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In Vitro ^{31}P-Magnetic Resonance Spectroscopy of Muscle Extracts in Malignant Hyperthermia-susceptible Patients

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***Background*: It was recently suggested that malignant hyperthermia-susceptible (MHS) patients could have an elevated peak of phosphodiesters in leg muscles using *in vivo* phosphorus magnetic resonance spectroscopy. In the current study, analysis of the phosphodiesters of muscle extracts of MHS and malignant hyperthermia-negative patients was performed using *in vitro* phosphorus magnetic resonance spectroscopy to chemically identify and to compare the muscle concentrations of water-soluble compounds between the two groups with respect to the muscle fiber type composition.**

***Methods*: Perchloric acid extracts of the *vastus medialis* muscle of seven MHS patients and ten malignant hyperthermia-negative patients on the basis of the European malignant hyperthermia contracture test were subjected to *in vitro* phosphorus magnetic resonance spectroscopy carried out at 9.4 T. In addition, chemical identification of the phosphodiester region and histologic examination of the muscle specimens were performed.**

***Results*: The peak in the phosphodiester region was assigned to glycerophosphorylcholine. Muscle perchloric acid extracts of MHS patients had a significantly ($P < 0.05$) higher glycerophosphorylcholine to the sum of phosphocreatine and inorganic phosphate (glycerophosphorylcholine/[phosphocreatine +inorganic phosphate]) value than those of malignant hyperthermia-negative patients. Neither a difference in the fiber type composition between the two groups nor any specific myopathy were found.**

***Conclusions*: In the absence of histologic differences between muscle specimens of MHS and malignant hyperthermia-negative patients, these results could suggest that glycerophosphorylcholine could be a marker of an impairment in the phospholipid metabolism in the skeletal muscle of MHS patients. (Key words: Hyperthermia: malignant. Measurement techniques: phosphorus magnetic resonance spectroscopy. Muscle: perchloric acid extracts.)**

METABOLIC abnormalities in malignant hyperthermia-susceptible (MHS) patients have been reported in skeletal muscle using *in vivo* phosphorus magnetic resonance spectroscopy (^{31}P-MRS).[1,2] It was recently reported that MHS patients also exhibited a higher phosphodiesters to phosphocreatine (PDE/PCr) ratio than did malignant hyperthermia-negative (MHN) patients in ^{31}P spectra of gastrocnemius muscle at rest.[3,4] The significance of such a finding remains unknown. An increased PDE/PCr ratio also has been reported in patients with other myopathies using *in vivo* ^{31}P-MRS.[5,6] *In vitro* ^{31}P-MRS studies of the PDE compounds from normal muscle perchloric acid (PCA) extracts have identified the presence of glycerophosphorylcholine (GPC),[7,8] which is one water-soluble product of phospholipid catabolism.[9] Glycerophosphorylcholine content was increased in dystrophic chicken pectoralis muscle[7] as well as in slow twitch muscle containing type I fibers (*e.g.*, in soleus muscle).[8]

The aim of the current *in vitro* ^{31}P-MRS study was to analyze the PDE region from the muscle PCA extracts of vastus medialis of MHS and MHN patients with respect to the muscle fiber type composition. A higher GPC content in muscle PCA extracts of MHS patients could be a result of either a predominance of type I

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muscle fibers or an impairment of membrane phospholipid metabolism.

Materials and Methods

Human Muscle Biopsy

Muscle specimens from 17 patients referred to the Grenoble MH Diagnosis Center were studied. Muscle biopsy was performed because of a family history of MH or symptoms of MH during anesthesia. Open surgical biopsies of the vastus medialis muscles were performed under regional anesthesia. One or two muscle specimens 2–2.5 cm long and weighing 440 mg (270–1067 mg) were quickly frozen and stored in liquid nitrogen until the PCA extraction procedure. Other muscle specimens were processed for histologic analysis and for the contracture test, which was performed according to the European MH Group protocol.[10] The test was considered positive when the muscle specimens produced a sustained increase in baseline tension with an amplitude ≥ 0.2 g during application of both $\leq 2\%$ halothane and ≤ 2 mM caffeine. The test was equivocal when a contracture was elicited by only one test substance. Other results were deemed MH-negative.

