NMR LIPID ANALYSIS OF PLATELET MEMBRANES OF PATIENTS WITH CORONARY ARTERY DISEASES: CASE STUDIES

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ABSTRACT

A high-resolution proton nuclear magnetic resonance (Nmr) was used to investigate the platelet membrane lipid composition of subjects who have been definitively diagnosed as having various forms of coronary heart disease (CAD). The one dimensional (1D) proton spectra were recorded at a temperature of 298°K in Fourier transform(FT) mode with 16K data points, using a 45° detection pulse and 2.0 acquisition time with a solvent presaturation during relaxation to remove excess water signal. The major phospholipids and cholesterol present were identified and estimated and compared to those of normal individuals' platelet membranes. Platelet cholesterol content as analysed by the proton Nmr in all the 3 patients investigated were significantly elevated ($p<0.00001$). In contrast, arachidonic acid levels in the three patients were significantly reduced ($p<0.0002$) as well as phosphatidylcholine ($p<0.0408$) and phosphatidylethanolamine ($p<0.016$). The degree of unsaturation in the various structural phospholipids were also compared and was found to be less than those of the control subjects. Cholesterol: phospholipid ratio were also raised in the patients ($p<0.0016$). This work has shown that vital clinical information could be derived from lipid analysis by proton nuclear resonance which could be of clinical significance in the management of such patients.

Keywords: *proton nuclear magnetic resonance (Nmr); coronary heart disease; platelets; lipids.*

INTRODUCTION

The role of lipids and lipoproteins as risk factors in heart diseases continue to interest many researchers [1-3]. In addition, variations in concentration of lipids and other macro-molecules of human tissues have important consequences for the health of the individual, thus the successful isolation and quantitation of such substances are of paramount importance.

Nmr spectroscopy has been a powerful analytical technique capable of producing a great deal of information on major macro-molecules including lipids[4]. As a single method it can provide comprehensive information on the nature, composition and characteristics of existing lipid in cells, tissues or body fluids without subjecting them to chemical modifications prior to their analysis[5]. Assignments of 1D and 2D Nmr spectra of lipids from tissue extracts have been reported[6]. In our previous study, a combination of high resolution proton Nmr and HPLC methods were successfully employed to analyse and quantify the lipid composition in intact membranes[7].

In this study, we have attempted to use Nmr as clinical analytical tool to study both qualitatively and quantitatively the structural lipid composition of platelets from some patients with coronary artery disease and compared them to those of normal individuals.

MATERIALS AND METHODS

50 mls of citrated blood were obtained from volunteered out-patients from the cardiovascular unit of the Kings College Hospital, (King's Healthcare) London, UK. Blood samples were collected with the consent of the patients according to the Helsinki declaration. Patients had

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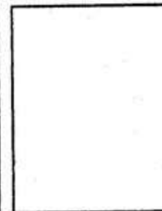

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to answer a questionnaire relating to their condition, medication, dietary and social habits. Individuals with lipid-related metabolic diseases were excluded in this study, besides, patients on medications known to affect lipid metabolism.

Collected blood samples were centrifuged at 1000rpm for 15 minutes to obtain a platelet-rich plasma (PRP). Platelets were sedimented by ultra-centrifugation at 20,000 rpm at 4^0C for half an hour. The platelet pellet was washed twice with Tris-buffered saline (140mM NaCl, 5mM glucose, 1mM EDTA and 15mM Tris-HCl, pH 7.4). Washed platelets were suspended in 0.25M sucrose and disrupted by sonication at 4^0C for 2 minutes and centrifuged at 18,500 rpm for 10 minutes to sediment the debris. The resultant supernatant was further centrifuged at 32,00rpm for one and half hours to pellet the membrane which was subsequently twice washed with Tris-buffered saline and solubilized in Tris buffer containing 0.5% of triton X100.

Lipid Extraction

This was carried out as previously described[7] using 10 mls of chloroform: methanol (2:1v/v). Extracted lipid was washed with 0.5MKCl in 50% methanol and dried under a stream of nitrogen. The extract was dissolved in 0.5 ml deuterated chloroform: perdeutero-methanol (2:1) (from Aldrich, UK), and transferred to a 5-mm Nmr tubes for analysis. Similar extracts were obtained from normal healthy individuals and used as controls.

