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Archives of Biochemistry and Biophysics 456 (2006) 71–78
ABB
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Fatty acid transport in articular cartilage

Kenton Paul Arkill *, C. Peter Winlove

Biomedical Physics Group, School of Physics, University of Exeter, Exeter, EX4 4QL, UK

Received 27 July 2006, and in revised form 13 September 2006
Available online 6 October 2006

Abstract

Articular cartilage extracellular matrix imposes a significant transport barrier to albumin, the principal carrier of fatty acids. It has not been previously established whether it also influences the transport of fatty acids important for chondrocyte metabolism. Albumin was labelled with rhodamine–maleimide and bound to NBD-labelled lauric acid. Plugs of fresh equine metacarpal–phalangeal cartilage and subchondral bone were incubated with the complex at 4 °C for 2–160 h. The fluorophore distribution was quantified using quantitative microscopy in histological sections. The fluorescence intensity of both fluorophores fell steeply over 300 μm below the articular surface and remained relatively uniform through the mid zone but the ratio of lauric acid to albumin was higher than in the incubation medium. The effective diffusivity of lauric acid in the mid zone was $(2.2 \pm 0.7) \times 10^{-12}\ m^2\ s^{-1}$ ($n = 33$), higher than that of the carrier albumin, suggesting dissociation in the surface layer. Lauric acid accumulated reversibly at the tidemark.

Keywords: Equine; Osteoarthritis; Diffusion; Fluorescence; Albumin; Fatty acid; Transport; Articular; Cartilage; Extracellular

Fatty acid metabolism in chondrocytes is, in several respects unusual [1]. In most cells metabolism involves in part *de novo* synthesis and in part uptake from the surrounding tissue. It has been suggested that the latter process is compromised because the chondrocytes have only limited access to essential fatty acids: the surrounding extracellular matrix is avascular and provides a major barrier to the transport of solutes [2]. There is indirect evidence that fatty acids are able to pass through the extracellular matrix at least to some degree, because dietary fatty acid intake influences the fatty acid composition of cartilage. However, the fatty acid composition of normal cartilage is close to that in other tissues when subjected to prolonged depletion of fatty acids [3]. The fatty acid content of chondrocytes changes with age and several authors have suggested relationships between abnormal fatty acid metabolism and osteoarthritis [4] or with the processes of vesicle formation and calcification [5,6]. Conversely, it has been suggested that dietary fatty acid manipulation may, at least in animal models, delay the progression of osteoarthritis [3,7,8].

Little is known about the actual mechanisms of transport of lipids in the extracellular matrix of cartilage. Histochemical methods provide information on the distribution of lipids in the tissue, showing prominent lipid staining just below the articular surface and more diffuse staining deeper in the tissue [9,10] but provide no indication of the source of these materials. The deep deposits are thought to arise from the lipid vesicles that are secreted by cells as part of the process of matrix calcification [5], but opinions differ as to whether the superficial lipid layer originates from within the cartilage [11] or from filtration of synovial fluid [12]. Support for the latter hypothesis derives from the only study of which we are aware of lipid transport in cartilage which showed that lipids injected into the synovial fluid are detectable in cartilage [13], but did not further quantify the transport processes.

The general features of molecular transport in the extracellular matrix have been established, generally in pursuit of the hypothesis that the transport of nutrients and metabolites through the extracellular matrix of cartilage is severely hindered and may be a factor in the development of

* Corresponding author. Fax: +44 1392 264111.
E-mail address: k.p.arkill@ex.ac.uk (K.P. Arkill).

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.09.014

osteoarthritis [10,14]. The extracellular matrix consists of a network of collagen fibres with a spacing of the order of 100 nm immersed in a finer matrix of proteoglycan aggregates whose pore size is estimated on structural grounds to be of the order of 6 nm [15]. Consistent with this picture is experimental evidence showing that small, hydrophilic solutes have access to over 90% of the interstitial water and their diffusivity is not markedly different to that in free solution. However, a protein such as albumin, with a hydrodynamic radius of 3.6 nm suffers steric hindrance sufficient to reduce its partition coefficient (the fraction of tissue water available to it at equilibrium) to 0.01 and its diffusivity by at least an order of magnitude compared to that in free solution [2]. Furthermore, recent studies using high resolution techniques such as fluorescence recovery after photobleaching have revealed great heterogeneities in transport properties within the tissue [16]. For solutes such as growth factors, partition coefficients and effective diffusivities are further influenced by specific interactions with matrix components (e.g. [17,18]).

The observations on albumin transport are highly pertinent to the question of fatty acid transport in cartilage because fatty acids are solubilised in blood either by incorporation into lipoproteins or binding to plasma proteins, principally albumin. Lipoproteins have been detected in synovial fluid (reviewed in [19]) but they are too large to penetrate the extracellular matrix of cartilage intact. They might exchange lipids at the articular surface, perhaps facilitated by interaction with glycosaminoglycans [20], but albumin appears to be the most likely of the blood constituents to be the carrier for fatty acids into the cartilage.

