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Inactivation of synovial fluid α_1-antitrypsin by exercise of the inflamed rheumatoid joint

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α_1-Antitrypsin (α_1AT) is known to be oxidised by reactive oxygen species both in vitro and in vivo, leading to its inactivation. We report here that synovial fluid (SF) α_1AT is inactivated during exercise of the knee-joints of rheumatoid arthritis (RA) patients. Sequential SF sampling from exercised RA patients showed a marked decrease in the mean activity of α_1AT after exercise with no change in the molecular forms of α_1AT. No such inactivation was found in the control (continuously resting) RA patients. We suggest that oxidation may contribute to α_1AT inactivation as a consequence of 'hypoxic-reperfusion' injury after exercise of the inflamed joint.

$\alpha_1$1-Antitrypsin; Rheumatoid arthritis; Serpin; Oxidation; Reactive oxygen species; Inflammation; Hypoxic reperfusion

1. INTRODUCTION

The inflamed human knee-joint provides an example of hypoxic reperfusion injury [1]. This form of injury occurs when tissue ischaemia is followed by the restoration of the blood supply, and involves the generation of reactive oxygen species (ROS) [2]. In rheumatoid arthritis (RA), joint hypoxia is caused, in part, by occlusion of the synovial capillary bed during exercise, whilst subsequent rest results in reperfusion. An hypoxic-reperfusion event may result in ROS generation due to activation of the xanthine–xanthine oxidase system within the synovium [3] or stimulation of the NADPH oxidase activity of neutrophils sequestered in the inflamed joint [4]. ROS-damaged biomolecules, such as IgG [5] and lipids [6], have been identified in the knee-joint synovial fluid (SF) of RA patients. Increases in the levels of these products are detectable following exercise-induced hypoxic reperfusion injury [1,7].

Human α_1-antitrypsin (α_1AT) is a member of the *serpin* (*ser*ine *p*roteinase *in*hibitor) superfamily of proteins [8]. It is an acute phase reactant and is used as an index of disease activity. α_1AT is the natural inhibitor of neutrophil elastase, which may contribute to connective tissue destruction in several diseases, such as pulmonary emphysema and RA [9]. The reactive centre of α_1AT contains a Met^{358}–Ser^{359} peptide bond at its reactive centre, which is located in a highly stressed exposed loop [10]. α_1AT (54 kDa) inhibits neutrophil elastase by rapidly forming a stable one-to-one complex (83 kDa), which remains tightly bound until removed from the circulation [10]. However, α_1AT can be rendered inactive in terms of its inhibitory capacity towards elastase by two different mechanisms: either oxidation of the reactive centre Met^{358} to methionine sulphoxide by ROS [10], or cleavage of any one of a number of peptide bonds close to the reactive centre by proteinases, including matrix metalloproteinases [11].

A proportion of α_1AT is present in an inactive form in RA SF [12] and in the bronchoalveolar lavage fluid of pulmonary emphysema patients [13]. Oxidised [14] and cleaved [15] forms of α_1AT have been found in RA SF. We have shown previously that, on average, 41% of total α_1AT in RA SF is inactive in an ambulatory rheumatoid population [16], although there was a large variation between the individuals in this population. We hypothesised that the presence of oxidised α_1AT and the variability of oxidation might both be the consequence of exercise-induced oxidative damage. We therefore examined the effect of exercise on the inactivation of SF α_1AT in the rheumatoid joint, and subsequently determined whether the dominant mechanism was proteolysis or oxidation.

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Abbreviations: α_1AT, α_1-antitrypsin; ROS, reactive oxygen species; RA, rheumatoid arthritis; SF, synovial fluid.

