Volume 332, number 3, 221–225 FEBS 13097 October 1993

A comparative evaluation of the metabolic profiles of normal and inflammatory knee-joint synovial fluids by high resolution proton NMR spectroscopy

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Received 25 August 1993

High resolution ^{1}H NMR spectroscopy has been employed to investigate the metabolic profile of heatlhy human knee-joint synovial fluid (SF) and the biochemical data acquired have been compared with those of matched serum, and inflammatory knee-joint SF samples. Results obtained indicate that the healthy human knee-joint has a hypoxic status (high lactate level when expressed relative to that of paired serum) that is milder than that of the inflamed human knee-joint. Moreover, normal SF differs from that of inflammatory SF in that it contains little or no NMR-detectable lipoprotein-associated fatty acids and 'acute-phase' glycoproteins, an observation reflecting the limited passage of these macromolecules from plasma into the synovial space in healthy subjects.

Normal synovial fluid; ^{1}H NMR spectroscopy; Inflammatory joint disease; Hypoxia; Low-density-lipoprotein; Acute-phase glycoprotein

1. INTRODUCTION

Much interest has been shown in the measurement of endogenous metabolite levels in both serum and knee-joint synovial fluid (SF) samples obtained from patients with inflammatory joint diseases. High resolution proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy permits the rapid, non-invasive multicomponent analysis of a wide variety of molecularly mobile biofluid metabolites [1–3], and Williamson et al. [4] have previously employed this technique to investigate potential diagnostic indicators of rheumatoid arthritis (RA) and osteoarthritis (OA). Indeed, ^{1}H NMR data obtained by these researchers indicated a shorter mean triacylglycerol chain length in the OA sample group, and measurements made on serial aspirations obtained from one RA patient suggested that alterations in the SF concentrations of triacylglycerols, *N*-acetylated 'acute-phase' glycoproteins (APGs) and creatinine correlated with those of serum α_2-glycoprotein, a common marker of disease activity. Moreover, we have recently applied high-field ^{1}H NMR analysis to demonstrate the abnormal metabolic status of SF collected from patients with RA [5]. Relative to paired sera, RA SFs contain (i) substantially elevated lactate levels, (ii) low glucose concentrations, (iii) markedly reduced levels of chylomicron- and very-low-density-lipoprotein (VLDL)-associated triacylglycerols which appear to have a shortened mean chain length, and (iv) high concentrations of ketone bodies (3-D-hydroxybutyrate, acetoacetate and acetone). Although the elevated RA SF lactate concentrations reflect the high level of anaerobic metabolism conducted in the inflamed joint, the high concentrations of ketone bodies detectable appear to arise from an increased metabolic consumption of lipids therein. These observations are consistent with those of Dunham et al. [15] who suggested that an enhanced intra-articular utilisation of fats for energy occurs in the inflamed joint, an environment where glucose levels are often low.

To date, only matched serum samples have served as controls for biochemical investigations of inflammatory SFs since the difficulties associated with the aspiration of sufficient quantities of this biofluid from normal knee-joints has hampered such studies. However, we have recently been successful in obtaining adequate volumes of normal SF (up to 0.3 ml) from a range of healthy male volunteers, and here we report for the first time a high-field ^{1}H NMR evaluation of the metabolic status of this biofluid. Appropriate comparisons between the ^{1}H NMR profiles of healthy human SFs and those of matched serum, and inflammatory SF samples, have also been conducted.

2. MATERIALS AND METHODS

2.1. *Sample collection and preparation*

Six healthy male volunteers (age range 25–42 years) were rested for

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a period of 30 min and SF samples were then aspirated from their knee-joints. These samples were collected using a 23-gauge needle connected to a 2 ml syringe via the standard clinical approach for inflamed joint aspiration. Where required, the passage of SF across the lateral joint space and supra-patellar pouch was assisted. Typically, volumes ranging from 0.1–0.3 ml were obtainable. Patients with moderately severe rheumatoid arthritis (n = 22, age range 40–67 years) were also rested for 30 min prior to the aspiration of SF samples.