The evidence therefore suggests that if the fatty acid remains bound to the albumin its movement in cartilage is severely restricted. However, it is possible that other carrier mechanisms are utilised within the tissue, which might overcome this apparently undesirable situation. We set out to quantify fatty acid transport and thereby to distinguish between these hypotheses. Our approach was based on methodology developed in [21] and cartilage plugs were exposed *in vitro* to fluorescently labelled fatty acid bound to albumin carrying a second fluorophore label, enabling us to examine the co-transport or otherwise of the two species in the cartilage matrix.

Methods

Tissue preparation

Metacarpal–phalangeal joints were obtained from an abattoir from horses approximately 7 years old, suffering from no known joint disorders. The intact joints were obtained immediately after slaughter and transported to the laboratory at 4 °C. In total 13 cartilage-subchondral bone plugs ($5 \times 5 \times 2.5$ mm deep) were excised, using a jeweller's saw from the load-bearing areas in the mid section of the condyles [22]. The uncalcified cartilage is approximately 1 mm in thickness and the depth of plugs ensured that the calcified cartilage and some subchondral bone were intact, in contrast to many previous preparations in which only uncalcified cartilage was studied [21,23–25].

Tracer preparation

Fatty acid-free bovine serum albumin (Sigma) was fluorescently labelled using rhodamine–maleimide (Molecular Probes). The maleimide reacts with thiol groups on the albumin. This reaction is thought to give a more stable product than the more widely used isocyanate reaction. A weight ratio of 80:1 albumin:maleimide was used (i.e. a slight excess of reagent, assuming one thiol group is labelled per albumin molecule) in phosphate-buffered saline (PBS)[1], pH 7.3. The solution was treated with shaking for 1 h at 4 °C, followed by elution through a salt trap (Sephadex 25, Sigma). Nitrobenz-2-oxa-1,3-diazole (NBD)-labelled lauric acid of molecular weight 378 Da (Molecular Probes) was bound to the albumin using the technique of Stremmel and Theilmann [26]. 2.2 mg Lauric acid was dissolved in 100 μl 0.1 M NaOH and mixed at 37 °C for 1 h with fluorescent albumin solution (4%w/v) at a 1:1 molar ratio. Fluorophore and fatty acid–albumin binding were checked by elution on a Sepharose CL4B column.

Tracer uptake measurements

The full depth cartilage-bone plugs, taken from the dorsal condyles, were incubated with fatty acid–albumin (4% albumin w/v) in PBS pH 7.3, in the dark at 4 °C then randomly selected for incubation time periods of 2, 5, 10, 16, 26, and 160 h. Control plugs were incubated in PBS containing albumin but without fluorescent tracer for measurement of tissue autofluorescence. Upon completion of incubation the plugs were removed from solution, excess liquid was dabbed off with a tissue and the samples were wrapped in cling-film to prevent drying and then snap frozen. Each cartilage plug was serially sectioned (20 μm thickness) perpendicular to the articular surface using a freezing microtome. No embedding medium was used in order to avoid leaching of tracer. Representative sections were washed extensively in PBS before microscopic examination to remove diffusible tracer and thereby to quantify binding of albumin and fatty acid to the tissue.

The relative fluorescence intensity of albumin and lauric acid in the stock solution was determined from microscope images of 1 mm internal diameter glass capillary tubes filled with the stock solution. At the end of the incubation the medium was passed though the salt trap once again to determine the amount of free tracer.

In light of preliminary experiments, the sections were examined dry to minimise tracer redistribution [27], using a 2.5× objective under epi-illumination (Leica DMLFS microscope equipped with I3 and N.2.1 filters for the two fluorophores). Twenty-four-bit colour digital images were acquired using a video camera (JVC KY-5F55B) and Image grabber PC software (Acquis Bio, Synoptics Ltd.). The camera provided a spatial resolution of 1.27 μm with the 2.5× objective. Between 5 and 12 sections were analysed for each time point. In previous studies [27], inter-subject, inter-plug and intra-plug variability have been extensively studied. The latter was found to be the largest, therefore in the present study all data were pooled and the mean and standard error are reported. Fluorescence intensity profiles were constructed along a line normal to the articular surface through the cartilage and into the subchondral bone. In light of preliminary experiments a line width of 50 μm was chosen as providing adequate signal to noise but avoiding unnecessary spatial averaging. The mid portion of each tissue section was selected for analysis to avoid the effects of lateral diffusion from the cut edges which was observable within 1 mm of the edge. A representative profile is shown in Fig. 1.

Quantification of tracer distribution in tissue sections

Quantification of tracer distribution was performed using a purpose written program (Multiview, developed by Dr C.G. Phillips, Centre for Biological and Medical

[1] *Abbreviations used:* PBS, phosphate-buffered saline; NBD, nitrobenz-2-oxa-1,3-diazole.