2. MATERIALS AND METHODS

2.1. *Synovial fluid and plasma*

Following local ethical committee approval for the clinical protocol ten patients were selected for study. All conformed to the American Rheumatism Association criteria for 'definite RA', and had active disease for 0.5 to 22 years. They were between 35 and 77 years of age. Each patient was randomly divided into one of two groups, 'exercised'

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or 'continuously rested'. For both groups, patients rested supine for a minimum period of 20 min. At the end of this period, at least two baseline SF samples were obtained by aspirating at −4, −2 or 0_{pre} min. Following the final baseline sample the five patients designated for 'exercise' walked as briskly as possible for 10 min. The five patients designated for 'continuous rest' remained recumbent for a further 10 min. Samples were then taken from both groups immediately after exercise/rest and at defined intervals until the knee was dry (0_{post}, 2, 5 min, and so on). A plasma sample was also obtained from each patient immediately prior to the start of the exercise/continuous rest protocol. Samples were centrifuged (2,000×g) at 4°C for 15 min and analyzed within 6 h of collection.

2.2. *Immunochemical determination of SF α_1AT concentration*

The total amount of α_1AT in each SF sample was quantified by single dimension immunoelectrophoresis [16]. The within-batch coefficient of variation for this method was 1.8% (n = 8), and the between-batch coefficient of variation was 5.1% (n = 6). The molar concentration of α_1AT was calculated (M_r = 54 kDa) and this value was used to calculate the number of moles of active α_1AT per mole of total α_1AT as described below.

2.3. *Kinetic spectrophotometric measurement of SF α_1AT activity*

The activity of porcine pancreatic elastase (Sigma, Dorset) was determined according to the method of Beatty et al. [17] and the preparation of elastase was found to contain 0.64 moles of active elastase per mole of total elastase. For each SF and plasma sample, five different volumes of SF, containing known amounts of SF α_1AT from the immunochemical determination, were used in kinetic measurements at 405 nm. The initial rate of elastase activity thus obtained was plotted (y-axis) as a function of the molar ratio of α_1AT to active elastase (x-axis). By extrapolation of the curve obtained to an intercept on the x-axis, the number of moles of α_1AT needed to completely inhibit one mole of porcine pancreatic elastase was calculated. The specific activity of SF α_1AT, defined as the number moles of active α_1AT per mole of total α_1AT, was calculated for each SF sample at each exercise/rest time point. The percentage change in specific activity from the initial (−4 min) baseline value (see Fig. 1) was then calculated (Δ specific activity, %).

2.4. *SDS-PAGE and Western blotting*

The molecular forms of SF α_1AT in each sample were examined by 10–20% gradient SDS PAGE followed by Western blotting [15]. This technique detects three molecular forms of α_1AT in the SF from RA patients (Fig. 2): (i) the 54 kDa form of α_1AT, which may be either native or oxidatively inactivated, (ii) the 50 kDa form, a major fragment of α_1AT proteolysis, and (iii) the 83 kDa form, a complex of native α_1AT with neutrophil elastase. The cleaved N-terminal fragment of SF α_1AT (4 kDa) could not be detected on the blot, presumably due to a lack of immunochemical reactivity with the antiserum used. The intensities of α_1AT bands were quantified by the Seescan digital imaging system (Cambridge, UK).

2.5. *Statistical analysis*

Statistical analysis was by Student's t-test. Results were expressed as the mean ± 1 S.D. A P value of < 0.05 was considered to be statistically significant.

3. RESULTS

The mean concentration of α_1AT in the ten patients studied was significantly higher ($P < 0.01$) in the plasma than in the first of the SF samples collected from each patient (3.02 ± 0.86 compared to 1.72 ± 1.15 mg/ml), in agreement with earlier results [18]. In addition, the mean specific activity of α_1AT was significantly higher ($P < 0.05$) in the plasma than the SF (0.83 ± 0.22 compared to 0.58 ± 0.21 moles of active α_1AT per mole of total α_1AT). Comparing the group of patients (n = 5) who exercised with the group of patients (n = 5) who rested continuously, there was no significant difference between the mean plasma values for either α_1AT concentration or specific activity. There was also no significant difference between the corresponding mean values for the first of the SF samples collected from each patient.