Perchloric Acid Extracts

The PCA extracts from muscle specimens were prepared as described previously,[11] within a 3-month period after biopsy. Briefly, the frozen biopsy specimen was pulverized with a liquid nitrogen-chilled mortar and pestled to a fine powder. The tissue was transferred and homogenized in a Potter-Elvehjem homogenizer with 0.6 mol l^{-1} of PCA (10 ml g^{-1} wet weight). After centrifugation at 41,400*g* for 10 min at 4°C, the *p*H of the supernatant was adjusted to 6.6–7.0 with potassium hydroxide. This procedure was repeated twice, and the resulting supernatant was lyophilized. The powder was then resuspended in D_2O solution (Laboratories Merck-Clévenot, Dardilly, France), centrifuged at 12,500*g* for 10 min and the supernatant was lyophilized. All the samples obtained after this PCA extraction procedure were stored in liquid nitrogen. Before the MRS experiment, the extract was resuspended in 350 μl D_2O solution at *p*H 8.4 and centrifuged at 12,500*g* for 10 min.

^{31}P-Magnetic Resonance Spectroscopy Study

Magnetic resonance spectroscopic experiments were performed with a Bruker AM400 spectrometer (Bruker Spectrospin, Wissembourg, France) equipped with a 9.4-T, 89-mm diameter vertical bore magnet. The ^{31}P spectra were acquired using a 5-mm diameter multinuclear probe tuned to ^{31}P frequency (162.4 MHz) under the following conditions: single-pulse sequence of 15.5 μs pulse length with a composite proton sequence decoupling; 4.05 s repetition time; 6 or 7 blocks of 1,600 scans; 16,384 data points per free induction decay; 6410-Hz spectral width; 3-Hz line broadening. The PCr was chosen as reference of chemical shift (0 ppm). The quantification was based on the integration of the peaks of phosphorylated compounds using UXNMR software (Bruker, Rheinstetten, Germany). Results were expressed as areas relative to the sum of PCr and inorganic phosphate (Pi) areas (PCr+Pi) or to the sum of the areas of all phosphorylated compounds visible by ^{31}P-MRS (ΣP).

Chemical Identification of the Phosphodiesters Peak

The following criteria were used to identify the chemical compounds present in the PDE region: (1) the correspondence between the observed chemical shifts in the muscle PCA extracts and the known chemical shifts for pure compounds at different *p*H values (titration experiment), (2) the persistence of a single spectral line of enhanced intensity on addition of a standard at a final concentration of 5 mg ml^{-1} to the PCA extract at more than one *p*H value (coresonance experiment). The standard compounds were L-α-glycerophosphoryl-ethanolamine, -inositol, -serine, -choline (GPC; Sigma, Saint-Quentin Fallavier, France).

Histologic Analysis

Sixteen muscle specimens were analyzed histologically by optical microscopy. Sections were cut on a freezing microtome and stained histochemically for myosin ATPase at *p*H 4.35 to perform the fiber type composition. The counting of fiber types and their mean diameters were determined using an image analyzer equipped with the Histo 200 software (Biocom, Lyon, France). The mean cross-sectional area of each muscle fiber type was then automatically calculated.

Statistical Analysis

Data were expressed as medians and ranges. The ^{31}P-MRS and histologic parameters of MHS and MHN groups were compared using the nonparametric Mann-Whitney *U* test at the 0.05 level of significance.