Nmr analysis of lipids

Spectra of the various extracted lipids were recorded on a Bruker AM 500 nmr spectrophotometer at a temperature of 298^0K in Fourier transform(FT) mode with 16K data points using a 45° detection pulse and 2.0s acquisition time[8] with a solvent presaturation during relaxation to remove excess water signal. The data were multiplied with a square sine-bell function prior to transformation. Chemical shifts were referenced to the residual methanol resonance at 3.31 parts per million (ppm). Characteristic chemical shifts of individual lipids were identified and integrated by a method adopted in a previous study[9] after applying a baseline correction to the spectrum. The number of diagnostic protons in each lipid class was used in the overall quantitation of each lipid moiety and expressed as a molar percentage composition. All data are displayed as mean $\pm$ standard deviation of 10 control patients, or individual values for subjects identified as patients 1-3. Statistical data was compiled using the Ronstats application package.

RESULTS

The proton Nmr spectrum of the lipids extracted from individual patients and the controls are shown in Fig.1-2 , whilst the comparison of the mean lipid distribution of normal subjects and those of the patients investigated are presented in Table 1&2

Patient 1 was a 40-year old man with a history of ischaemic heart disease who was diagnosed with unstable angina, with a trial fibrillation. He was an ex-heavy smoker with a high blood pressure. His Nmr platelet lipid profile reveals an elevated cholesterol and fatty acids (mainly arachidonic and doco-sahexanoic acid). Cholesterol was identified by its characteristic C-18 methyl singlet at 0.68ppm. The fatty acids, arachidonic and deco-sahexanoic acids were estimated from their C:4 and C:6 resonances at 1.70 and 2:40 ppm. Other special features also observed included an unsaturation index of less than unity (0.72) $p<0.0001$. This was estimated from the difference between the total vinyl groups in all the fatty acids and that arising from cholesterol as described in our previous study[7] There was an elevated Cholesterol: Phospholipid ratio of 0.76 ($p<0.0016$).

Patient 2 was a 70-year old man with primary coronary disease. He was an ex-heavy drinker. His platelet membrane lipid profile reveals an elevated cholesterol content (56.76%), a decrease in the phospholipids(mainly PC and PE) and a decrease in the fatty-acids (arachidonic acid and decosahexaenoic acid). Among other special characteristics observed were a significantly low unsaturation index (0.44), and an elevated cholesterol:phospholipid ratio (1.3). The cholesterol moiety was quantified from its C-18 methyl moiety at about 0.68 ppm with a

characteristic multiplet. An elevated linoleic acid was estimated at its specific methylene proton signal at 2.75

Patient 3 was a 62-year old man who was diagnosed as having atherosclerosis with a history of primary coronary disease. His Nmr platelet lipid membrane profile revealed a high cholesterol content 54.3%, a low arachidonic and docosahexaenoic acids (Table.1). His unsaturation index was significantly lower compared to those of the controls ($p<0.0001$) and cholesterol: phospholipid ratio was elevated (1.2) $p<0.0016$.

DISCUSSION

At this stage of our investigation it was difficult to attribute the observed differences in the platelet membrane lipid between the patients investigated and those of the normal individuals to solely pathological causes, however, it is worthwhile to comment on their possible clinical implication, especially in coronary artery disease.

Coronary artery disease(CAD) is the most common heart disease[8] and is almost always the result of atherosclerosis - hardening of the arteries. The primary physiological event leading to the formation of atherosclerotic plaque is the increased deposition of macro molecules (including cholesterol) within the endothelial cells of the arteries[8]. Factors that influence the nature or the rate of lesion development at all subsequent stages include; hypercholesterolemia, with an associated excess of LDL (low density lipoproteins), and disturbances in platelet function, homeostasis and thrombosis[8]. The accelerating factors exert their influence by stimulating to varying degrees the synthesis by smooth muscle cells of collagen, elastin and the glycosaminoglycans[9].

It has been shown that platelets cannot synthesise cholesterol[10], therefore the cholesterol contents observed in these patients reflect the initial composition of the megakaryocyte. Studies have also shown that exchange of cholesterol with plasma lipoproteins may result in remodelling of the endogenous cholesterol content of platelets. It therefore stands to reason that the apparent elevated cholesterol as estimated among these patients could reflect a contribution of that which is exchanged with those of their plasma lipoproteins. The mechanism for such an exchange was however not addressed in this study. It may be via a receptor-mediated process. Due to the positive correlation between blood cholesterol and increased risk for coronary heart disease, it could be quite likely that the high platelet cholesterol content could be an indication of elevated plasma cholesterol levels among these patients, which may be secondary to high dietary cholesterol.

Reported platelet membrane of normal individuals have a much lower cholesterol: phospholipid ratio of approximately 0.5[11].which compares favourably with the 0.53 value obtained from this study. However, it was clear from this study that cholesterol:phospholipid ratios in all the patients studied were significantly greater than the control which could be of clinical importance. Patients with type II hyperlipoproteineamia whose platelet demonstrate hypersensitivity tend to have an elevated cholesterol:phospholipid ratio[12]. This phenomenon of an induced hypersensitivity possibly explains the observed elevated ratios in these patients investigated. However, it is not certain from this study whether such a hypersensitive response in these patients would be secondary to increase thrombotic activities of their platelets.