Sterile knee-joint SF samples collected from each group of subjects were drawn into plastic tubes and transported to the laboratory on ice immediately after collection. These samples were centrifuged at 7,000 × g for 1 h to remove cells and debris, when present, and then stored at −20°C for a maximum duration of 72 h prior to ^{1}H NMR analysis. Control experiments established that none of the criteria investigated changed significantly during these periods of storage. Non-heparinized blood was drawn from all subjects at the same time points as the SF samples. These samples were allowed to clot at ambient temperature and the resulting serum was immediately centrifuged and stored as described above.

2.2. *Proton NMR measurements*

^{1}H NMR measurements on biofluids were conducted on Bruker WH400 or AMX-600 (University of London Intercollegiate Research Services (ULIRS), Queen Mary and Westfield College facilities, University of London, UK) spectrometers operating in quadrature detection mode at operating frequencies of 400.13 and 600.13 MHz, respectively, for ^{1}H. For normal SFs, the volume collected was measured and these samples were then made up to 0.60 ml with ^2H_2O, the latter providing a field frequency lock. For inflammatory SFs, 0.60 ml of sample was directly added to 0.07 ml of ^2H_2O. Samples were placed in 5-mm diameter NMR tubes, and ^{1}H NMR spectra acquired at a probe temperature of 20°C, or, where specified, 45°C. The broad protein resonances were suppressed by the Hahn spin-echo sequence (D[90°x-t-180°y-t-collect]) which was repeated 128 times with t = 68 ms. The intense water signal was suppressed by gated irradiation, and chemical shifts were referenced to external sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (δ = 0.00 ppm). The methyl group resonances of lactate (δ = 1.050 ppm), alanine (δ = 1.487 ppm) or valine (δ = 1.050 ppm) served as secondary internal references for all biofluid spectra acquired.

The identies of components responsible for the resonances present in spectra of synovial fluid and serum samples were routinely assigned by a consideration of characteristic chemical shift values, coupling patterns and coupling constants. Where appropriate, standard additions of authentic standards were made to confirm assignments.

^{1}H NMR measurements on a commercially available sample of low-density-lipoprotein (LDL) were conducted on a JEOL JNM-GSX 500 (ULIRS, Biomedical NMR Centre, Birkbeck College, University of London, UK) spectrometer operating in quadrature detection mode at an operating frequency of 500.16 MHz for ^{1}H. Spectra were recorded at probe temperatures of 20 and 45°C. 0.60 ml of an aqueous solution of human LDL (6.0 mg protein/ml) containing 0.15 M NaCl and 270 μM EDTA (Sigma, UK) was placed in a 5-mm diameter NMR tube, and 0.07 ml of ^2H_2O was added to provide a field frequency lock. Hahn spin-echo spectra were acquired using the above pulse sequence which was repeated 128 times with t = 60 ms. The intense water signal was suppressed by pre-saturation with gated decoupling during the delay between pulses, and chemical shifts were referenced to external TSP.

3. RESULTS AND DISCUSSION

The high-field (aliphatic) regions of 500 MHz ^{1}H Hahn spin-echo NMR spectra of isolated, matched normal SF and serum samples (Fig. 1) contain resonances attributable to a wide variety of low-molecular mass components (including isoleucine, leucine, valine, threonine, alanine, glutamine, glycine, 3-D-hydroxybutyrate, lactate, acetate, pyruvate, citrate, glucose, creatinine and creatine) together with those assignable to the molecularly mobile portions of macromolecules, i.e. lipoprotein-associated fatty acids and the branching carbohydrate side chains of APGs. The corresponding low-field (aromatic) regions of these spectra contained only weak signals arising from tyrosine, histidine and formate (data not shown).