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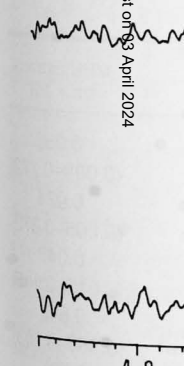

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Results

The contracture tests revealed that seven patients (median 37 yr, range 16–63) were MHS and ten patients (median 23 yr, range 12–51) MHN. None were equivocal for responses to halothane or caffeine. Figure 1 shows the PDE region in muscle PCA extract from an MHS patient, before and after sequential addition of PDE compounds. In the PDE region, a major peak was detected at 3.0 ppm. When standard compounds were added to the muscle PCA extract, there was an exact coincidence of the PDE peak only with the GPC standard (coresonance experiment). In addition, there were similar changes in the *p*H-dependent chemical shifts of the PDE peak of the muscle PCA extract spectrum, the pure GPC peak in D_2O solution and the GPC peak when added to the muscle PCA extracts (fig. 2; titration experiment). The major peak in PDE region was thus assigned to GPC.

Typical ^{31}P spectra of muscle PCA extract obtained from MHN and MHS patients are shown in figure 3. In these spectra, the GPC/(Pi+PCr) ratio was measurably higher in the muscle PCA extract of the MHS patient. For the two groups, the phosphomonoesters (PME) to ΣP (PME/ΣP), GPC/ΣP and GPC/(Pi+PCr) ratios were significantly higher in MHS muscle extracts than in MHN extracts (fig. 4 and table 1). The mean of the sum of ranks was 6.3 *versus* 12.8 for the PME/ΣP ratio, 6.8 *versus* 12.1 for the GPC/ΣP ratio, and 6.7 *versus* 12.3 for the GPC/(Pi/PCr) ratio in the MHS and MHN groups, respectively. There was no difference between the muscle specimens of MHS and MHN patients concerning the presence of type I fibers (43% [27.9–52.8] *vs.* 41.4% [28.3–50.5], respectively) and the mean cross-sectional area of type I fibers (3,019 μ^2 [2206–4657] *vs.* 2,734 μ^2 [1,257–3,217], respectively). None of the patients had specific myopathy on histologic examination of muscle specimens.

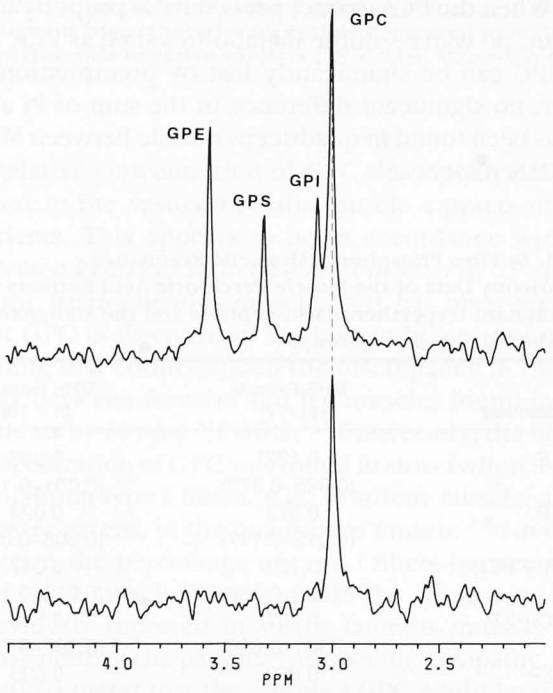


Fig. 1. A coresonance experiment from a malignant hyperthermia-susceptible muscle muscle perchloric acid extract before (*A*) and after (*B*) sequential addition of phosphodiester standard compounds to assign the resonance of the phosphodiester region. The ^{31}P spectra were acquired at 9.4 T with a 4.05-s pulse repetition rate. GPE = glycerophosphorylethanolamine; GPI = glycerophosphorylinositol; GPC = glycerophosphorylcholine; GPS = glycerophosphorylserine.

