Vol. 180, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1991 Pages 216-222

ENERGY STATE OF CHONDROCYTES ASSESSED BY ^{31}P-NMR STUDIES OF PREOSSEOUS CARTILAGE

Piero Pollesello[1], Benedetto de Bernard[2], Micaela Grandolfo[2], Sergio Paoletti[2], Franco Vittur[2] and Bjarne J. Kvam[1]

[1] POLY-biòs Research Center, LBT - Area di Ricerca, Padriciano 99, I-34012 Trieste, Italy

[2] Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, via Alfonso Valerio 22, I-34127 Trieste, Italy

Received August 28, 1991

Summary. The energy state of resting and hypertrophic chondrocytes from growth plate was studied by ^{31}P-NMR spectroscopy of superfused cartilage slices. The presence of phosphocreatine was demonstrated in both cell types, using a repetition time of 3 s. By comparing the decline in the nucleoside triphosphate level after adding blockers of the glycolysis or of the mitochondrial respiration, it was deduced that resting and hypertrophic chondrocytes use both metabolic pathways for energy production, but the glycolysis dominates. Hypertrophic cells rely more on the mitochondrial respiration than the resting cells.

The aim of the present work was to apply comparative ^{31}P-NMR studies of the energy state of growth cartilage in the different stages of maturation. Chondrocytes in the growth plate are differentiating cells (1-3). Their morphology and functions change progressively from the resting to the ossifying zone of the tissue, where the cells become hypertrophic (4), produce matrix vesicles (5,6) and eventually die. Studies on chondrocyte energy requirement have focussed on the oxidative and glycolytic activities (7,8) and on the pentose phosphate shunt metabolism (9) in the different zones of growth cartilage. The ATP/ADP ratios (10) and NTP concentrations (11,12) have been measured at various stages of the chondrocyte maturation. It has been suggested that chondrocytes obtain their metabolic energy by both glycolysis and oxidative mitochondrial processes, but the hypertrophic cells seemed to rely more on the nonoxidative metabolism and to have a low energy charge (12). All these

Abbreviations: DPDE, diphosphodiesters; GPC, glycerophosphorylocholine; GPE, glycerophosphorylethanolamine; HC, hypertrophic chondrocyte; NADs/FADs, pyridine and flavin nucleotides; NDP, nucleoside diphosphates; NTP, nucleoside triphosphates; PCr, phosphocreatine; P_i, intracellular inorganic phosphate; PC, phosphocholine; PE, phosphoethanolamine; PDE, phosphodiesters; PME, phosphomonoesters; RC, resting chondrocyte.

0006-291X/91 $1.50

studies were performed either by biochemical assays on isolated cells, or by analysis of metabolites in freeze-trapped sections.

To our knowledge, no ^{31}P-NMR studies of cartilage have appeared so far. This may be due to the low cell density and the low metabolic activity level as compared to other tissues, resulting in a low overall concentration of phosphorylated metabolites in the tissue. However, after initiating our work, various conference abstracts demonstrate that there is an increasing interest for cartilage and other connective tissues within the NMR milieu.

We have recently demonstrated (13) the feasibility of ^{31}P spectroscopy of cartilage. In the present work, the phosphate metabolites of resting and hypertrophic chondrocytes (RCs, HCs) were followed in superfused tissue in order to compare their energy production patterns. The NTP concentration level and the intracellular pH were followed after addition of metabolic inhibitors, acting either on the glycolytic pathway (iodoacetate) or on the mitochondrial respiration (KCN).

MATERIALS AND METHODS

Tissue preparation. Scapulas from 40-70 kg pigs were excised from the animals immediately after their death. Resting and hypertrophic regions were selected and dissected as reported previously (14). Thin cartilage slices (0.3 mm or less) were washed repeatedly under sterile conditions with phosphate-free Krebs-Ringers-Hepes buffer (KRH, 110 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM Hepes, pH 7.4) containing 10 mM glucose, 10 mM glutamine, 500 U/ml penicillin and 0.5 mg/ml streptomycin, and then kept for 30 min. at 37°C in the same buffer prior to NMR measurement or $HClO_4$ extraction.

Superfusion. Stable metabolic conditions was ensured by superfusion at 37°C as described previously (13). The tissue (ca 3 g) was immersed in KRH buffer in a 10 mm NMR tube. The buffer was saturated with air (chromatographic grade) prior to the flushing of the tissue (every 2 hours). The pH of the waste superfusion buffer remained between 7.35 and 7.40. Some experiments were performed by adding either 2 mM KCN or 10 mM iodoacetate to the superfusion buffer.

