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# Choline acetyltransferase and the nicotinic acetylcholine receptor AChRα7 in experimental myositis

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ABSTRACT

It is not known to what extent a non-neuronal cholinergic system is involved in myositis (muscle inflammation) evoked by marked muscle overuse. Therefore, in the present study, a recently established rabbit myositis model was used and the expression patterns of ChAT and nicotinic acetylcholine receptor AChRα7 (α7nAChR) were evaluated. Immunohistochemistry and in situ hybridization were used. The model leads to myositis including occurrence of muscle fiber necrosis. It was found that the infiltrating white blood cells as well the walls of small blood vessels exhibited immunoreactivity for both ChAT and α7nAChR. There was also pronounced immunoreactivity for these in the white blood cells that had coalesced within the necrotic muscle fibers. The findings show that there is a presence of a non-neuronal cholinergic system in the situation of muscle inflammation. Cholinergic effects may be highly involved in the inflammation-modifying events that occur in muscle overuse.


## 1. Introduction

Severe muscle inflammation (myositis) is a troublesome inflammatory condition. It leads to muscle pain and weakness. Myositis can occur in response to marked overuse or muscle injury [1] but can also be related to autoimmune disease [2]. Experimentally, myositis is reported to develop in response to experimental crush-injury [3]. We in our laboratory have developed a rabbit model of marked muscle overuse that leads to a significant myositis in the triceps surae muscle [4]. The resultant muscle pathology shows resemblances to that seen in overuse musculoskeletal disorders [1].

We noted that the tachykinin, TNF-alpha and glutamate systems seemed to be involved in the myositis in our rabbit model. There was thus a marked expression of the neurokinin 1-receptor (NK-1R) [4], which is the substance P preferred receptor, and of TNF-alpha [5] in the myositis processes. Furthermore, we observed that the cells of the inflammatory infiltrates within the muscle tissue exhibited expressions of the glutamate receptor NMDAR1 and the vesicular glutamate transporter 2 (VGluT2) [6]. The rabbit muscle overuse model has been found to be suitable for the evaluation of the sequence of processes that proceed in muscle damage and inflammation [4,7].

Acetylcholine (ACh) is produced in non-neuronal cells [8]. That includes inflammatory cells [9]. ACh also acts on these cells via auto- and paracrine loops [10]. It has been observed that ACh hereby can have anti-inflammatory effects, but acute ACh stimulation can also lead to pro-inflammatory effects [9]. [11]. Thus, the existence of a non-neuronal cholinergic system is well recognized. A dampening of inflammation can occur via cholinergic effects, termed the "cholinergic anti-inflammatory pathway" and which is mainly related to nerve-associated effects, for example via the vagal nerve [11]. However, dampening effects can occur independently of actions via cholinergic nerves, namely via the non-neuronal cholinergic system [10].

In principle nothing is known concerning the non-neuronal cholinergic system in myositis. The information that exists for muscle tissue are the findings that the white blood cells infiltrating muscle tissue in mdx dystrophic mice, an animal model of human Duchenne muscular dystrophy, express the nicotinic acetylcholine receptor AChRα7 (α7nAChR) [12]. There is no information at all for the non-neuronal cholinergic system in myositis evoked by marked muscle overuse. We have therefore in the present study further used our rabbit muscle overuse model in order to examine the importance of the non-neuronal cholinergic system during development of myositis. The aim of this study was to determine the expression of the ACh-producing enzyme choline acetyltransferase (ChAT) and that of the α7nAChR. We focused on the soleus part of the triceps surae muscle, examining the invading white blood cells, the muscle fibers and the blood vessels.

## 2. Material and methods

### 2.1. Animals

The experiments were performed on New Zealand adult white female rabbits with an age ranging from 6 to 9 months and a weight of approximately 4 kg. In total, 24 animals were included. Six belonged

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to a control group without any type of intervention. Six were exposed to an exercise procedure, which is further described below, for 6 weeks. The other twelve animals were exposed to the exercise procedure for one week but received in parallel injections of substances having pro-inflammatory effects adjacent to the Achilles tendon. For further information, see below.

### 2.2. Ethics statement

The present study has been carried out according to national and international guidelines, including in accordance with EU Directive 2010/63/EU for animal experiments. The study protocol was approved by the local ethical committee at Umeå University (A34/07).

### 2.3. Exercise procedure

Muscle overuse and myositis were induced using a laboratory model, a so-called "kicking machine". This model has been shown in previous studies to lead to marked overuse of the triceps surae muscle [4–7]. The apparatus is designed to generate repetitive passive flexion/extension of the right ankle joint via a pneumatic piston. The left leg was not attached to the kicking machine and the pelvis was strapped down. In addition to this passive movement, an active contraction during the plantar flexion was achieved via electrical stimulation induced by surface electrodes (pediatric electrodes 40 426A, Hewlett Packard, Andover, MA, USA) placed over the right-sided triceps surae muscle. For further details on operating modes, see [4–7].