The time-course plots for the changes in specific activity of SF α_1AT in the exercised and continuously rested patients were paired for ease of presentation (Fig. 1). In the continuously rested group, the changes were no greater than those during the baseline period. The mean value for the lowest specific activity of the post-rest samples was 0.55 ± 0.15 moles of active α_1AT per mole of total α_1AT. This value was not significantly different from the mean activity of α_1AT in the baseline SF samples (0.55 ± 0.19 moles of active SF α_1AT per mole of total SF α_1AT). In contrast, the exercised group showed a significant fall after the exercise programme was completed. The mean value for the lowest specific activity of SF α_1AT occurring in the exercised RA patients was significantly lower than the mean of the initial baseline values in the same group (0.33 ± 0.18 compared to 0.62 ± 0.24 moles of active α_1AT per mole of total α_1AT; $P < 0.05$). The mean value for the maximum decrease in specific activity of SF α_1AT after exercise was 50.0 ± 10.7% (Fig. 1).

When the molecular form of α_1AT in SF during the exercise and resting protocol was examined by SDS PAGE and Western blotting, there was no significant change in any of the three α_1AT bands (see section 2) in either the exercised or continuously rested patients (Fig. 2). In the patients studied here, the ratio of the cleaved α_1AT band intensity to the native α_1AT band intensity agreed with previously published data [15].

4. DISCUSSION

The specific activity of SF α_1AT was employed as an index of protein damage within the inflamed human joint, and was used to monitor the effect of hypoxic reperfusion injury within the joint as a consequence of joint exercise. The results demonstrated that exercise promoted SF α_1AT inactivation, whilst patients sampled sequentially during continuous rest showed no such alteration in functional α_1AT.

It has been demonstrated, in vivo, that the inflamed rheumatoid joint has a positive synovial cavity pressure that rises further with exercise [19]. This pressure rise exceeds the capillary perfusion pressure, and causes a temporary occlusion of the superficial capillary bed (hypoxia). With cessation of exercise, the blood supply is restored (reperfusion). Hypoxia followed by reperfusion within the synovium will activate the xanthine dehydrogenase/oxidase system, localised in the synovial

