MECHANISMS OF INHIBITION OF CREATINE KINASE AND ASPARTATE AMINOTRANSFERASE ACTIVITY OF RAT SKELETAL MUSCLES BY NEUTROPHILS

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The inflow of leukocytes and, in particular, of neutrophils into skeletal muscles after intensive muscular activity is a well established fact [2, 8, 9, 12, 13]. Definite regions of skeletal muscles, needing elimination, evidently undergo infiltration. Neutrophils finding their way into the tissue may remove healthy tissue also along with parts of the muscle requiring destruction. The destructive potential of the neutrophil is largely associated with its proteolytic enzymes and with myeloperoxidase [14]. Meanwhile the role of these factors during interaction between neutrophils and skeletal muscles after muscular activity is unknown.

We decided to study this process in vitro by carrying out combined incubation of a skeletal muscle (the rat m. soleus) and neutrophils.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 200-220 g. The soleus muscle was isolated from rat hind limbs, placed in Eagle's medium, and kept on ice until use (about 2 h). The neutrophils were collected by flushing out the peritoneal cavity of rats with cold Hanks' solution without phenol red, after preliminary intraperitoneal injection of 0.3% of starch suspension [5]. Contaminating erythrocytes were hemolyzed by hypotonic shock for 30 sec, after which the physiological NaCl concentration was restored. The neutrophils were sedimented by centrifugation, suspended in Hanks' solution, and after determination of the cell concentration, the required dilution of the suspension was obtained. Characteristics of the neutrophil preparations were described previously [2]. Neutrophils were activated with zymosan, opsonized with mixed rat serum, washed, and suspended in Hanks' solution in a concentration of 10 mg/ml.

Tests were carried out in three incubation systems, containing: 1) m. soleus; 2) m. soleus and neutrophils, not activated by zymosan; 3b) m. soleus and neutrophils, activated by zymosan. The control systems contained m. soleus and zymosan, neutrophils not activated by zymosan, and neutrophils activated by zymosan. The incubation systems were set up on ice. The total volume of the system was 6 ml, the complete system containing m. soleus, neutrophils ($8 \cdot 10^6$ cells/ml), zymosan (0.5 ml) and antibiotics (penicillin 100 U/ml, streptomycin 200 U/ml). Samples were incubated at 37°C for 24 h. During incubation aliquots measuring 0.6 ml were taken after 0, 1, 3, 6, 8, and 24 h, and centrifuged (3000*g*, 15 min); the supernatants were collected, frozen, and kept at −20°C until analysis. The

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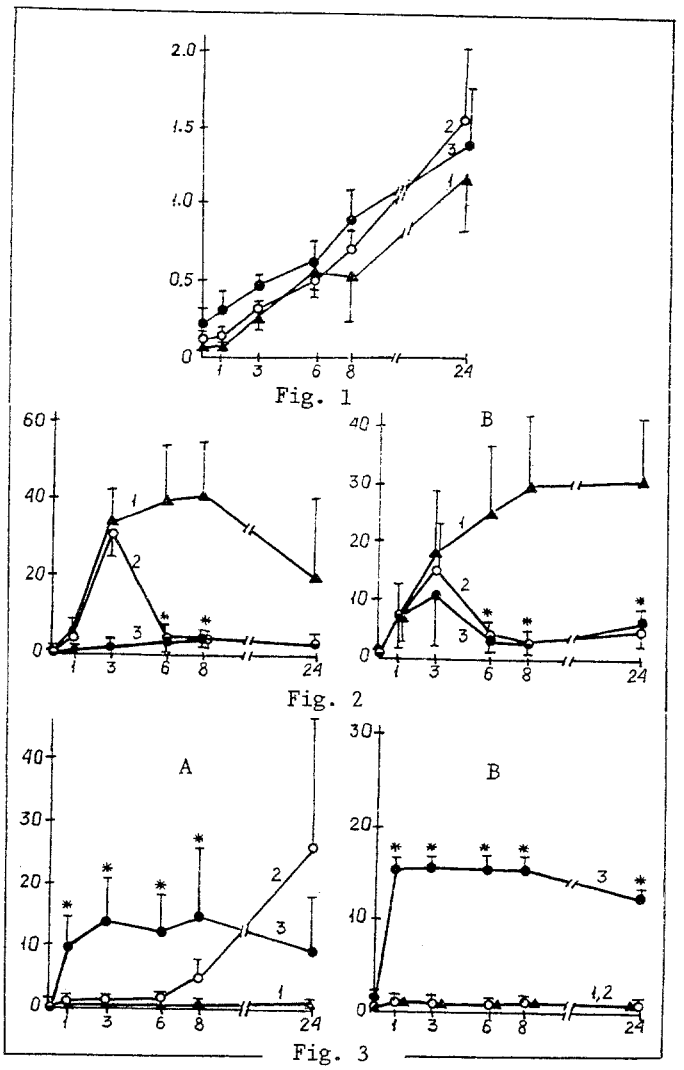

Fig. 1. Protein concentration in incubation medium during incubation of m. soleus (a), m. soleus and neutrophils, not activated (b) and activated (c) by zymosan. Abscissa, incubation time (in h, the same in Figs. 2 and 3); ordinate, protein concentration (in mg/ml). Here and in Figs. 2 and 3, values shown are M ± m (n = 5, five parallel tests in each experiment). Asterisk indicates significant difference from control ($p < 0.05$).