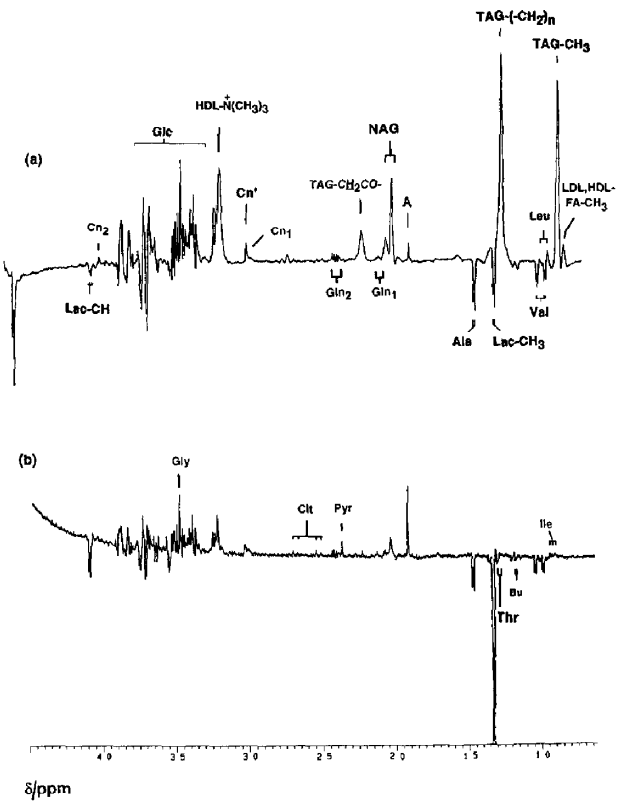

Fig. 1. High-field regions of 400 MHz ^{1}H Hahn spin-echo (t = 68 ms) NMR spectra of (a) normal blood serum and (b) matched knee-joint synovial fluid. Typical spectra are shown. A, acetate-CH_3; Ala, alanine-CH_3; Bu, 3-D-hydroxybutyrate-CH_3; Cit, citrate-CH_2; Cn′, creatine N-CH_3; Cn_1 and Cn_2, creatinine N-CH_3 and N-CH_2-CO-, respectively; Glc, glucose ring protons; Gln_1 and Gln_2, β and γ-CH_2 groups of glutamine, respectively; Gly, glycine-CH_2; Ile, isoleucine-CH_3; Lac-CH_3 and CH, lactate-CH_3 and -CH groups; Leu, leucine-CH_3; Pyr, pyruvate-CH_3; Thr, threonine-CH_3; Val, valine-CH_3; NAG, -NHCOCH_3 group protons of N-acetylsugars present in the branching carbohydrate side chains of acute-phase glycoproteins (predominantly α_1-acid glycoprotein); LDL- and HDL-FA-CH_3, acyl chain terminal-CH_3 groups of low-and high-density lipoprotein-associated fatty acids (predominantly core cholesterol esters), respectively; TAG-CH_3 and -(-CH_2-)$_n$, predominantly chylomicron- and very-low-density-lipoprotein (VLDL)-associated triacylglycerol acyl chain terminal-CH_3 and bulk-CH_2- groups, respectively; TAG-CH_2CO, predominantly chylomicron- and VLDL-associated triacylglycerol -CH_2CO- groups. HDL-$\overset{+}{N}(CH_3)_3$, choline head groups of high-density-lipoprotein-associated phospholipids.

A major difference between spectra of normal SF and paired serum samples is the reproducibly higher concentrations of lactate detectable in the former (inverted doublet and quartet resonances located at 1.33 and 4.13

