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Detection of the exercise-associated changes in serum proteins by two-dimensional electrophoresis under non-denaturing conditions

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ABSTRACT

The changes in serum proteins caused by physical exercise (10-km run) were examined using two-dimensional electrophoresis under non-denaturing conditions. Two specific proteins were found to increase remarkably in amount. Both proteins had a slightly higher molecular mass than albumin, which suggests that they are albumin-bound with large amounts of sugars or lipids. However, these proteins were not adsorbed on an anti-albumin affinity column, and they were not stained by either periodic acid–Schiff base or Sudan Black. The molecular mass was determined to be 25 000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The changes of the specific proteins in competitors in a triathlon race were also examined.

INTRODUCTION

Exercise is thought to be an essential factor in human health and physical fitness. However, the reasons for this, and the relationship between exercise and other factors (such as nutrition and rest), are not yet fully understood. In order to answer these questions, many biological and morphological changes under various physical conditions have been extensively investigated [1–5].

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Few studies have been made concerning the changes in proteins, however, because of the insufficient resolution of the analytical techniques available.

Two-dimensional electrophoresis [6] is a technique that can be used to analyse proteins under non-denaturing conditions. As this technique does not employ denaturing agents such as sodium dodecyl sulphate (SDS) or urea, it is possible to detect the proteins based on their biological activities [7,8]. The method has been used to elucidate the changes in serum proteins caused by some diseases [9–13].

We have been studying the changes in blood properties and components caused by various types of physical exercise [14,15]. This paper describes the use of two-dimensional electrophoresis to study the changes in serum proteins resulting from a 10-km run.

EXPERIMENTAL

Reagents

Ampholines (pH range 3.5–10 and 4–6.5) were obtained from LKB (Bromma, Sweden). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (all of special-reagent grade for electrophoresis), Tris, glycine, sucrose, hydrochloric acid and ammonium persulphate (all of analytical-reagent grade) were from Wako (Osaka, Japan). Coomassie Brilliant Blue R-250 was purchased from Sigma (St. Louis, MO, U.S.A.).

Exercise and sample preparation

Six healthy volunteers (male, athletic students of physical education, 19–22 years) undertook a 10-km run (trial time 45–50 min). Blood was obtained conventionally at 30 min before and 5 min after the run, left to stand at 4 °C for 2 h and centrifuged at 3000 *g* for 10 min. The serum samples were stored at −20 °C. As a control, sera of another six volunteers (male, non-athletic students, 19–22 years) were used.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed as described previously [13]. First-dimension isoelectric focusing was performed on a 4% cylindrical polyacrylamide gel (14.5 cm × 0.5 cm) containing 2% Ampholine. The cathode solution was 0.1 *M* sodium hydroxide and the anode solution was 0.01 *M* phosphoric acid. The electrophoresis was run at a constant current of 2 mA per gel for ca. 90 min (until the voltage reached 460 V), then at a constant voltage of 460 V for 20 h at 4 °C.

Second-dimension polyacrylamide gel electrophoresis (PAGE) was performed on a 4–21% linear gradient (containing a 0–10% sucrose linear gradient) slab gel of 14 cm long, 16 cm wide and 0.4 cm thick. The electrophoresis

was run at a constant current of 36 mA per gel at 4 °C in 0.05 *M* Tris–0.38 *M* glycine buffer (pH 8.3).

Gels were stained overnight in 0.05% Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (50:7:43, v/v). Destaining was carried out in methanol–acetic acid–water (30:7:63, v/v).

Determination of pI value and molecular mass

The p*I* and molecular mass under non-denaturing conditions were estimated as described previously [13]. The molecular mass under denaturing conditions was determined by SDS-PAGE, using phosphorylase *a* (94 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (30 000) and cytochrome *c* (12 400) as standard proteins.

