Changes in Contractile Characteristics of Rat Skeletal Muscles Associated with P2-Receptor Activation After Spinal Cord Transection

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Abstract

Introduction. *Traumatic spinal cord and peripheral-nerve injury is associated with release of proinflammatory cytokines and chemokines, which may stimulate neuronal activity. Adenosine triphosphoric acid (ATP) is an important pain mediator involved in the acute and chronic neuropathic pain development. Its excessive release from primary injured tissue leads to activation of P2-receptors, which may further start secondary injury mechanisms. Although the effects of ATP on the peripheral nervous system are relatively well studied, the pathophysiological role of purinergic signaling after spinalization remains unclear.*

The study was ***aimed*** *at assessing the post-spinalization effects of P2-receptors on the contractile characteristics of rat skeleton muscles.*

Materials and methods. *The objects of the study were the soleus muscle, the extensor digitorum longus (EDL) muscle, and diaphragm in intact rats and spinalized rats. Seven days after laminectomy followed by spinal cord transection, animals were anesthetized, exsanguinated, and their muscles with nerve stumps were isolated. Contractile response parameters were recorded using mechanomyography (MMG). To study effects of ATP on ligand binding, ATP was added to a bath and mechanical responses in the rat muscles were assessed 7 min after. After washing with Krebs–Henseleit solution, the preparations were incubated with suramin solution for 20 min with subsequent ATP application. Then the mechanical responses in the muscles were again recorded. Statistical significance was assessed using Student's t-test for independent (unpaired) and paired samples.*

Results. *We found a significant ($p < 0.05$) decrease in the modulating activity of ATP, as the main endogenous signaling agent, in the cholinergic synapse of the soleus muscle from 32.4 to 5.8% and from 13.7 to 5.6% for the EDL muscle after the spinalization (spinal cord injury at the Th6–Th7 level) compared with intact animals. No such dramatic changes were observed in the diaphragm.*

Conclusions. *Abnormal ATP-mediated modulation of neuromuscular transmission demonstrated in this study supports the involvement of purinergic signaling in the neurotrophic control and functioning of various motor units.*

Keywords: *spinalization; ATP; P2-receptors; skeletal muscles; post-traumatic movement disorders; synapse; suramin*

Ethics approval. The research protocol was approved by the Ethics Committee of the Kazan Federal University (protocol No. 30, June 28, 2021).

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Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

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Динамика сокращений скелетных мышц крысы при активации P2-рецепторов после перерезки спинного мозга

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Аннотация

Введение. *Травма спинного мозга, периферических нервов сопровождается выделением провоспалительных цитокинов и хемокинов, которые могут усиливать активность нейронов. Среди медиаторов повреждения особо можно выделить аденозинтрифосфорную кислоту (АТФ), которая вовлечена в процессы формирования острой и хронической нейропатической боли, и чрезмерное её высвобождение травмированной тканью вызывает активацию P2-рецепторов, что может повлиять на механизмы вторичного повреждения тканей. При общей изученности эффектов АТФ на периферическую нервную систему патофизиологическая роль пуринергического сигнального звена при спинализации не раскрыта.*

Цель *исследования – оценка динамики сокращений скелетных мышц крысы при воздействии на P2-рецепторы после спинализации.*

Материалы и методы. *Объектом исследования выступали камбаловидная мышца, длинный разгибатель большого пальца и диафрагма интактных крыс и животных после спинализации. Через 7 сут после ламинэктомии с последующей перерезкой спинного мозга животных наркотизировали, обескровливали и выделяли мышцы с культями нервов. Параметры сократительных ответов регистрировали механомиографическим методом. Для оценки эффектов лигандов в ванночку добавляли АТФ и через 7 мин оценивали механические ответы мышц. После отмывки раствором Кребса инкубировали с раствором сурамина в течение 20 мин с последующим добавлением АТФ и вновь регистрировали механические ответы мышц. Статистическую значимость оценивали с помощью критерия Стьюдента для независимых и попарно сопряжённых выборок.*