The experiment was performed for 2 h and was repeated every second day. During the experiment, the rabbits were anesthetized by i.m. injections of fentanylfluanison (0.2–0.3 ml/kg) and diazepam (0.2 ml/kg). Fentanylfluanison (0.1 ml/kg) was further given each 30–45 min to maintain anesthesia. Buprenorphine (0.01–0.05 mg/kg) was injected s.c. postoperatively. Between the experiments, the rabbits were kept in ordinary cages where free movement was possible.

### 2.4. Injection treatments

In previous studies in our laboratory it was shown that the exercise protocol for 6 weeks leads to marked structural changes in the muscles, including pronounced inflammation (myositis) in parts of the muscle tissue [4,5]. We also noted that in principle similar changes occurred in response to a one-week exercise protocol if injection treatments leading to pro-inflammatory effects were given in parallel [13]. Therefore, animals exercised for 6 weeks plus animals that were exercised for one week and that were given proinflammatory injections were used in the present study.

The substances that were injected were as follows: Substance P (10–8 μmol/ml, 1 ml; S6883, Sigma) and the endopeptidase inhibitors Captopril (30 μmol/kg, 1 ml; c4042, Sigma) and DL-Thiorphan (500 μg/ml, 0.02 ml; 6031, Sigma). The injections were given into the loose connective tissue (paratenon) surrounding the Achilles tendon of the exercised leg. Six animals were given all three substances and three were given Captopril and DL-Thiorphan. Six animals received only Captopril injections. All variants of pro-inflammatory injections have been seen to give principally similar effects on the muscle tissue [13].

### 2.5. Grouping of animals according to morphology

The morphology of the muscle tissue of the animals subjected to a 6 week period of exercise has in principle been found to correspond to that seen for animals exercised for only 1 week but that in parallel was given local injections of substances having pro-inflammatory effects [5,13]. Therefore, all these animals are further on in the present study grouped together and defined as "myositis animals".

### 2.6. Sampling, fixation, sectioning

One day after the last exercise session, the animals were anesthetized by an intraperitoneal injection of pentobarbital natrium (60 mg/kg) and were then sacrificed with an overdose of this substance. The right triceps surae muscle was dissected out and transported to the laboratory. The time for transportation was 10–15 min. They were always transported on ice. For this study, tissue specimens from the soleus muscle were immediately fixed overnight at 4 °C, in 4% formaldehyde in 0.1 M phosphate buffered solution (PBS), pH 7.0. The following steps concerning washing, mounting and freezing were as previously described [5–7]. The specimens were serially cryosectioned (5–8 μm thickness) on to chrome-alum gelatine pre-coated slides. The specimens were then used for demonstration of morphology, for immunohistochemistry and for in situ hybridization.

### 2.7. Staining for demonstration of morphology (H&E)

In order to visualize the morphology, the sections were stained with hematoxylin and eosin (H&E). This was performed according to a previously described routine process [6].

### 2.8. Immunofluorescence processing

Staining for ChAT and α7nAChR immunoreactivity was performed. The full staining procedures for detecting ChAT and α7nAChR were extensively described in previous studies in our laboratory [14,15]. FITC-conjugated AffiniPure donkey anti-goat IgG (705-095-147) was used as secondary antiserum.

The ChAT antibody used was raised in goat (AB144P, Chemicon, Temecula, USA) and used in dilutions of 1:10–1:50 when incubation with primary antibody was performed for 1 h and in dilution 1:250 if overnight incubation was made. The α7nAChR antibody used was an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of α7nAChR of human origin (sc-1447, Santa Cruz Biotechnology, dilution used for 1 h incubation 1:100).

Desmin staining was performed as the reaction pattern for Desmin reveals degenerative features for muscle fibers. Necrotic fibers show thus typically a lack of Desmin staining [16], which is in accordance with previous findings of ours for such fibers in the myositis process [7,17]. The antibody against Desmin (D33, DAKO Cytomation, Denmark) was a monoclonal antibody and was used in a concentration of 1:1000 (overnight incubation), using protocols previously described [7,17].

### 2.9. Control stainings

Control stainings using PBS buffer instead of the primary antibody and preabsorption of the ChAT antibody with ChAT antigen (AG220, Chemicon, USA) and preabsorption of α7nAChR antibody with α7nAChR (sc-1447P; Santa Cruz) have been performed. The specific reactions were abolished in these stainings. The characteristics of the ChAT and α7nAChR antibodies used have been well described in previous studies in our laboratory [14,15] and the Desmin antibody has also been evaluated [7,17]. The usefulness of the α7nAChR antibody has also previously been evaluated via the preabsorptions that were made in studies on human synovial tissue [15]. In this previous study, it was shown to demarcate the same types of cells that are stained in the human synovial tissue via the use of other types of α7nAChR antibodies used by other research groups [18,19].

### 2.10. Double stainings

The initial procedures conformed to the procedures for staining with ChAT or for α7nAChR [14,15]. That included the use of FITC-conjugated AffiniPure donkey anti-goat IgG (705-095-147) and rinsing in PBS

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