RESULTS

Before examing the changes in serum proteins caused by a 10-km run, we determined the proteins in the sera of six healthy volunteers (male, non-athletic students, 19–22 years) by two-dimensional electrophoresis, which confirmed that there were no significant differences in the two-dimensional electrophoretic patterns. The changes in serum proteins caused by the run were then examined. Serum samples obtained before and after the run were subjected to two-dimensional electrophoresis and the protein distributions were compared. Fig. 1 is an example of the patterns of serum proteins before and after the run. (These protein distributions have been identified by Manabe et al. [16]. The positions of some major proteins are indicated on the figure.) Remarkable changes in amount were observed for two proteins slightly above albumin (indicated by arrows). The p*I* values and apparent molecular masses of these proteins were measured as described in Experimental, and were estimated as follows (expressed as p*I*/molecular mass): 4.8/70 000 and 5.1/70 000, respectively (for convenience, these were called the p*I* 4.8 species and the p*I* 5.1 species).

Fig. 2 shows the gel sections containing the specific proteins in all volunteers before and after the run. A remarkable increase in the specific proteins was observed in all volunteers after the run. The volunteers can be divided roughly into two types: in type I the p*I* 4.8 species increased but the p*I* 5.1 species did not change (subjects H.S., T.O. and T.H.); in type II the p*I* 5.1 species increased but the p*I* 4.9 species did not change (subjects Y.I., N.K. and N.S.).

As shown in Figs. 1 and 2, the location of these specific proteins on the two-dimensional electrophoretic gel was slightly higher than the region of albumin, and the electrophoretic shape was unique ("rocket" shape). From these observations, it was suspected that the specific proteins might be bound to albumin with considerable amounts of identified metabolites, such as sugars or lipids. Therefore, the characteristics of these specific proteins were investi-