Результаты. *Выявлено значимое ($p < 0,05$) снижение модулирующей активности основного эндогенного агента – АТФ в холинергическом синапсе камбаловидной мышцы с 32,4 до 5,8% и с 13,7 до 5,6% для длинного разгибателя большого пальца вследствие спинализации (повреждения спинного мозга на уровне Th6–Th7) в сравнении с интактными животными. На диафрагме столь драматических изменений не наблюдалось.*

Заключение. *Продемонстрированная нами аномальная модуляция АТФ нервно-мышечного перехода предоставляет доказательства вовлечённости пуринергического звена в нейротрофический контроль и функционирование различных двигательных единиц.*

Ключевые слова: *спинализация; АТФ; P2-рецепторы; скелетные мышцы; травматический двигательный синдром; синапс; сурамин*

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Introduction

Traumatic spinal cord and peripheral nerve injuries are not unusual among people of working age and can be accompanied by severe and often irreversible motor disorders. Traumatic spinal cord injury (SCI) is characterized by immediate and irreversible tissue loss at the injury site followed by the secondary injury in adjacent tissues over time. Traumatic peripheral nerve injury is known to cause various changes in the expression of intracellular signaling molecules in the spinal cord [1], primarily in response to increased release of various mediators in activated spinal cord microglia [2], which may play an important role in the neuropathic pain development and maintenance [3].

Microglia activated by a trauma produces and releases pro-inflammatory cytokines and chemokines [4], which can stimulate neuronal activity. Adenosine triphosphoric acid (ATP) is an important pain mediator involved in the development of acute and chronic neuropathic pain after an injury [5]. Its excessive release by injured tissue activates high-affinity purinergic receptors in microglial cells, which may further affect the mechanisms of additional tissue damage, known as secondary injury [3].

Although the effects of ATP on the peripheral nervous system are relatively well understood, the pathophysiological role of purinergic signaling associated with spinalization remains unclear. So, the objective of the study is to evaluate the changes in contractile characteristics of rat skeletal muscles associated with P2-receptor activity after spinalization.

Materials and methods

Male Wistar rats aged 9–12 months, weighing 160–240 g, were used for the experiments. The objects of the study were the pelvic girdle and lower limb muscles, which are fundamentally important for motor activity (slow-twitch muscles [soleus muscle, *m. soleus*], fast-twitch muscles [*extensor digitorum longus muscle, m. extensor digitorum longus, EDL*], and functionally distinct respiratory muscle [*diaphragm, m. diaphragma*] with their corresponding neuromuscular synapses) isolated from intact rats and spinalized animals.

One week prior to and during the experiments, rats were housed in individual cages at room temperature of 22ºC with a 12 h/12 h light/dark cycle, access to water and food *ad libitum*. All manipulations were performed at the same time of a day. Rats were divided into 2 groups of 12 animals each: the control group included intact animals and the spinalization group included animals after spinal cord transection.

The surgery was performed under aseptic conditions and combined intramuscular analgesia using zoletil (Zoletil 50, Virbac) at a dose of 0.5 mg/kg and xylavet (XylaVET, Pharmamagist Ltd.) at a dose of 0.5 ml/kg. After dissection of Th6–Th7 vertebrae, a laminectomy was performed to expose the spinal canal with subsequent transection of the spinal cord at this level (Fig. 1).