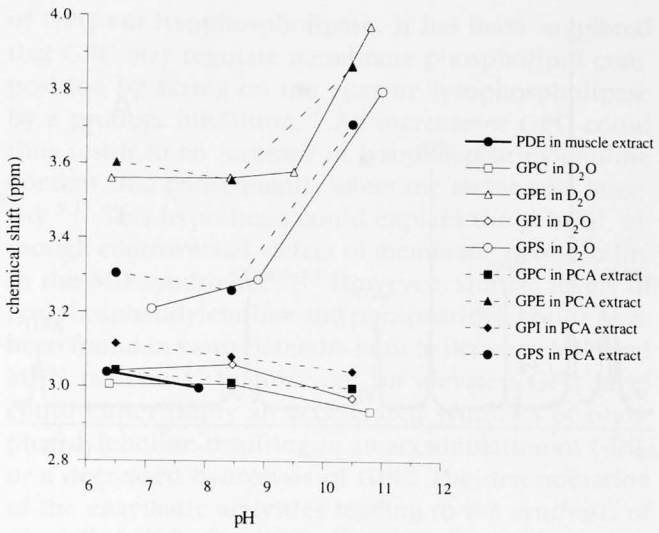
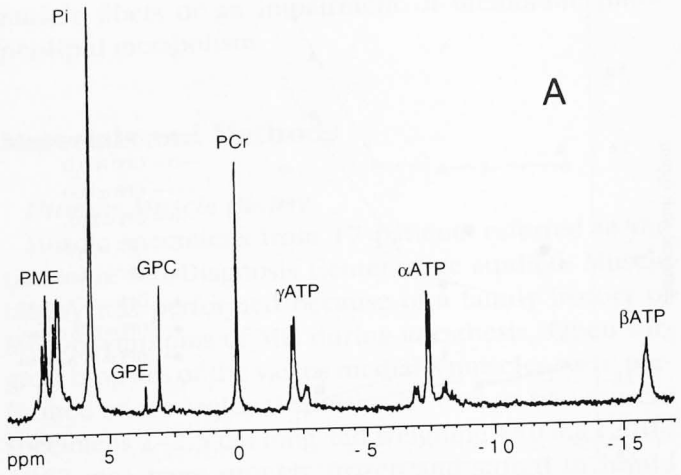


Fig. 2. A titration experiment with the determination of chemical shifts, relative to the phosphocreatine as reference, of the phosphodiester compounds at different *p*H values, to assign the resonance of the phosphodiester region with respect to the *p*H-dependent chemical shifts. The chemical shifts of the major phosphodiester peak of the muscle extract spectrum were measured as well as those of pure phosphodiester compounds peaks in D_2O solution and those when addition of phosphodiester standard compounds to the muscle perchloric acid extract.

Discussion

A significantly higher relative concentration of GPC was found in muscle PCA extracts of the MHS patients as compared to the MHN patients. In the absence of



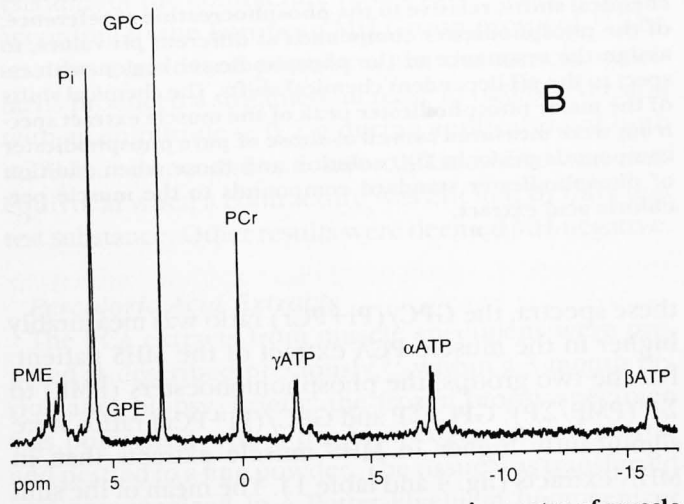


Fig. 3. ^{31}P-Magnetic resonance spectroscopic spectra of muscle perchloric acid extract from malignant hyperthermia-negative patient (*A*) and malignant hyperthermia-susceptible patient (*B*). Spectra were acquired at 9.4 T in 7 × 1,600 scans (*A*) and in 6 × 1,600 scans (*B*) with a 4.05-s pulse repetition rate and processed with a 3-Hz exponential line broadening.

