifts and signal intensities of all muscles in the central part of the calf were measured by surface coil localization with a simple one-pulse sequence. Three FID spectra with NOE enhancement (TR=5s, AC [the number of acquisitions]=16, number of points: 512, spectrum width = 4000 Hz, signal-to-noise ratio [$S/N_{(PCr)}$]=130±30) were obtained (see Fig. 1).

Method II.

^{31}P MR spectra from specific muscles, see Fig. 2, were obtained using the 2D-CSI technique with 16x16 phase encoding steps (TR=323 ms, TE=3 ms, flip angle=90°, AC=32, slice=40 mm, FOV=320 mm, digitally increased resolution in k-space up to 32x32 resulting in a VOI (volume of interest) of 4 ml, number of points: 512, spectrum width=4000 Hz, $S/N_{(PCr)}$=40±20).

Automatic and manual global shim of water signal was used and the shim was characterized by the line width of the water signal in the range of 9-13 Hz.

Spectral analysis and data processing

Three spectra were measured by Method I and processed using standard Siemens Numaris software. The following steps of spectral evaluation were performed for all measured spectra: zero filling: 2048 points (digital resolution 0.076 ppm**),** exponential apodization (center 0.0, width 100 ms), Fourier transformation, zero and first order phase correction, time domain baseline correction and deconvolution. Deconvolution to obtain chemical shifts and signal intensities was performed step by step to each peak; the starting points for deconvolution were chemical shifts of individual signals relative to P_{Cr} which was kept at 0.00 ppm. Signals of P_i (≈ 5 ppm), P_{Me} (≈ 6,7 ppm), and P_{De} (≈ 3 ppm) were approximated by a single Lorentzian and/or Gaussian line. Doublet and triplet of γATP (≈ -2.7 ppm) and βATP (≈ -16.5 ppm) signals were optimized by three Lorentzian peaks with J=18 Hz. The signal in the αATP region (≈ -7.3 ppm, J=17 Hz) was fitted by the doublet representing αATP and by the singlet to cover the contribution of the NADP signal (-8.3 ppm) which exists in the right shoulder of the signal (see Fig. 3). Spectra acquired using Method II were processed in the same way. To exclude a partial volume effect from overlaying areas, only VOIs from the gastrocnemius and soleus muscles were chosen for the study.

The deconvolution was repeated three times, and the mean values of chemical shifts and signal intensities were used to calculate the signal of intensity ratios and δ_{Pi} and $\delta_{\alpha\beta obs}$. Constants used in equations (4) and (5) were applied according to literary data (Thompson *et al.* 1995): δ_{ATP}=10.6 ppm, $K_H=3.4x10^{-7}$ M, $K_D=9.0x10^{-5}$ M, δ_{ATPMg}=8.165 ppm, δ_{ATPH}=11.66 ppm, pK_P = 6.75, δ_{HP}=3.27, δ_P=5.69. Statistical evaluation of data was performed by t-tests and U-tests using Quatro for Windows program.

Subjects

The group of patients (JHP) included 11 asymptomatic individuals (8 M, 4 F, age range 17-23 years; mean age 19±2 years) with a randomly diagnosed primary form of hypertension (renovascular, endocrine, cardiac or neurogenic in nature). The interpretation of current blood pressure (BP) values in the case of individuals below 18 years of age was undertaken according to distribution graphs (Report of the Second Task Force 1987), which represent the development of BP from childhood to adulthood. In adults, the criteria as defined by WHO guidelines were applied (borderline BP and mild elevation ≥ 140/90 mm Hg, significant hypertension BP ≥ 165/95 mm Hg). Mild hypertension was diagnosed in 10 individuals, while the BP of one subject was above the significant value. The BP of patients on the day of MR examination and biochemical tests was within the range of ±10/5 mm Hg of their mean BP.

Eight patients received dietary treatment and increased physical activity for body weight reduction; in three patients, antihypertensive therapy had been discontinued 3 weeks before MR and the biochemical examinations. Only in one case was a dermatological drug employed, with no effect on mineral metabolism. A group of 19 healthy controls (HC) (8M, 11F) aged 25±9 years consisted of subjects with recalled and current physiological BP values. The exclusion criterion for the selection of the control group depended on any present symptoms or past history of a afflictions influencing BP and mineral metabolism.

Biochemical and MR examinations of patients and controls were performed under the same protocol after an overnight fast (minimum 6-8 hours) (Resnik *et al.* 1991).

Volunteers and patients were informed about the examination protocol in accordance with local ethics committee standards.