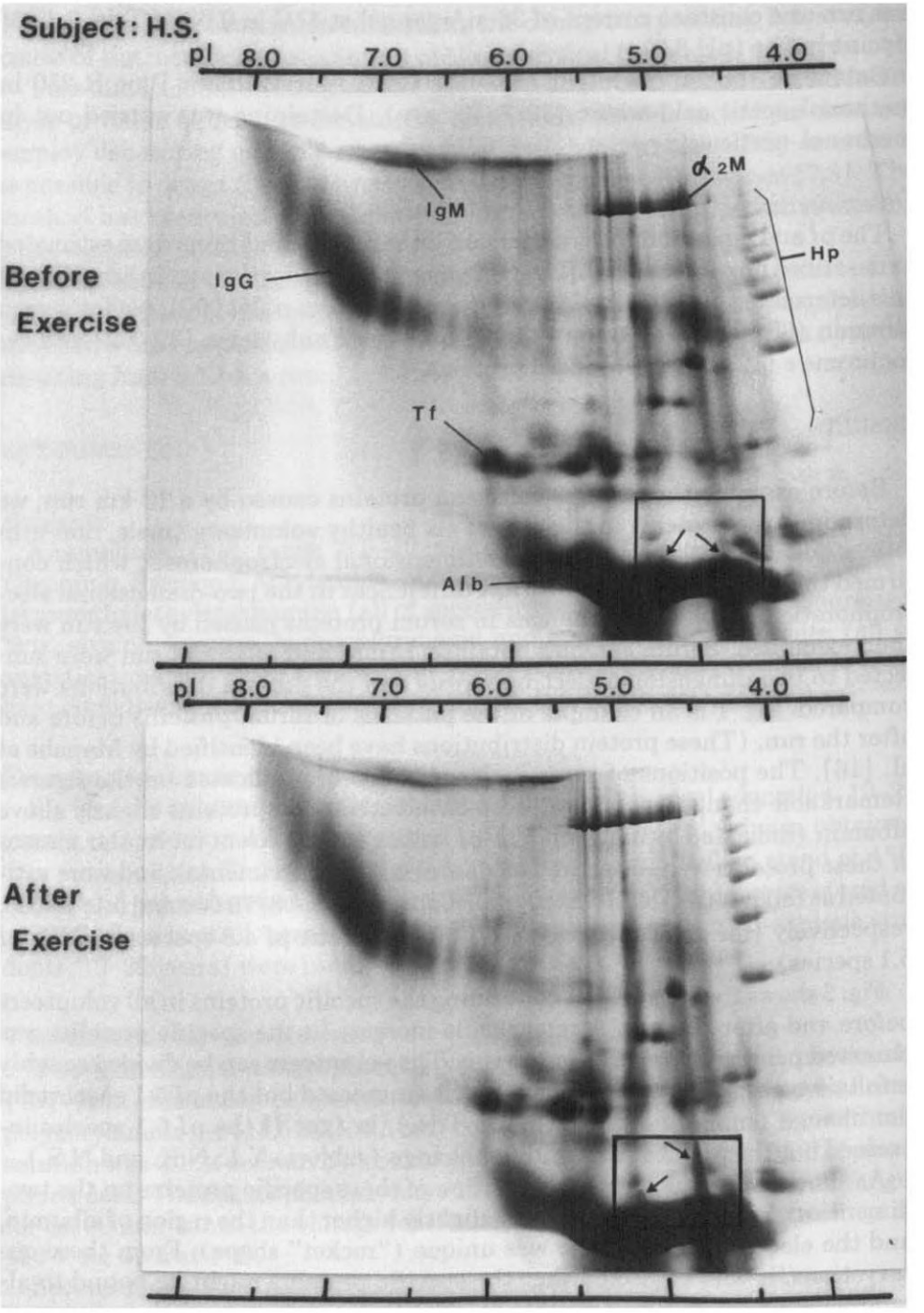

Fig. 1. Example of the two-dimensional electrophoretic patterns of human serum proteins: 50 μl of serum were used for each electrophoresis. IgM = immunoglobulin M; IgG = immunoglobulin G; α_2M = α_2-macroglobulin; Hp = haptoglobin; Tf = transferrin; Alb = albumin.