histologic differences between muscle specimens of the two groups, these results suggest that GPC could be a marker of an impairment in the phospholipid metabolism in the skeletal muscle of MHS patients. However, there was an overlap between MHS and MHN values (fig. 4), limiting diagnostic potential of these *in vitro* results.

Each phosphorus-containing chemical group has a characteristic chemical shift position, which is determined by the molecular environment of the phosphorus atom contained within different phosphorylated compounds. However, ionic strength, *p*H, cationic, and protein concentration in cell compartments may complicate the assignment of a resonance peak recorded from intact tissue. Therefore, coresonance experiments at different *p*H levels are usually done to assign the resonances observed in ^{31}P spectra of PCA extracts.[11,12] According to the PCA extract procedure, GPC was clearly identified as the primary component of the PDE peak in muscle PCA extract of MHS patients. The other PDE compounds appeared to be of minor contribution in muscle PCA extracts, as previously reported in normal muscle.[8] However, no absolute concentrations of GPC and other phosphorylated compounds were provided in the current study because of the absence of an external standard. In addition, the muscle specimens of different weights were resuspended in the same volume of D_2O solution, leading to different signal-to-noise ratio between PCA extracts (figs. 3A and 3B). Internal references were thus chosen such as ΣP and Pi+PCr, because there is a stoichiometric change between the decrease in the PCr peak intensity and the increase in the Pi peak intensity, so the sum Pi+PCr remains constant. When the PCA extract procedure is properly carried out, no water-soluble metabolites such as PCr, Pi, and GPC can be significantly lost by precipitation.[13] Finally, no significant difference in the sum of Pi and PCr has been found in quadriceps muscle between MHS and MHN patients.[14]

Table 1. *In Vitro* Phosphorus Magnetic Resonance Spectroscopy Data of the Muscle Perchloric Acid Extracts in the Malignant Hyperthermia-susceptible and the Malignant Hyperthermia-negative Groups

MRS Parameter	MHS Extracts (n = 7)	MHN Extracts (n = 10)
PME/ΣP	0.132* (0.065–0.373)	0.053 (0.001–0.172)
GPC/ΣP	0.073* (0.012–0.177)	0.024 (0.006–0.072)
GPC/(Pi + PCr)	0.163* (0.026–0.360)	0.047 (0.013–0.145)
(Pi + PCr)/ΣP	0.467 (0.301–0.696)	0.542 (0.360–0.763)
βATP/ΣP	0.063 (0.023–0.124)	0.095 (0.018–0.153)

Values are median (ranges).

MRS = magnetic resonance spectroscopy; MHS = malignant hyperthermia-susceptible; MHN = malignant hyperthermia-negative; PME = phosphomonoesters; GPC = glycerophosphorylcholine; Pi = inorganic phosphate; PCr = phosphocreatine; ΣP = sum of all phosphorylated compounds.

* Significantly different from MHN extracts ($P < 0.05$) by Mann-Whitney test.