Table 1. ^{31}P MR of signal ratios and calculated concentrations of [fMg^{2+}] and pH in control and patient groups, as measured by Method I

Group	P_{Cr}/P_i	$P_{Cr}/_{PATP}$	P_{Cr}/P_{Me}	P_{Cr}/P_{De}	$P_{Cr}/P_{\beta ATP}$	P_i/P_{De}	$P_{De}/P_{\beta ATP}$	P_i/P_{ATP}	[fMg^{2+}] μmol/l	pH
PJH	8.7±1.6	1.5±0.1	10.8±3.3	6.1±1.5	7.1±0.9	0.7±0.2	1.2±0.3	0.2±0.03	788±33	7.05±0.02
HC	5.6±1.0	1.4±0.2	11.9±3.1	6.0±1.5	5.9±0.8	1.1±0.3	1.05±0.3	0.25±0.04	776±21	7.06±0.01
U	**	**	**	-	**	**	**	**		

*U: U test: ** significant difference ($p<0.01$), P_{Cr} - phosphocreatine, P_i - inorganic phosphate, P_{ATP} = αATP + βATP + γATP, P_{Me} - phosphomonoesters, P_{De} – phosphodiesters*

Results

The concentrations of Mg in the serum (0.83±0.04 mmol/l), the concentrations of Mg in the urine (4.4±1.3 mmol/l), and the concentrations of high-density lipids (1.3±0.2 mmol/l) were within the standard reference range. The results of [fMg^{2+}] concentrations and pH calculations with the help Equations (4) and (5) using spectroscopic data obtained by Method I are summarized in Table 1. The data obtained in our patients were compared with those of the controls and no significant difference between control and patient groups was found in pH or [fMg^{2+}] values. Measurements evaluated by Method I gave a mean value for the ratios from all muscles in the measured area. Chemical shifts and signal intensities obtained by Method II (which is volume sensitive) from the gastrocnemius and soleus muscles are summarized in Table 2. No significant difference was observed in either [fMg^{2+}] or pH when PJH patients and controls were compared. Furthermore, no significant difference was noted between the two muscle groups, in patients and the controls.

However, an unexpected significant difference between patients and controls was found in the relative concentrations of phosphorus metabolites (see Tables 1 and 2). P_{Cr}/P_i, $P_{Cr}/P_{\beta ATP}$, $P_{De}/P_{\beta ATP}$ were increased while P_i/P_{De} and P_i/P_{ATP} were decreased compared to the data from the controls. These changes indicate that the concentrations of creatine/phosphocreatine are increased and concentrations of inorganic phosphate are decreased.

Discussion

Determination of [fMg^{2+}] and pH in muscles

The concentrations of [fMg^{2+}] in control groups has been previously described (Widmaier *et al.* 1996, Ward *et al.* 1996, Ryschon *et al.* 1996, Isrish *et al.* 1997, Sebekova *et al.* 1999) and lies in the range of 0.460 to 0.929 μmol. Our results, which are in the middle of the range, fit well with these data. To our knowledge, only two groups of authors have described [fMg^{2+}] concentrations in patients with hypertension. In both cases, no significant changes in [fMg^{2+}] concentrations were demonstrated (Isrish *et al.* 1997, Sebekova *et al.* 1999). A similar result was reported in a group of females where no change in [fMg^{2+}] was observed across the menstrual cycle (Rosenstein *et al.* 1995). In our case, both biochemical tests and ^{31}P MR spectroscopy showed no significant difference in the concentrations of [fMg^{2+}] and pH between patients and controls (Tables 1 and 2). The result of MR spectroscopy is independent of the experimental method, i.e. use of the simple pulse (Method I) or CSI (Method II) techniques. In a VOI, no significant difference in [fMg^{2+}] was observed between control and patient groups or between gastrocnemius and soleus muscles when Method II was used. The results of ^{31}P CSI show a slightly greater difference between the concentrations of [fMg^{2+}] in the measured muscles. This is probably due to the decreased S/N ratio, and is in agreement with data analysis performed by Golding (Golding *et al.* 1996) who demonstrated an increasing error in the calculation of [fMg^{2+}] concentrations with a decreasing S/N ratio. Using Method I, the S/N ratio is approximately 150 compared to the S/N of spectroscopic imaging which is about 30. Although the S/N ratio of spectroscopic imaging is less than a quarter of that obtained using Method I, [fMg^{2+}] and pH are not significantly different. We noted no significant difference between the muscles, similar to previously described results (Widmaier *et al.* 1996).

As mentioned above, the [fMg^{2+}] concentration in the present study was approximately in the middle of published values. The difference between the absolute concentrations can be attributed to the application of

Table 2. ^{31}P MR ratios, pH and [fMg^{2+}] concentrations in m.gastrocnemius (G) and m.soleus (S) of healthy volunteers (HC) and in patients with primary juvenile hypertension (PJH). The spectra were measured from a 4 ml volume. Groups of patients and controls: healthy volunteers n=17, juvenile hypertension patients n=5