Fig. 2. Activity of CK (A) and AsAT (B) in incubation medium. Ordinate, enzyme activity (in I.U.; remainder of legend as to Fig. 1).

Fig. 3. Proteolytic activity (A) and incorporation of ^{125}I into acid-insoluble fraction of incubation medium (B). Ordinate, proteolytic activity for A (in relative units/h); ratio for B (in percent of radioactivity of acid-insoluble fraction of aliquot to its total radioactivity). Remainder of legend as to Fig. 1.

protein concentration [11], activity of creatine kinase (CK) [4], aspartate aminotransferase (AsAT) [15], and proteinases, using FITC-casein (1 mg/ml) as the substrate [3], were determined in the supernatants. Myeloperoxidase activity was estimated by measuring incorporation of ^{125}I into the acid-insoluble fraction [1]. In this case $6 \cdot 10^5$ cpm of $Na^{125}I$ was added to the incubation system. The experimental results were subjected to statistical analysis by parametric tests. The significance of differences was assessed by Student's t test.

EXPERIMENTAL RESULTS

Incubation of m. soleus led to gradual release of muscle proteins into the incubation medium after a short latent period of not more than 1 h. Combined incubation of m. soleus and nonactivated neutrophils for 6 h caused little change in the dynamics of total protein release compared with the system including m. soleus. Only after this time did a more rapid increase in protein concentration take place in the system containing m. soleus and non-activated neutrophils. Addition of zymosan stimulated protein release from neutrophils as early as in the initial period of incubation (Fig. 1). In the control experiments no effect of zymosan on protein release from m. soleus could be detected.

Against the background of increased release of total protein from m. soleus, in the course of 6-8 h there was a sharp increase in activity of CK and AsAT in the incubation medium. During subsequent incubation, CK activity fell whereas AsAT activity remained unchanged. On determination of CK and AsAT activity in systems containing m. soleus and neutrophils, a decrease in activity of these enzymes was found at the end of 3-6 h of incubation. Inactivation of CK and AsAT was more marked in the presence of cells activated by zymosan. Inhibition of CK activity in this case was quicker and more complete than that of AsAT, probably evidence of differences in resistance of the enzymes to the damaging factors produced by neutrophils. Toward 6 h of incubation CK and AsAT activities were at a very low level in systems containing both activated and nonactivated neutrophils, and subsequently the dynamics of inactivation of the enzymes was similar in character in both incubation systems (Fig. 2). Incidentally, during incubation of neutrophils, neither CK nor AsAT were found in the incubation medium, so that the activity of these enzymes appearing during incubation of m. soleus and neutrophils can be attributed entirely to m. soleus.

Neutrophils evidently inhibit activity of both CK and AsAT in the absence of zymosan through the action of proteinases released during degranulation, but in the presence of activation by zymosan, their activity is further inhibited by chemically reactive oxygen derivatives produced by these cells. Under the influence of zymosan the membrane NADPH-oxidase of the neutrophils is activated, triggering O_2^- production. The superoxide anion is next converted into H_2O_2, which is a component of the myeloperoxidase reaction [10, 14]. As a result of this reaction, in the presence of halogens powerful oxidizing agents are formed: hypohaloid acids (HOCl, HOBr, HOI), which can make a considerable contribution to the damaging action of neutrophils [6, 7, 14].

To assess the possible role of proteinases and myeloperoxidase in the inhibition of CK and AsAT activity of m. soleus, activity of the proteinases in the incubation medium and incorporation of ^{125}I into the acid-insoluble fraction were investigated.

During incubation of m. soleus proteolytic activity was virtually absent during 3 h of incubation, after which it began to appear, although it remained at a low level throughout the period of incubation compared with systems including neutrophils (Fig. 3a). During incubation of nonactivated neutrophils, proteolytic activity appeared after 3 h of incubation, and thereafter increased. Activation of neutrophils by zymosan led to a marked increase in the rate of release of proteinases. The presence of m. soleus did not significantly change the dynamics of proteolytic activity in incubation systems containing nonactivated and activated neutrophils.

Investigation of myeloperoxidase activity, based on incorporation of radioactive iodine into the acid-insoluble fraction, showed that activity of the enzyme was observed only in incubation systems containing neutrophils activated by zymosan (Fig. 3). Incorporation of ^{125}I took place quickly and reached a maximum after only 1 h of incubation.

The rapid release of active proteinases and myeloperoxidase from the neutrophils in the presence of zymosan suggests that these enzymes of the neutrophils are involved in inactivation of CK and AsAT of m. soleus. In this case AsAT is more resistant to damage by proteinases and by myeloperoxidase than CK.

Our experimental approach did not reveal any direct damaging action of neutrophils on m. soleus. However, the above mechanism of inhibition of CK, AsAT and, probably, of other enzymes also, is observed on interaction of neutrophils with skeletal muscles after intensive muscular activity, when leukocytes infiltrate the skeletal muscles which have carried the load.

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