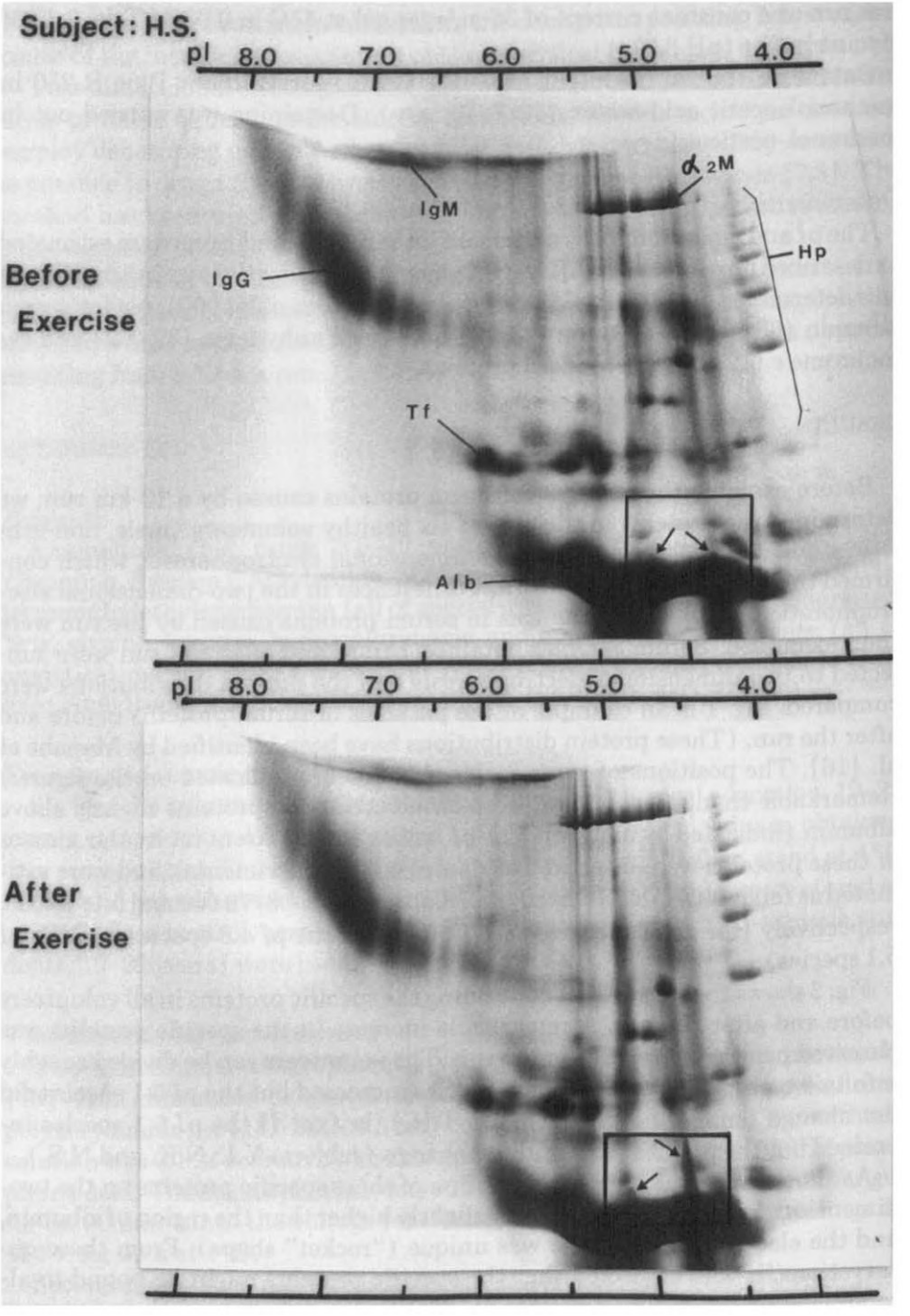

Fig. 2. Enlargements of the boxed area in Fig. 1, showing how two specific proteins increased significantly after a 10-km run, for six subjects.

gated. Serum samples obtained after the run were applied to an anti-albumin affinity column, and then the proteins in the non-adsorbed fraction were subjected to two-dimensional electrophoresis. Fig. 3 is the two-dimensional electrophoretic pattern of the non-adsorbed fraction. The specific proteins were recovered in the non-adsorbed fraction even when the greater part of the albumin was removed by affinity chromatography. In addition, these specific proteins were not stained by either periodic acid–Schiff base (PAS) or Sudan Black.

The specific proteins were further characterized. Stained proteins on the two-dimensional electrophoretic gel (indicated in Figs. 3 and 4) were punched out and homogenized with 0.1 *M* sodium hydroxide solution containing 2% β-thiodiglycol. The supernatant was centrifuged at 3000 *g* for 10 min, then dia-