Muscle	Group	P_{Cr}/P_i	PCr/P_{ATP}	P_{Cr}/P_{Me}	P_{Cr}/P_{De}	$P_{Cr}/P_{\beta ATP}$	P_i/P_{De}	P_i/P_{ATP}	Muscle	Group	[fMg^{2+}] µmol/l	pH	S/N
G	PJH	6.6±1.4	0.8±0.1	16.5±6.7	6.0±1.6	2.2±0.2	0.9±0.2	0.12±0.02	G	PJH	811±133	7.06±0.02	30±14
G	HC	4.8±1.6	0.7±0.2	10.8±7.5	7.0±4.7	2.0±0.6	1.4±0.8	0.15±0.05	G	HC	737±132	7.05±0.02	38±5
		*	-	-	-	*	*	-					
S	PJH	6.6±0.8	0.9 ±0.1	15.2±4.4	7.0 ±1.4	2.6 ±0.2	1.0±0.2	0.13±0.01	S	PJH	936±433	7.06±0.04	42±20
S	HC	5.1±1.1	0.7 ±0.1	13.0 ±5.7	9.4 ±6.0	1.8 ±0.4	1.8±1.1	0.14±0.03	S	HC	794±130	7.05±0.01	51± 8
		*	*	-	-	*	*	-					

*U: U test: *- significant difference (p< 0.05)*

different equilibrium constants, which are obtained from titration curves, again underlining the importance of spectral analysis (see Fig. 3). The signal of αATP is known to be superimposed upon the smaller signal of NADP. Both signals are represented by doublets and the center of NADP has a chemical shift smaller than αATP. Because the chemical shift of NADP is smaller, the deconvolution of αATP and NADP is better represented by the superposition of two doublets. If this superposition is not taken into account and the chemical shift of αATP is calculated only from a single doublet, smaller $\delta_{\alpha ATP}$ and $\delta_{\alpha\beta ATP}$ are obtained. The result then indicates higher concentrations of [fMg^{2+}].

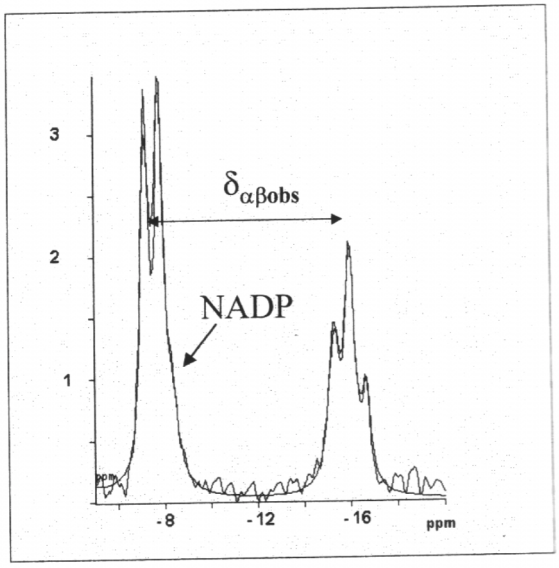

Fig. 3. *Detail of the deconvolution of the ^{31}P MR spectrum from Figure 1 in the range of -7 to -20 ppm. The αATP signal range was approximated by the doublet of the αATP and NADP signal at -8.3 ppm and by the singlet with a relative intensity of about 10 % of αATP. The signal of NADP is visible as the shoulder of the main signal. The signal of βATP was fitted by a triplet.*

Other errors which can influence the calculation of [fMg^{2+}] arise from an incomplete description of the equilibrium system and from the selection of incorrect equilibrium constants. In living tissue, many cations participate in the biochemical reactions, and a rapid exchange with ATP sites can be expected. Iotti (1996) described several systems which should, at least theoretically, be taken into consideration.

Results of assessment of the relative ratio of signals

The results in Tables 1 and 2 show a significant difference in the relative amount of high-energy phosphates in PJH patients compared with healthy controls, particularly the ratios of signal intensity of P_{Cr}/P_i, $P_{Cr}/P_{\beta ATP}$, and P_i/P_{De}. Two independent methods of ratio measurement indicate that the intensity of signals P_{Cr} and P_{De} is enhanced in the muscles of PJH patients. According to our experience obtained from hundreds of ^{31}P MRS examinations of calf muscles performed in our laboratory (Kurková *et al.* 1997), a significant elevation of the P_{Cr}/P_i ratio towards higher values is rare and may be due to hypophosphate anemia whose origin is not clear.

These results indicate that [Mg^{2+}] concentrations, measured by ^{31}P MR spectroscopy as well as by serum and urine extracellular Mg concentrations, are not significantly different between patients with primary juvenile hypertension and the controls. This is in accordance with literary findings where examinations based on [fMg^{2+}] measurements during exercise and at rest in patients with juvenile dermatomyositis and healthy controls gave similar results (Niermann *et al.* 2000).

Our study is an extension to previous reports on hyperinsulinemia in adult individuals with hypertension and, particularly, to our own results emerging from our research projects. These have repeatedly confirmed significant hyperinsulinemia in pediatric and adolescent hypertensive patients (Palyzová *et al.* 1996, 2001). The ion hypothesis on the early coexistence of hypertension and metabolic disorders thus assumes specific functional consequences, e.g. vasoconstriction and an increase in BP (Resnick 1993). An impaired biological response to insulin may also entail impaired phosphorylation processes and alteration of cellular cation metabolism (Sowers *et al.* 1998).

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