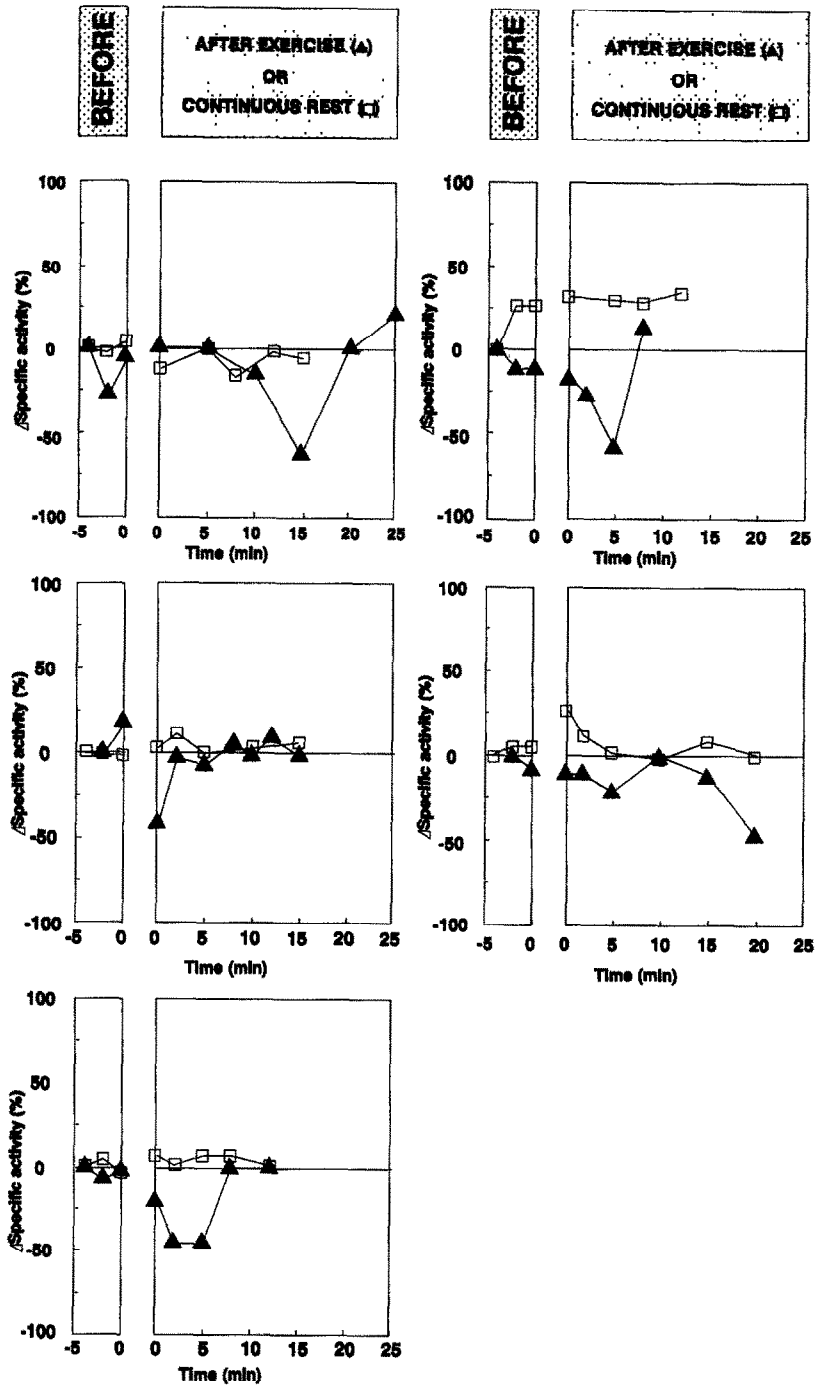

Fig. 1. Change in SF α_1AT specific activity in 'exercised' and 'continuously rested' RA patients. The specific activity was determined as described in section 2. For each SF sample, the total concentration of α_1AT was determined by immunoelectrophoresis. Agarose gel (1%) containing anti-human α_1AT antiserum (0.5%, Sigma, Dorset) was made up in 0.0315 M sodium barbital/5.7 mM diethyl barbituric acid/0.187 M Tris/0.375 M glycine/5 mM NaN_3, pH 8.6. The gel was run for 4 h at 50 mA/50 cm^2. After electrophoresis the plate was squash blotted, washed in phosphate saline buffer (PBS) and stained with Coomassie brilliant blue. Four standards of purified α_1AT (25, 50, 100 and 200 ng/μl; Calbiochem, CA) were employed. The negative time-points represent times prior to the start of exercise or continuous rest. The solid triangles represent exercised patients, and the open squares continuously rested patients. Measurements are the means of duplicates (duplicates differed by less than 6%).

capillary endothelium of both healthy and diseased synovium [3]. Activation generates both the superoxide anion radical and H_2O_2. In the presence of catalytic iron salts, these ROS will react to generate the hydroxyl radical via the Haber-Weiss reaction [20]. ROS generation by the synovium during 'hypoxic reperfusion' cycles has been demonstrated by electron spin resonance with spin trapping [21]. ROS have the capacity to cause