Perchloric acid extraction. Resting cartilage was extracted essentially as described by Munch-Petersen et al. (15). The tissue (15-20 g) was ground to a fine powder under liquid nitrogen, ice-cold perchloric acid was added (70%; 0.3 ml/g of tissue) and the mixture was reground and freeze-thawed 3 times. Following centrifugation at 2000xg at 4°C for 30 min, EDTA was added to the supernatant (final concentration 20 mM) and the pH was adjusted to 7.4 with K_2CO_3. The $KClO_4$ precipitate was removed by centrifugation (30 min.; 10.000xg; 4°C) and the supernatant was subjected to NMR analysis. The extract of the hypertrophic zone contained a large quantity of inorganic phosphate released from hydroxyapatite crystallites. The resulting ^{31}P spectrum was completely dominated by the strong P_i signal, and no other signals could be identified.

NMR measurements. ^{31}P-NMR spectra were obtained at 121.5 MHz on a Bruker AM 300 WB NMR spectrometer using 8.0 kHz spectral width. For the tissues, 6000 scans were aquired with 4 k data points, 62° flip angle, an acquisition time of 0.26 s and a repetition time of 3.0 s. Zero filling of 4 k data points and a line broadening of 30 Hz was employed. For the perchloric acid extracts, 30000 scans were aquired with 16 k data points, 2.6 s relaxation delay, 62° flip angle and a repetition time of 3.0 s. Spectra were ^{1}H decoupled and zero-filled to 32 k data points. Resolution enhancement was achieved by Lorentz-Gauss transformation. Tissue spectra were acquired at 37°C and the

extracts at 30°C. All chemical shifts are referred to phosphocreatine at pH 7.20 (0.00 ppm).

NTP quantification. The relative concentrations of NTP were obtained from the area of the β-peak of NTP, due to the lack of nearby signals.

Intracellular pH determination. Intracellular pH values were determined by utilizing the pH-dependence of the chemical shift of inorganic phosphate, δP_i (16,17). The intracellular pH follows a Henderson-Hasselbalch type equation (18): $pH = pK_a + \log ((\delta P_i - \delta_a)/(\delta_b - \delta P_i))$
where δ_a is the acid end-point and δ_b the basic end-point. The values of pK_a, δ_a and δ_b depend on the ionic strength and the concentration of some ionic species of the medium (18,19). Parameters determined previously for ^{31}P-NMR studies of cartilage (13) were therefore applied: pK_a=6.86, δ_a=3.18 and δ_b=5.88.

RESULTS AND DISCUSSION

Recently, we have demonstrated that ^{31}P-NMR can be successfully applied to study superfused cartilage (13), despite the low cell density of the tissue. The choice of repetition time was also found to be critical due to the slow longitudinal relaxation of some phosphorylated compounds (T_1 = 1.5-3.9 s)(20,21). In the present work we have chosen a rather long repetition time (3 s), to ensure the detection of molecules, like e.g.guanido-phosphates, that may remain undetected with a rapid cycling.

^{31}P spectra of superfused hypertrophic and resting cartilage (Fig. 1 A and B, respectively) showed peaks characteristic of mammalian cells. Phospho-mono- and diesters, inorganic phosphate and nucleotides are indicated. The spectrum of the neutralized perchloric extract of the resting zone is shown in Fig.1 C, and its assignment, as reported previously (13), allowed an easier interpretation of the superfused tissue spectra.

The phosphomonoesters (PME) appear in the region 5-7 ppm and their major components are phosphocholine (PC) and phosphoethanolamine (PE) (see Fig. 1C). The phosphodiester peaks (PDE), with glycerophosphorylcholine (GPC) and glycerophosphoryl-ethanolamine (GPE) as the most abundant compounds, are seen close to the inorganic phosphate peak (P_i)(see Fig. 1C). The PME, P_i and PDE concentration levels differ significantly in the two zones. In the HCs (Fig.1A), P_i is more abundant with low PME and PDE levels and vice versa (Fig. 1B). In the spectrum of the extract (Fig.1 C), the two NDP peaks can be recognized, although in general they are not detectable in spectra of living tissues (22). In the range from -8 to -10 ppm, the diphosphodiester (DPDE) peaks appear, including pyridine and flavin nucleotides. From these basic spectra, the intracellular pH values of HCs and RCs can also be estimated (see Table I, controls).