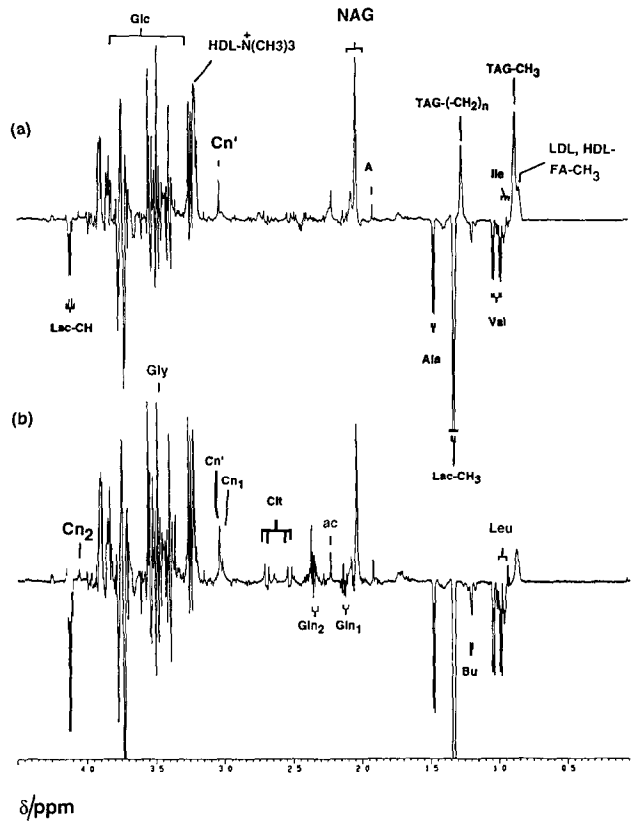

Fig. 2. High-field regions of 600 MHz ^{1}H Hahn spin-echo (t = 68 ms) NMR spectra of (a) blood serum and (b) matched knee-joint synovial fluid samples obhtained from a patient with rheumatoid arthritis. Typical spectra are shown. Abbreviations are as in Fig. 1; ac, acetone-CH_3.

ppm, respectively), i.e. 1.5–3.1 mM [6] which are approximately 2-fold greater than those of healthy human serum samples. However, in ^{1}H Hahn spin-echo spectra of RA SF (Fig. 2b), the lactate resonances are more prominent, reflecting the presence of extremely high levels of this product of anaerobic metabolism (ca. 5–12 mM [5,7]). Substantially elevated lactate concentrations detectable in RA SF are a characteristic feature of the hypoxic and acidotic conditions which prevail within the inflamed rheumatoid joint, and data obtained here establish that a similar, albeit milder, physiological environment is present in the healthy intra-articular space.

In addition, major broad resonances attributable to lipoprotein-associated fatty acids (i.e. chylomicron and VLDL triacylglycerols, low- and high-density-lipoprotein (LDL and HDL, respectively) cholesterol esters, and HDL phospholipids) present in ^{1}H Hahn spin-echo spectra of healthy human serum were either of very low intensity or undetectable in corresponding spectra of paired SF (Fig. 1), an observation that clearly reflects the poor filtration of these macromolecular species through the generally intact healthy synovial membrane. Indeed, comparison of normal SF spectra with those of SFs obtained from patients with inflammatory joint diseases (Fig. 2) confirmed that the latter group of samples contained higher levels of these lipoprotein-associated fatty acids that were readily detectable by ^{1}H NMR analysis. Detection of LDL in intact inflammatory SF samples was greatly facilitated by increasing the temperature at which spectra were accumulated from 20 to 45°C (Fig. 3). Indeed, LDL- and, to a much lesser extent, HDL-associated lipids make substantially greater contributions to the line-shape and intensity of the composite SF lipoprotein fatty acid acyl chain terminal-CH_3 and bulk $(-CH_2-)_n$ resonances at higher temperatures than they do at lower ones, a phenomenon attributable to the core cholesterol esters of these lipoproteins undergoing an order–disorder phase transition throughout this temperature range [8]. The corresponding signals of the triacylglycerol-rich chylomicron and VLDL particles do not undergo this phase transition, consistent with their core lipids being in a liquid-like state at temperatures ≥ 20°C. Non-invasive evaluations of the nature and levels of LDL cholesterol esters in this manner are particularly applicable to inflammatory SF samples in view of the low concentrations of this lipoprotein present when expressed relative to those of matched serum samples (approximately 42%) [9].

The intensities of resonances attributable to the choline head groups of LDL- and especially HDL-associated phospholipids located at 3.25 ppm in Hahn spin-echo spectra of inflammatory SFs were also markedly increased on elevating the acquisition temperature from 20 to 45°C (Fig. 3).