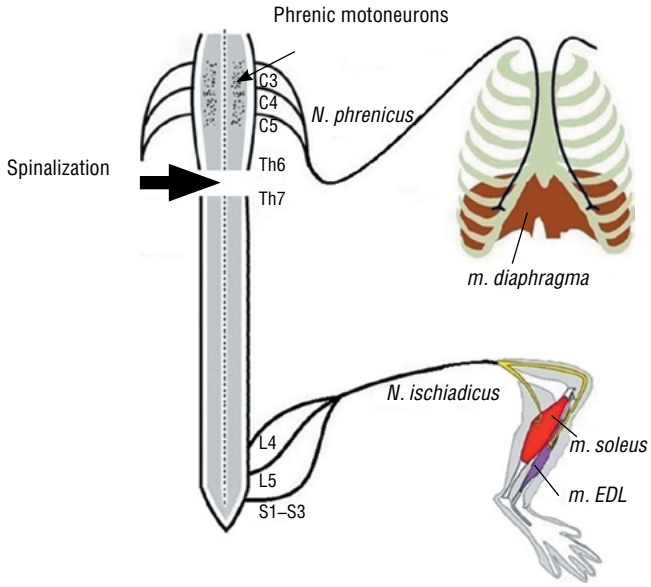

Fig. 1. Schematic diagram of spinalization at Th6–Th7.

Seven days after the surgery, the animals were anesthetized with sodium ethaminal (40 mg/kg intraperitoneally) and exsanguinated. *M. soleus, m. EDL, m. diaphragma* were isolated with nerve stumps fixed by both tendon ends, immersed in 10 ml beakers filled with Krebs–Henseleit solution [6].

The nerve stump of the isolated muscle was placed in a special nerve stump suction electrode for electrical stimulation [7]. Rectangular pulses of 10 V amplitude and 0.5 ms duration at a frequency of 0.1 Hz were applied for 2 min using D330 MultiStim System. Contractile force was recorded with a force displacement transducer (Linton FSG-01), the analog signal was digitized and processed using a Biopack MP100WSW data acquisition system.

The initial load on the myoneural preparations was 1 g on *m. soleus* and *m. diaphragma* and 0.5 g on *m. EDL*. The muscle preparations were kept in the solution for half an hour for adaptation, then the stability of the contractile responses was assessed twice at 5-minute intervals [8].

To study the effects of purinergic agonists and antagonists, 100 μM ATP was added to the bath and the muscle mechanical responses were assessed 10 minutes after. Further, after 20-minute washout of muscle preparations with Krebs–Henseleit solution, the electrical stimulation was repeated. To confirm the ATP effects on synaptic transmission, the muscles preparations were maintained in 100 μM suramin

(non-selective P2-receptor antagonist) for 20 min, followed by the addition of 100 μM ATP (P2-receptor agonist), and mechanical muscle responses were again recorded. In control experiments, contractile responses to indirect electrical stimulation were recorded after 20-minute neuromuscular tissue incubation with 100 μM suramin [9].

The responses recorded within 2 min (12 contractions) were averaged and processed as a single value in % of the baseline results obtained at the beginning of the experiment. Statistical data analysis was performed with SPSS Statistics software. Conformity to normal distribution was checked using the Kolmogorov criterion. Statistical significance was assessed using multivariate analysis of variance (MANOVA) for independent and paired samples. The differences were considered significant at $p < 0.05$.

Results and discussion

After the spinalization, contractile responses in *m. soleus* and *m. EDL* changed divergently in contractile force and in time parameters (Fig. 2; Table 1). In contrast, amplitude-time parameter values in *m. diaphragma* remain stable, perhaps due to a higher position of phrenic motor neurons, which were less affected by spinalization.

Application of 100 μM ATP to muscle preparations of intact rodents modulates the contractility parameters: a 10-min exposure to ATP decreased the contractile force of locomotor *m. soleus* and *m. EDL* and increased the contractility of respiratory *m. diaphragma*. ATP had virtually no effect on the neuromuscular preparations from the spinalized animals. Only *m. diaphragma* remained sensitive to the study nucleotide (see Table).

Suramin (100 μM) as a non-competitive inhibitor of P2-receptors showed no significant effects. In the presence of suramin (100 μM), exogenous ATP (100 μM) activity was completely inhibited in all study objects (see Table).

Our findings demonstrate a significant suppression of the peripheral nervous system activity in the SCI animal model. Changes in synaptic signaling indicate axon degeneration after the injury of the spinal cord at its upper levels.

Understanding mechanisms underlying suppression of the peripheral nervous system is important to prevent functional decline and maintain a high potential for motor function recovery, especially with cellular therapies aimed at SCI repair.