The presence of phosphocreatine in both RCs and HCs is a major point of interest. This high-energy compound is typical for cells that rely mainly on glycolysis for their energy supply. Nevertheless, both RCs and HCs contain pyridine and flavin nucleotides, in accordance with the hypothesis that energy to some extent may be obtained also by aerobic metabolism (12). The high

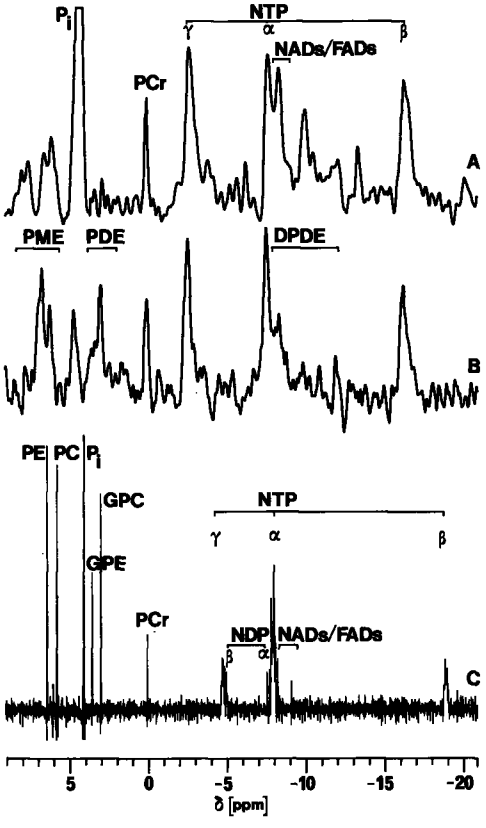

Fig.1. ^{31}P-NMR spectra of superfused cartilage and its perchloric acid extract. (A), hypertrophic zone and (B), resting zone from growth plate, in the stable metabolic state obtained during superfusion at 37°C. (C), neutralized perchloric acid extract of resting zone. The P_i-peak in (A) is truncated to 50% of actual height.

content of pyridine and flavin nucleotides (with respect to NTP) in the HCs as compared to the RCs (Figs. 1A and B) confirms the results obtained by other techniques regarding concentrations of NAD^+, NADH (8) and ATP (11) in the different zones.

Table I

Effect of KCN on the intracellular pH of resting and hypertrophic chondrocytes. The values of the controls were obtained from spectra of tissues superfused with phosphate-free KRH buffer (pH 7.4, supplemented with 500 U/ml penicillin, 0.5 mg/ml streptomycin, 10 mM glucose, 10 mM glutamine). The pH values represent averages of 5 hours.

	pH(*)	
	Resting	Hypertrophic
Control	7.10±0.05	7.00±0.05
After addition of 2 mM KCN	6.64±0.05	6.72±0.05

(*) the error limits reflect uncertainties in the determined chemical shifts of the P_i-signal.

The ^{31}P spectrum of the HCs shows a rather intense P_i signal (Fig. 1A). The sample was washed exhaustively prior to the measurements, and the observed P_i-shift corresponds to a pH value of 7.0, which differs from that of the superfusion buffer (7.4). Contributions from extracellular free inorganic phosphate can therefore be neglected. It was already known that the total P_i content of resting and hypertrophic regions is different (23). However, no specific evidence regarding intracellular concentrations has hitherto been presented. The high content of free inorganic phosphate together and the low NTP/P_i ratio in HCs (Fig. 1A and B), suggest that they are characterized by an impairment of the energy restoration, as compared to RCs. Similar conclusions have been drawn from a totally different approach (11).