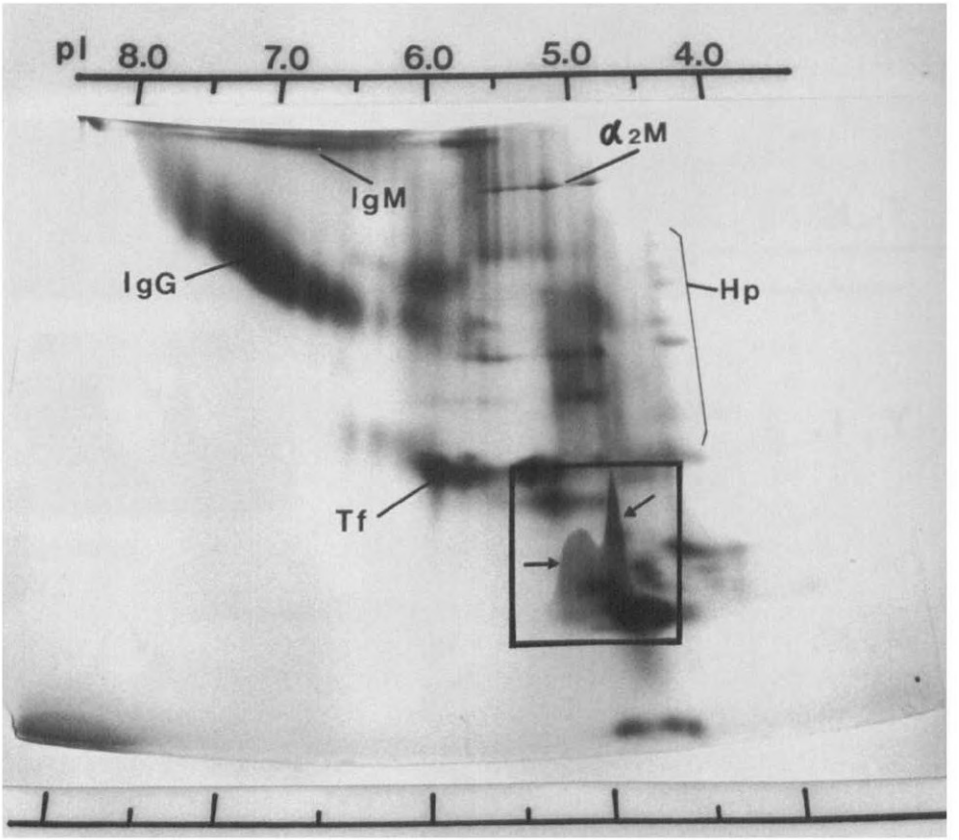

Fig. 3. Two-dimensional electrophoretic pattern of serum proteins after partial removal of albumin. Serum obtained after a 10-km run was applied to an anti-human albumin-Sepharose affinity column, and the proteins in the non-adsorbed preparation were analysed by two-dimensional electrophoresis.

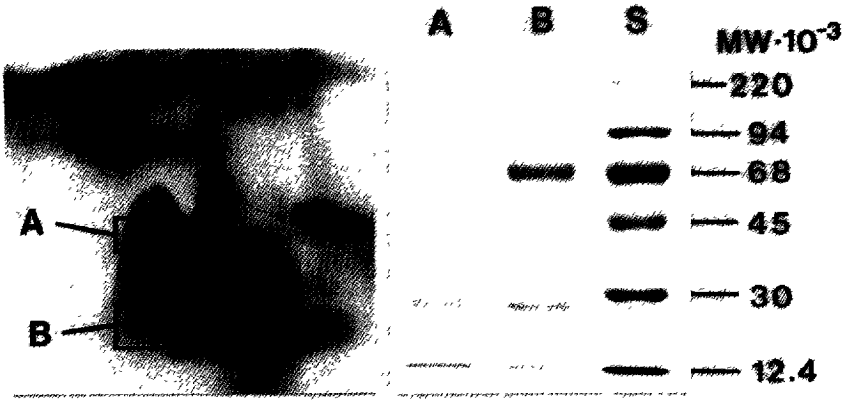

Fig 4. SDS-PAGE analysis of the specific proteins. Left, an enlargement of the boxed area in Fig. 3, right, the SDS-PAGE pattern. Gel pieces containing the specific proteins (A and B in left figure) were punched out from the two-dimensional gel. The proteins were extracted by homogenization with 0.1 *M* sodium hydroxide solution containing 2% β-thiodiglycol and analysed by SDS-PAGE. S = standard proteins.

