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Compression-induced ATP release from rat skeletal muscle with and without lengthening contraction

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Abstract

Adenosine triphosphate (ATP) is well known to be released from injured or inflamed tissues, and to excite/sensitize nociceptors in response to heat and mechanical stimulation. To determine whether muscle releases ATP when it is compressed, we measured ATP release from the extensor digitorum longus muscle (EDL). In addition, we investigated whether there is any difference in ATP release from the EDL of rats 2 days after lengthening contraction (LC), since the condition of the muscle is different, i.e., mechanically hyperalgesic and swollen. The EDL was put in a small chamber and superfused with Krebs–Henseleit solution equilibrated with a gas mixture of 95% oxygen and 5% carbon dioxide. The muscle was quantitatively stimulated with a servo-controlled mechanical stimulator. Reproducibility of ATP release was examined with stimulation using a 20*g* force. Stimulus intensity-dependency of ATP release was also examined with 5 time compression with intensities of 5, 10, 20 and 40*g* force. Bioluminescent determination by the luciferin–luciferase method was used to quantify ATP in the sample. The ATP release was decreased by repetitive mechanical stimulation of the EDL with 30 min intervals, and it was stimulus intensity (5–40*g* force)-dependent. The amount of ATP released from the muscle preparations was not different between the non-treated control and the LC group. These results provide clear evidence that ATP is released from rat skeletal muscle by compression.

Keywords: Adenosine triphosphate (ATP); Lengthening contraction; Mechanical hyperalgesia; Mechanical stimulation; Delayed onset muscle soreness (DOMS); Rat

A millimolar level of adenosine triphosphate (ATP) is present in the cytosol of cells [5]. In muscle, ATP plays a pivotal role in contraction as well as in the relaxing process. Recent studies revealed that ATP was released from activated muscle fibers to extracellular spaces [14] and that interstitial ATP concentrations linearly increased with contracting muscle tension [8]. ATP is also released from cells as a result of tissue damage or inflammation, and this ATP may stimulate nociceptors: myelinated and non-myelinated thin fiber muscle receptors are activated by extracellulary applied ATP [6,13,19] and sensitized to mechanical stimulation [9]. In behavioral experiments ATP-induced signs of mechanical hyperalgesia when injected into the neck muscle in mice [10] and the masseter muscle in rats [15]. It is known that uroepithelial cells release ATP upon strong stretching of the urinary bladder, and this ATP stimulates afferents with P2X3 receptors, the terminals of which are located near epithelial cells; thus, ATP plays an important role in mechanotransduction in the bladder [4].

We have reported that, 2 days after lengthening contraction (LC), the extensor digitorum longus muscle (EDL) of rats is mechanically hyperalgesic (delayed onset muscle soreness, DOMS) [18], and that the C-fiber receptors recorded from this hyperalgesic muscle have facilitated sensitivity to mechanical stimulation [19]. The mechanism for this increased sensitivity to mechanical stimulation remains unknown. Lengthening contraction leads to much stronger micro-damage of muscle fibers than does isometric or shortening (concentric) contraction [11], and results in more severe subsequent soreness [12]. Although there is no evidence that exercise-induced muscle soreness is directly associated with muscle damage [1], there is a possibility that ATP released by micro-injury after exercise may sensitize nociceptors to mechanical stimulation with increased expression

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of P2 receptors, or alternatively, that the release of ATP induced by mechanical stimulation may be enhanced after exercise, thus sensitizing nociceptors to mechanical stimulation. To test the latter possibility we measured ATP release induced by servo-controlled mechanical stimulation from muscle that underwent LC, and compared it with the ATP release from non-exercised control preparations.

Twenty-four male Sprague–Dawley rats (SLC Inc., Japan) weighing 350–435 g were used in this study. Twelve of them received lengthening contraction (LC) 2 days before measurement of ATP (LC group), and the rest served as the control (CTR group). The animals were kept 2–3 per cage under a 12 h light/dark cycle (light between 07.00 and 19.00 h) in an air-conditioned room (22–24 °C). Free access to food and water was available. All experiments were conducted according to the Regulations for Animal Experiments in Nagoya University, and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions in Japan.