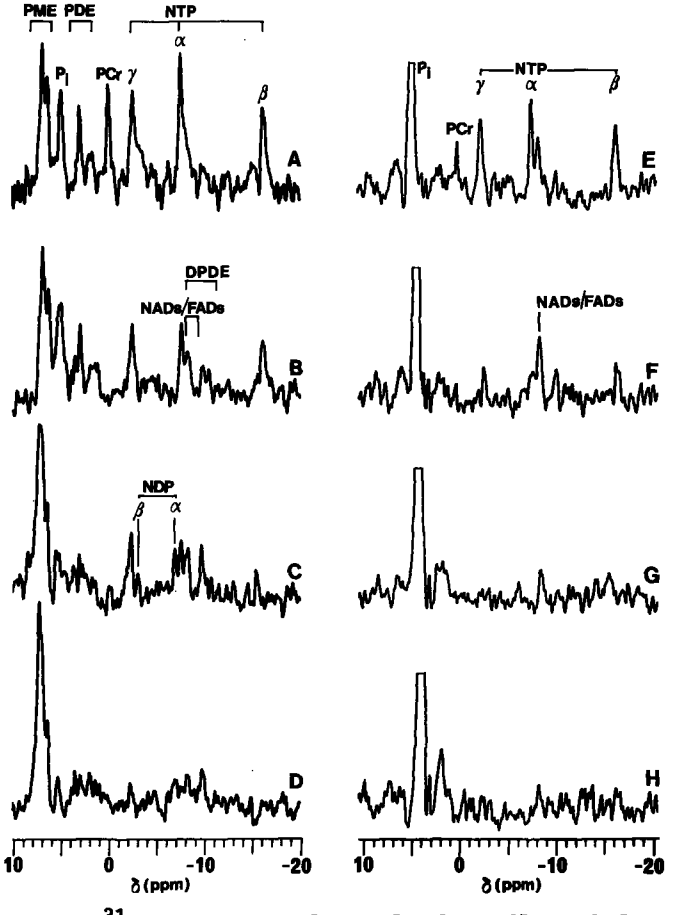

Fig.2. Series of ^{31}P-NMR spectra of superfused cartilage before and after addition of 2 mM KCN. (A-D), spectra of the resting zone, and (E-H) of the hypertrophic zone. (A and E), control spectra representing the stable metabolic state in KRH buffer (pH 7.4, supplemented with 500 U/ml penicillin, 0.5 mg/ml streptomycin, 10 mM glucose, 10 mM glutamine). The P_i-peak in (E) is truncated to 33% of actual height. (B-D) and (F-H), series of spectra acquired consecutively after addition of 2 mM KCN to the superfusion medium. Each spectrum was acquired in 5 hours.

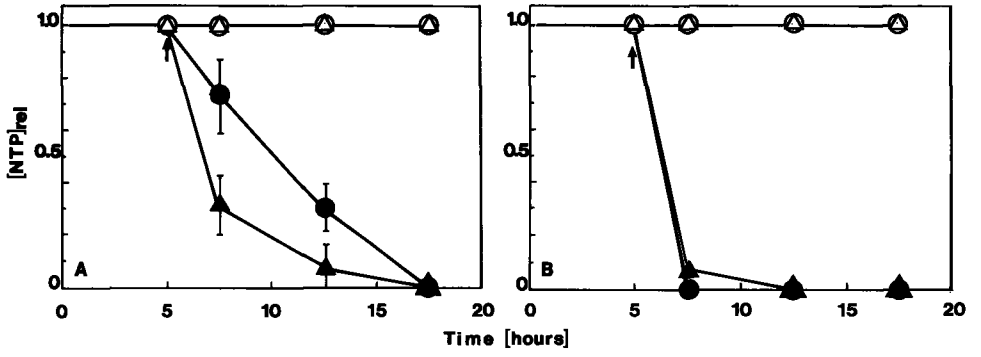

Fig.3. Relative NTP concentrations in resting and hypertrophic chondrocytes after addition of metabolic inhibitors.
(●), resting and (▲), hypertrophic chondrocytes after addition of blockers of (A) mitochondrial respiration (2 mM KCN), and (B) glycolytic pathway (10 mM iodoacetate). An arrow indicates the addition of the inhibitor. Control values obtained without addition of inhibitors are shown by open symbols. Data are the average of 3 different experiments ± s.d.

Further information regarding the metabolic activity was obtained by adding blockers depressing the NTP content by inhibiting the synthesis of ATP by mitochondrial respiration (KCN) or by glycolysis (iodoacetate). KCN resulted in a drop in the intracellular pH for both cell types (Table I) and the NTP concentration level was significantly affected, as seen in Fig. 2A-D. The fast drop in the phosphocreatine signal intensity and the progressive rise of NDP peaks are also evident. Signals related to pyridine and flavin nucleotides remain stable during the first 10 h of inhibition of the mitochondrial respiration (Figs. 2B and C). Similar trends were observed for the HCs (Fig. 2E-H). The decline in the relative NTP level with time after addition of KCN was faster in HCs (Fig. 3A), suggesting that they rely more than RCs on an oxidative metabolism. This finding fits well with the higher mitochondrial weight fraction in HCs as compared to RCs, which reflects the index of enzymatic activities (24).