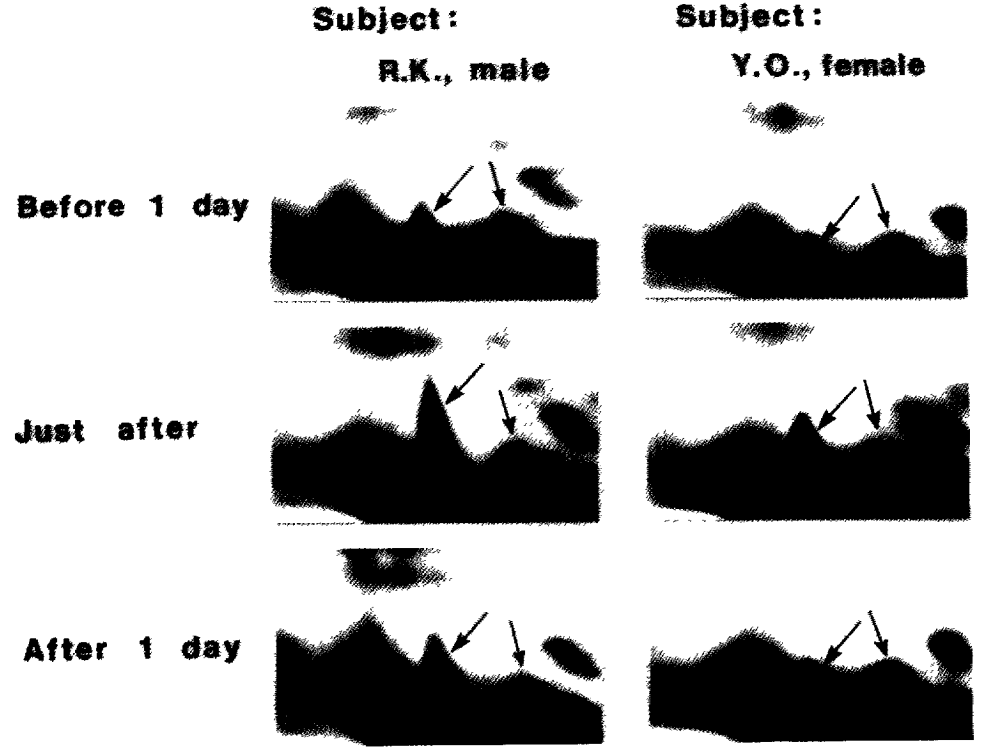

Fig. 5. Changes of the specific proteins caused by a triathlon race. Serum proteins obtained one day before, and 5 min and one day after were analysed by two-dimensional electrophoresis. Gel sections containing the specific proteins are shown, and 50 μl of serum were used for each electrophoresis. The areas and the positions in the two-dimensional electrophoretic pattern are the same as in Fig. 2.

lysed against distilled water, lyophilized and analysed by SDS-PAGE in the presence of 2-mercaptoethanol. The results indicated that the specific proteins formed a single band at a position corresponding to a molecular mass of ca. 25000 (Fig. 4).

The changes of the specific proteins in a triathlon race (3-km swim, 180-km bicycle ride and 42.195-km marathon run) were examined. Serum samples obtained one day before, and 5 min and one day after the race were subjected to two-dimensional electrophoresis. Fig. 5 shows the time-courses of the specific proteins in two triathlon participants. In both cases, the specific proteins, especially the p*I* 5.1 species, increased remarkably just after the race and returned to their former level after one day.

DISCUSSION

In order to define the relationship between human health and exercise, the biological and morphological changes brought about by various types of physical exercise have been extensively investigated [1–5]. However, few studies have been performed on the changes in protein because of the insufficient resolution of the analytical technique available. As shown in Figs. 1, 2 and 5, two-dimensional electrophoresis could clearly detect the specific proteins in serum after the 10-km run and the triathlon race. As this technique does not employ denaturing agents such as SDS or urea during the electrophoresis, it is possible to determine both the p*I* and the molecular mass of the proteins under native conditions. Further, determination of the molecular mass of the stained proteins under denaturing conditions is also possible using SDS-PAGE.

The electrophoretic shape of the specific proteins detected after the run was unique, and it was suspected that these proteins were albumin-bound with other molecules such as sugars of lipids. However, these proteins could not be stained with either PAS or Sudan Black. In addition, they were not adsorbed on an anti-albumin affinity column. The molecular mass was estimated to be ca. 25 000 by SDS-PAGE. These observations clearly indicate that the specific proteins were not albumin-bound with a substantial amount of sugars or lipids.

It is well known that the serum concentrations of myoglobin and creatine kinase increase markedly after exercise [17]. Therefore, we examined their location on the two-dimensional electrophoretic gel by immunochemical staining, as described previously [13]. Results confirmed that these specific proteins were neither myoglobin nor creatine kinase.

The function and the role of these specific proteins are still unclear. However, their increase was observed in all cases examined after exercise, and they could not be detected in non-athletic students (data not shown). Therefore, we suggest that these proteins have a close relationship with exercise, and their

quantification may prove useful in estimating health or the amount of exercise. Further investigations are under way in our laboratory.

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