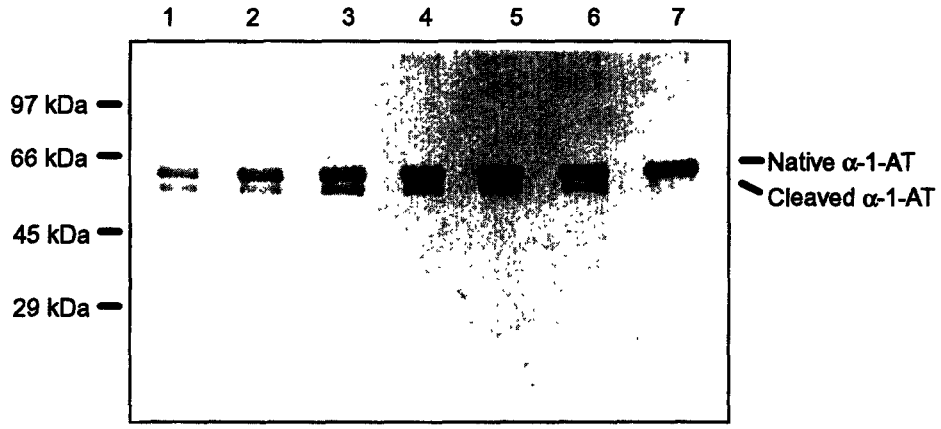

Fig. 2. Typical Western blot showing the molecular forms of α_1AT in RA SF obtained before and after exercise (patient represented in Fig. 1b). SF α_1AT was run on 10%–20% gradient SDS PAGE, and then Western-blotted to nitrocellulose in 30 mM Na_2CO_3/ 10 mM $NaHCO_3$/20% methanol (pH 9.9) at 400 mA for 2–3 h. The blot was blocked with 5% (w/v) bovine serum albumin in PBS, and a monospecific sheep anti-human α_1AT antiserum (Unipath, Bedford, UK) was then incubated with the blot. After washing away the unbound first antibody, the blot was reprobed with a peroxidase-conjugated rabbit anti-sheep immunoglobulin (Dakopatts, High Wycombe, UK). The α_1AT bands were visualised by developing with 0.05% (w/v) diaminobenzidine tetrahydrochloride and 0.002% (v/v) H_2O_2 in PBS. Lane 1, −4 min; lane 2, 0_{pre} min; lane 3, O_{post} min; lane 4, 2 min; lane 5, 5 min; lane 6; 8 min; lane 7, purified α_1AT (native form).

direct oxidative damage to biomolecules, including α_1AT. Active α_1AT has a methionine residue at its reactive centre, which can be readily oxidised to methionine sulphoxide by ROS [8,10]. When the methionine at the reactive centre is oxidized, the activity of α_1AT against elastase is diminished.

The inactivation of SF α_1AT after exercise was transient. This might be due to the rapid ingress and egress of α_1AT across the synovial membrane during exercise, since both the concentration and specific activity of α_1AT in plasma was significantly higher than in SF. Although the passage of plasma proteins across the synovial membrane is a slow process in normal situations, the joint physiology may be different immediately after exercise of the inflamed joint, perhaps as a consequence of a reactive hyperaemia. It is noteworthy that the concentrations of both total SF α_1AT and total protein varied substantially in samples derived from the repeated aspiration of SF after exercise (data not shown). The fluctuations were greater than the 'noise' of the assay itself, but there was no obvious pattern in the changes. This suggests that bidirectional protein exchange between SF and synovium may have occurred following exercise, with the net effect of the observed fluctuating increases and decreases of α_1AT concentration. However, movement of α_1AT between the blood and SF compartments could not account for the observed decrease in α_1AT specific activity because in RA the specific activity of plasma α_1AT was higher than that of SF α_1AT. Another possibility is that α_1AT may be reactivated by methionine sulphoxide-peptide reductase. This enzyme has been detected in human lung homogenate and in human peripheral blood neutrophils [13], but, to our knowledge, there are no reported studies of its activity within the joint.

In this study, no further proteolysis or α_1AT-neutrophil elastase complex formation was detected over the time-period studied. In vitro, ROS have been shown to activate latent metalloproteinases, such as human neutrophil collagenase [22]. Since a number of metalloproteinases are capable of degrading α_1AT [11,22], ROS may indirectly promote the proteolytic inactivation of α_1AT and cartilage destruction. α_1AT proteolysis due to ROS-mediated metalloproteinase activation may occur over a longer time-course than that studied here. Certainly, α_1AT proteolysis does occur, since a cleaved form of α_1AT has been detected in RA plasma and SF (see Fig. 2), being present as a significantly higher proportion of total α_1AT in RA SF [15]. We infer then that short-term damage to α_1AT in the exercised inflamed human joints was not due to proteolysis, and therefore oxidative damage is implicated.

In conclusion, our study demonstrates that SF α_1AT is inactivated by exercise of the inflamed rheumatoid joint, which we believe is attributable to the production of ROS. Many previously reported studies have attempted to relate disease activity to biochemical parameters in SF. It is clear that brief exercise might considerably influence such measurements and we know of no studies where this effect has been considered.

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