The NTP pool of both cells is dramatically reduced by iodoacetate in a shorter time, as seen in Fig. 3B. This observation confirms that the major part of the energy in any case is obtained from glycolysis.

In conclusion, ^{31}P spectroscopy permitted the demonstration of significant amounts of PCr in cartilage and the dependence on both the glycolytic and the oxidative pathways has been confirmed. However, our results suggest that the OCs rely more than the RCs on the oxidative metabolism, whereas the opposite has been hypothesised previously (12).

ACKNOWLEDGMENTS

We wish to thank Istituto di Radiologia dell'Università di Trieste for the access to the Bruker AM 300 WB NMR spectrometer and the U.S.L. n°1 for a research grant to P.P. The technical assistance of C. Galbardi and of M.T. Nicodemo is gratefully acknowledged. We thank also Uanetto & Borghese Delicatessen factory for the generous supply of pig scapulas. This work was financially supported by C.N.R. and M.U.R.S.T., Italy.

REFERENCES

1. Robinson, R.A., Cameron, D.A. (1956) J. Biophys. Biochem. Cytol. 2 (Suppl.), 253-263.
2. Fitton-Jackson, S. (1957) Proc. R. Soc. Lond. Ser. B 146, 270-280.
3. Cameron, D.A. (1963) Clin. Orthop. Rel. Res. 26, 199-228.
4. Pal, S., Tang, L.H., Choi, H., Haberman, E., Rosenberg, L., Roughley P. and Poole, A.R. (1981) Coll. Res. 1, 151-176.
5. Bonucci, E.J. (1967) Ultrastruct. Res. 20, 33-50.
6. Anderson, H.C. (1969) J. Cell. Biol. 41, 59-72.
7. Shapiro, I.M., Golub, E.E., Haselgrove, J., Havery, J., Chance, B., Frasca, P. (1982) Science 217, 950-952.
8. Kakuta, S., Golub, E.E., Haselgrove, J.C., Chance, B., Frasca, P., Shapiro, I.M. (1986) J. Bone Min. Res. 1, 433-440.
9. Silverton, S.F., Matsumoto, H., DeBolt, K., Reginato, A. and Shapiro, I.M.(1989) Bone 10, 45-51.
10. Iannotti, J.P., Brighton, C.T. (1989) J. Orthop. Res. 7, 511-518.
11. Shapiro, I.M., Golub, E.E., May, M., Rabinowitz, J.L. (1983) Bioscience Report 3, 342-351.
12. Matsumoto, H., DeBolt, K. and Shapiro, I.M. (1988) Journal of Bone and Mineral Research 3, 347-352.
13. Kvam, B.J., Pollesello, P., Vittur, F., Paoletti, S. (1991) Magnetic Resonance in Medicine, in press
14. Vittur, F., Pugliarello, M.C. and de Bernard, B. (1971) Experientia 27, 126-127.
15. Munch-Petersen, B., Trysted, G. and DuPont, B. (1973) Exp. Cell. Res. 79, 249-256.
16. Moon, R.B. and Richards, J.H. (1973) J. Biol. Chem. 248, 7276-7278.
17. Burt, C.T., Glonek, T. and Barany, M. (1976) J. Biol. Chem. 251, 2584-2591.
18. Roberts, J.K.M., Wade-Jardetzky, N., Jardetzky, O. (1981) Biochemistry 20, 5389-5394.
19. Seo, Y., Murakami, M., Watari, H., Imai, Y., Yoshizaki, K., Nishikawa, H. and Marimoto, T. (1983) J. Biochem. 94, 729-734.
20. Malloy, C.R., Cunningham, C.C. and Radda, G.K. (1986) Biochim. Biophys. Acta 885, 1-11.
21. Challis, R.A.J., Blackledge, M.J. and Radda, G.K. (1990) Biochem. J. 268, 111-115.
22. Gadian, D.G. and Radda, G.K (1981) Ann. Rev. Biochem. 50, 69-83.
23. Arsenis C. (1972) Biochem. Biophys. Res. Comm. 46, 1928-1935.
24. Stambough, J.L., Brighton, C.T., Jannotti, J.P. and Storey, B.T. (1984) J. Orthop. Res. 2, 235-246.