Previous studies have shown that platelet activation is associated with profound changes in lipid metabolism[13]. It is quite possible, that the indicated platelet lipid changes from the nmr profiling could be associated to an overall increase in lipid metabolic influx.

The low platelet unsaturation index observed in the patients investigated also provides vital clinical information since it has been recommended that a therapeutic diet for lower plasma cholesterol should have an unsaturation index of 1-1.2 implying, such patients should consume about 2 tablespoons of polyunsaturated fat or vegetable oil per day[14].

Despite the observed differences, the overall trend in the composition of the phospholipids were quite similar to those of the normal subjects, with PC being the predominant phospholipid followed by PE.

This work has shown that vital clinical information could be derived from lipid analysis by proton nuclear magnetic resonance which could be of immense clinical application in the management of patients with lipid-associated diseases.

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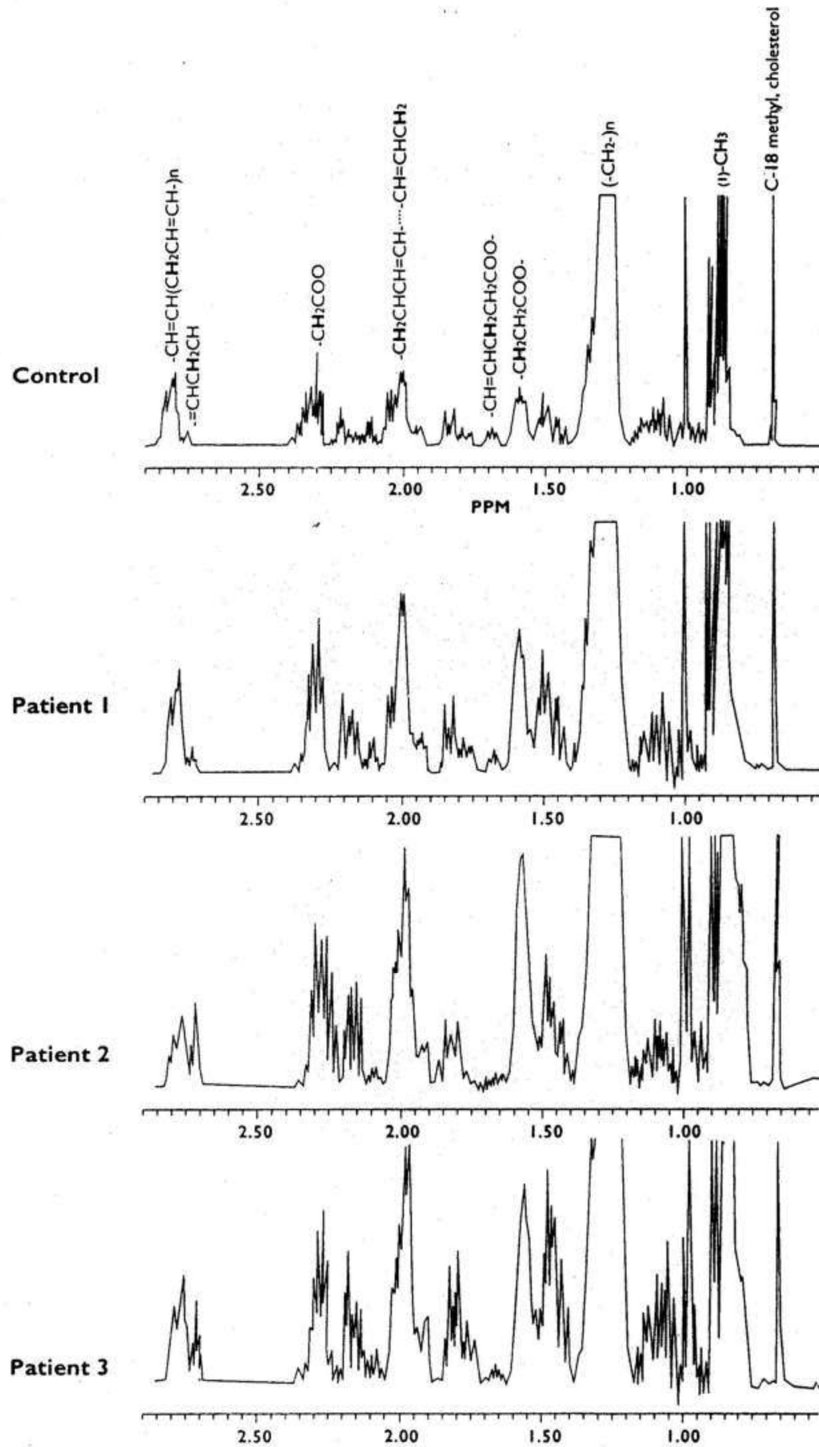