As expected, broad resonances assignable to the molecularly mobile APG carbohydrate side chain *N*-acetylsugars (*N*-acetylglucosamine and *N*-acetylneuraminate) centred at 2.04 and 2.08 ppm were only barely detectable in ^{1}H Hahn spin-echo spectra of normal SF, whereas these signals (predominantly attributable to α_1-acid glycoprotein [10]) were prominent features of those acquired on matched, healthy serum samples. Moreover, these APG signals were reproducibly of a much greater intensity in corresponding spectra of inflammatory SFs (Fig. 2). Increased levels of APGs in the blood serum of patients with inflammatory joint diseases have been determined previously by more conventional analytical techniques.

Interestingly, when normalised to resonances arising from low-molecular mass components (e.g. that of the alanine-CH_3 group), the intensities of the above APG signals in spectra of inflammatory SF were also found to increase on raising the acquisition temperature from 20 to 45°C (Fig. 3), demonstrating that the molecular mobility of their branching carbohydrate side chains increases with increasing temperature.

Data obtained from this investigation demonstrate that the metabolic status of healthy human SF is (i) markedly different from that of matched serum, and (ii) resembles that of inflammatory SF in terms of elevated lactate concentrations, but differs in that it contains very low levels of NMR-detectable lipoprotein-associ-