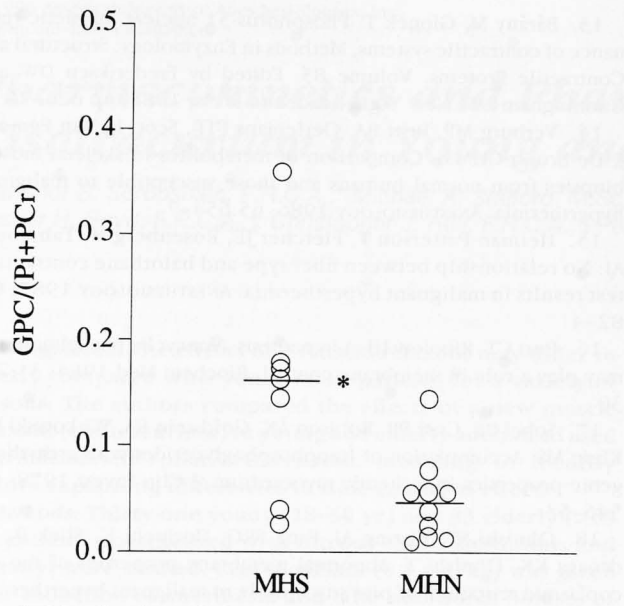


Fig. 4. Distribution of the glycerophosphorylcholine/(inorganic phosphate+phosphocreatine) ratios for malignant hyperthermia-susceptible and malignant hyperthermia-negative patients. The lines represent the median values for the two groups. There is a significantly higher glycerophosphorylcholine/(inorganic phosphate+phosphocreatine) ratio in malignant hyperthermia-susceptible compared to malignant hyperthermia-negative subjects (*$P < 0.05$, Mann-Whitney U test).

Relative concentration of GPC was significantly elevated in the vastus medialis muscle extracts of MHS patients. This appears to be in accordance with the elevated PDE/PCr ratio observed *in vivo* in ^{31}P spectra of the gastrocnemius muscle.[3,4] It has been reported that GPC is absent from the human biceps muscle,[7] a finding that could explain the discrepancy in the PDE peak between forearm and leg muscles found in MHS patients by *in vivo* ^{31}P-MRS.[1–4] Conversely, the highest concentration of GPC was found in slow twitch muscle containing type I fibers, *e.g.*, in soleus muscle, and to a lesser extent, in the quadriceps muscle.[7,8] No difference in the percentage of type I fibers between MHS and MHN muscles was found in the current study, as previously reported in vastus lateralis muscle.[15] Because none of the patients had specific myopathy, these results suggest that the elevated GPC could be related to the MH susceptibility.

Glycerophosphorylcholine is a water-soluble metabolite produced by the catabolism of the membrane phospholipids, from the pathway of the phosphatidylcholine deacylation: production of lysophosphatidylcholine *via* phospholipase A_2, followed by production of GPC *via* lysophospholipase. It has been suggested that GPC may regulate membrane phospholipid composition by acting on the enzyme lysophospholipase by a product inhibition.[16] An increase of GPC could thus result in an increase in lysophosphatidylcholine content, and consequently affect the membrane integrity.[9,17] This hypothesis could explain the general, although controversial, defect of membrane permeability in the MH syndrome.[18–20] However, similar levels of lysophosphatidylcholine and phosphatidylcholine have been found in vastus lateralis muscle between MHS and MHN patients.[21] In this way, an elevated GPC level could either imply an accelerated synthesis of phosphatidylcholine resulting in an accumulation of GPC, or a decreased hydrolysis of GPC. The determination of the enzymatic activities leading to the synthesis of phosphatidylcholine as well as to the degradation of GPC would thus be useful to elucidate the mechanisms that could account for the data.

The PME/ΣP ratio was higher in the muscle PCA extracts of MHS patients than those of MHN patients. The phosphomonoesters include a number of compounds such as triose phosphates, hexose aliphatic phosphates, pentose phosphates, inosine and adenosine monophosphate, choline, and phosphoethanolamine.[12] However, no chemical identification of the PME region was performed in the current study and the significance of such results remains unknown.

These *in vitro* ^{31}P-MRS results demonstrate that the skeletal muscle of MHS patients exhibits a higher relative GPC content than that of MHN patients. Because this difference cannot be explained by histologic changes between MHS and MHN muscle specimens, these results suggest that GPC could be a marker of an impairment in the phospholipid metabolism in the skeletal muscle of MHS patients.

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