Delayed onset muscle soreness (DOMS) was made by applying repetitive lengthening contraction (LC) to the EDL (LC group). The method for LC was the same as described in our previous study [18], as follows. The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). A pair of needle electrodes was transcutaneously inserted near the common peroneal nerve that innervates the EDL, and the location of the needles was assured by dorsi-flexion of the ankle joint and extension of the middle three toes upon electrical stimulation of the nerve. The parameters for electrical stimulation to induce repetitive contraction of the EDL were as follows: current magnitude was three times the twitch threshold, frequency was 50 Hz with pulse width of 1 ms, and stimulus period was 1 s. The ipsilateral foot was plantar-flexed with a servomotor to stretch the EDL in synchrony with electrical stimulation of the nerve over a 1 s period, and then returned to the starting position over a 3 s period. This pattern was repeated every 4 s for a total of 500 repetitions (thus overall exercise period was about 33 min). The animals without LC served as controls.

To confirm that the applied LC induced DOMS, we tested for the existence of edema, a sign of DOMS, after LC, by measuring the wet weight of the EDL. Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.) 2 days after LC (LC group) or on the day of ATP measurement (Control group). The EDLs of both sides were cut at the origin and at the insertion, the nerves and blood vessels attached were cut at the entrance to the muscle, and then the EDLs were isolated from other tissues and removed. They were rinsed in Krebs solution a few times, and then extra water was removed by gently rolling on filter paper, after which the weight of the muscle was measured with a scale. After measurement, 8 EDL muscles of the LC side (right side) of each group were used for ATP measurement.

The right EDL muscle of both LC and CTR groups was used for ATP measurement. The muscle preparations were put in a small chamber (volume: 1.8 ml) and superfused 3 ml/min with sterilized modified Krebs–Henseleit solution (containing (in mM) 110.9 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, and 20.0 glucose) continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. Temperature in the chamber was continuously monitored and kept constant (32 ± 0.2 °C). Before mechanical stimulation at least one hour was elapsed. The superfusate was sampled at a speed of 0.8 ml/min for 20 s using a peristaltic pump through piping with a metal tube (2 mm in diameter) at one end. The tip of the metal tube was placed downstream to and within 1 mm from the point to be mechanically stimulated, and this relation was fixed during one experiment. The delay time for passing through this piping was estimated as 20 s. Time zero of the sampling was defined at the point 20 s after the time when mechanical stimulation was finished, and the superfusate was sampled at −6, −3, 0, 0.5, 1, 2, 3 and 6 min. The ATP concentration was measured using the luciferin–luciferase method [14]. Briefly, 100 μl aliquot of the bath sample was added to 100 μl of ATP assay mix (Sigma, containing firefly luciferase and luciferin) and the luminescence was measured in a luminometer (Lumat LB 9507, Berthold Technologies, GmbH & Co. KG, Germany) for 10 s. Net ATP release in the sample was calculated by subtracting the basal concentration measured before stimulation from that induced by the mechanical stimulation.

Mechanical stimulation (compression) was applied with a servo-controlled mechanical stimulator (manufactured by Dr. S. Aizawa, Goto College of Medical Arts and Science, Tokyo, Japan). The stimulator had a plastic, cylindrical probe with a flat circular tip (tip size 2.28 mm^2). At first we measured ATP release by repetitive mechanical stimulation to the middle of EDL (a ramp of 20*g* force over 10 s × 3 trials with intervals of 30 min). All muscle C-fiber receptors recorded in our previous single-fiber experiments were excited with this stimulus intensity [19], suggesting that it is the suprathreshold intensity for muscle nociception. Second, stimulus intensity dependency of the ATP release was examined: 5, 10, 20 and 40*g* force with a speed of 10 g/s × 5 times that was proceeded by a slow increase of 2*g* force over 5 s to get better control of stimulation (see stimulus command in Fig. 1A). The four different intensities were randomly applied to four different points (2.5 mm apart from each other) in the muscle to avoid the effect of the order and the point stimulated.

Muscle wet weight was analyzed using paired t test. Baseline ATP concentrations in the perfusate between the CTR and the LC group were compared with unpaired t test. The Friedman test was used to examine the reproducibility, and the stimulus intensity dependency of the ATP release. The Mann–Whitney *U* test was used to compare the difference in the ATP release with each trial or stimulus intensity between the CTR and the LC group. $P < 0.05$ was considered as significant.

The wet weight of the EDL in the LC group was 209 ± 4 mg on the side ipsilateral to the exercise ($n = 12$), and it was significantly heavier than that on the contralateral side (201 ± 4 mg, $n = 12$, $P < 0.01$, paired *t* test). In the control group there was no difference in muscle weight between the right and left sides (194 ± 3 and 195 ± 3 mg, respectively, $n = 12$ each).

Baseline concentrations of ATP before the first mechanical stimulation were 25.3 ± 3.7 pM in the CTR ($n = 8$) and 19.7 ± 2.7 pM in the LC group ($n = 8$), which were not significantly different. The first mechanical stimulation (20*g* force