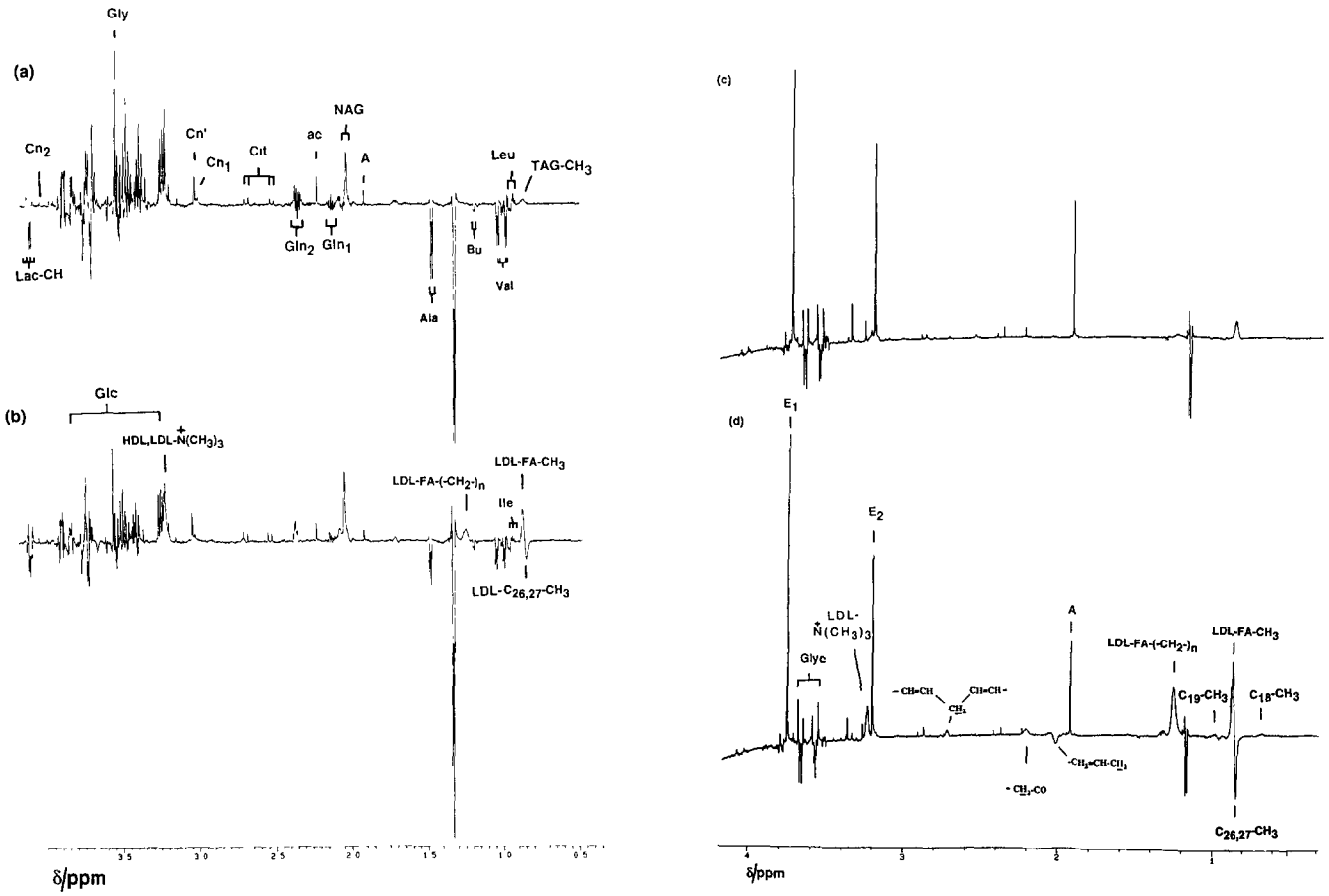

Fig. 3. High-field regions of 600 MHz ^{1}H Hahn spin-echo (t = 68 ms) NMR spectra of a rheumatoid knee-joint synovial fluid sample acquired at (a) 20°C and (b) 45°C. Typical spectra are shown. The high-field regions of 500 MHz ^{1}H Hahn spin-echo (t = 60 ms) spectra of an aqueous solution of a commercially available sample of human low-density-lipoprotein (6.0 mg protein/ml) containing 0.15 M NaCl and 270 μM EDTA (pH 7.4) acquired at 20 and 45°C are shown in (c) and (d), respectively. Abbreviations are as in Figs. 1 and 2; LDL C_{18}-CH_3, C_{19}-CH_3 and $C_{26,27}$-CH_3, the C-18, -19 and -26,27 position methyl groups, respectively, of the cholesterol moiety of low-density-lipoprotein (LDL)-associated cholesterol esters; E_1 and E_2, acetate and ethylenic protons, respectively, of EDTA; Glyc, glycerol-CH_2OH; LDL-$\overset{+}{N}(CH_3)_3$, choline head groups of LDL-associated phospholipids. The fatty acid acyl chain resonances of LDL-associated cholesterol esters are also indicated in spectrum (d). Glycerol and acetate detectable in spectra (c) and (d) are contaminants present in the commercially available LDL preparation utilised for these investigations. Further unassigned sharp resonances present in these spectra (e.g. inverted doublet located at 1.19 ppm) are also attributable to contaminants.

ated fatty acids and APGs. The second of these observations confirms that physiologically important macromolecules present in inflammatory SF arise from plasma via their passage into the synovial space, a phenomenon ascribable to the enhanced permeability of inflammatory synovium.

In view of established links between elevated circulating LDL concentrations and the development of atherosclerosis [11], the higher levels of LDL present in inflammatory SF over those of normal SF may render the inflamed joint more susceptible to the induction of localised atherosclerotic lesions, perpetuating the disease process. Indeed, a recent report has outlined the detection of foam cells containing oxidised LDL in synovial membrane samples obtained from RA patients [12]. Oxidation of LDL by chemically reactive oxygen radicals has been postulated to be a key step in the pathogenesis of atherosclerosis [13], and there is currently much evidence available for the deleterious role of such oxygen-derived radical species in contributing to the chronicity of RA, OA and allied conditions [14].

Acknowledgements: We are grateful to the Arthritis and Rheumatism Council (UK) (Grant B0141) and the Ministry of Agriculture, Fisheries and Food (UK) for financial support, the University of London Intercollegiate Research Services and the Science and Engineering Research Council for the provision of NMR facilities, and to Mr. P. Haycock for excellent technical assistance.

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