Fig. 1 1-dimentional proton nmr (3.0ppm-0.0ppm) of platelet total lipids of controls and patients

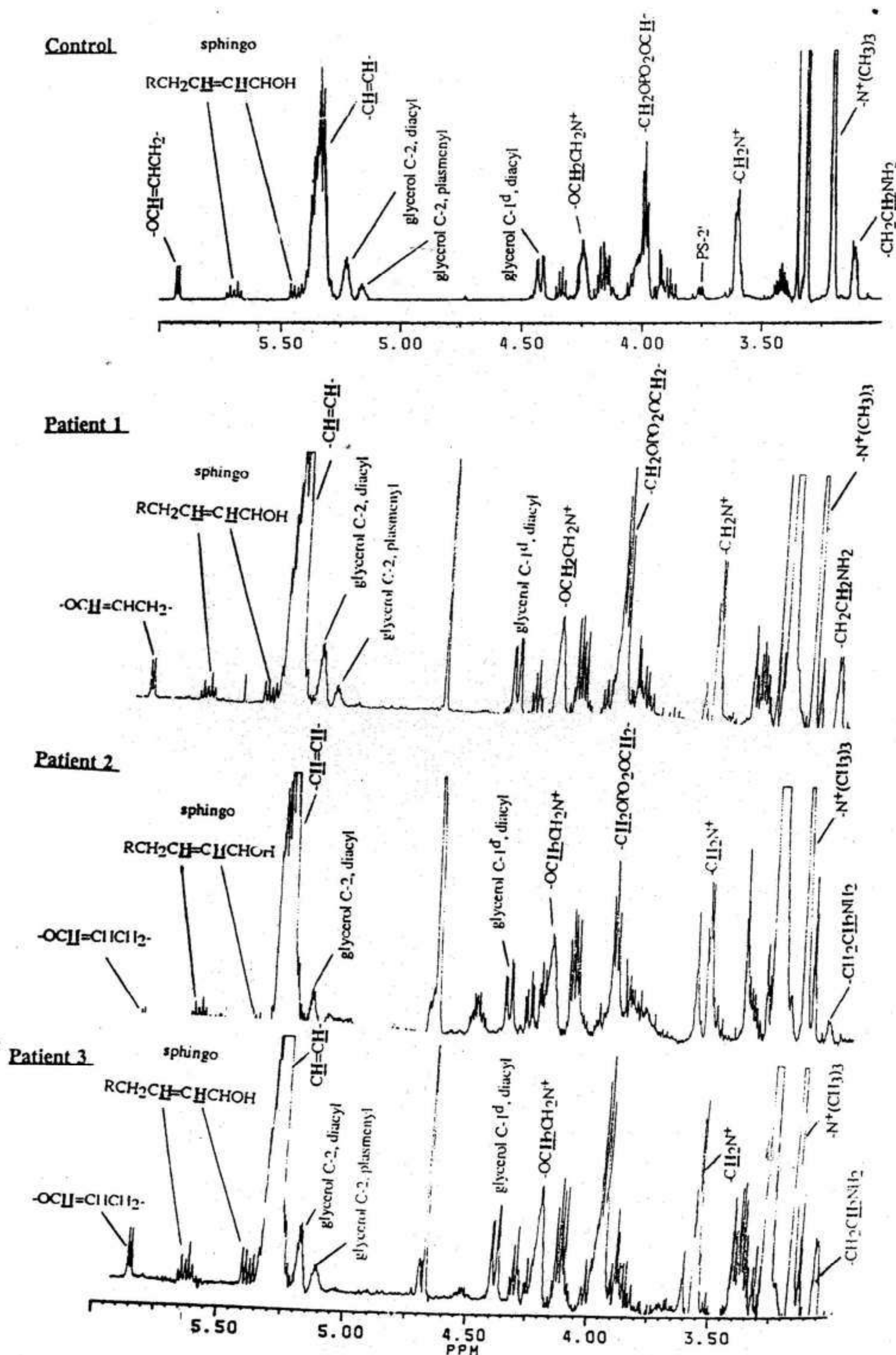

Fig. 2 1-dimentional proton nmr (6.0ppm-3.0ppm) of platelet total lipids of controls and patients