Disorders of muscle function caused by SCI can result from a mechanical injury and from secondary injury caused by pathophysiological response to the initial trauma. For example, there are studies demonstrating abnormally high and persistent ATP release levels in peritraumatic tissues in SCI rat models, indicating P2-signaling involvement in the cascade of degenerative events, known as secondary injury, and neurodegeneration after the initial injury [10].

This cascade of injury-associated events include extensive hemorrhage, necrosis of cellular components of the central and peripheral nervous systems. The subsequent activation of astrocytes and other cells located in close proximity to the injury site results in extremely unfavorable conditions for axon repair. The concurrent activation of the immune system leads to additional tissue damage at the injury site by attracting immune inflammatory cells, such as neutrophils and macrophages. On the other hand, macrophages and T-helpers provide trophic support to damaged components of the CNS. All of the above processes lead to axon degeneration and the loss of communication between neurons, which primarily results in various functional muscle disorders [11].

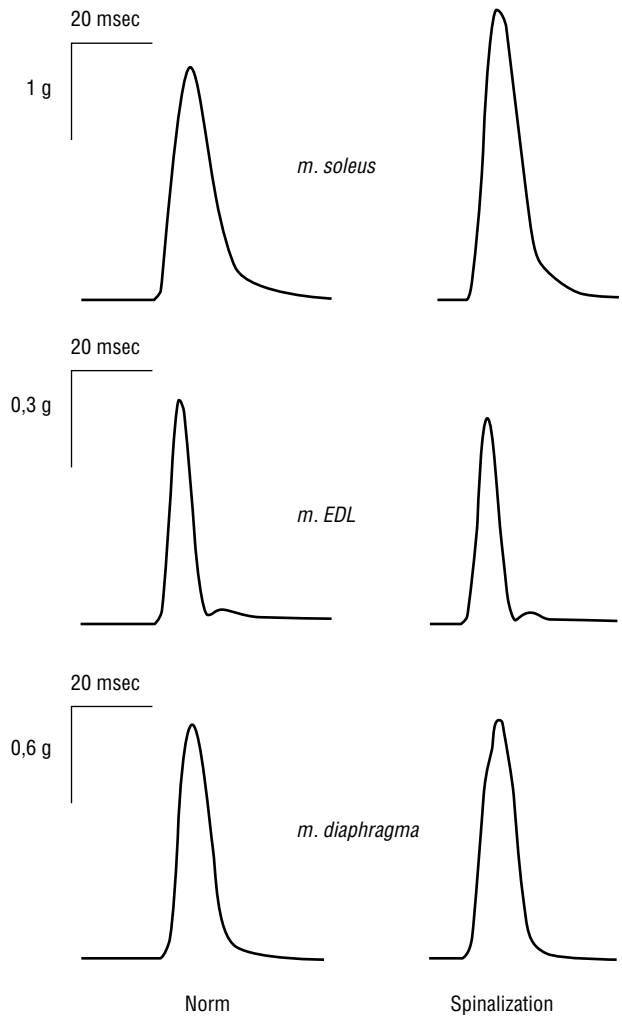

Fig. 2. Traces of single contractile responses of the isolated rat *m. soleus*, *m. EDL* and *m. diaphragma* evoked by electrical stimulation in controls and in spinalized rats (selected representative traces are presented).

Table 1. Parameters of rat muscle contractility evoked by electrical stimulation in different experimental conditions, n = 10–12

Experimental conditions	*n*	Parameter	Control	ATP, 100 μM	Suramin, 100 μM	Suramin, 100 μM + ATP, 100 μM
M. soleus						
Normal value	10	CF	100,0 ± 4,2	67,6 ± 5,2*	104,3 ± 3,9	98,5 ± 7,1
		CT	0,081 ± 0,004	0,083 ± 0,006	0,080 ± 0,004	0,079 ± 0,005
		RT/2	0,092 ± 0,007	0,105 ± 0,011	0,090 ± 0,006	0,093 ± 0,010
Spinalization	10	CF	119,8 ± 5,1#	114,0 ± 6,1#	120,2 ± 4,3#	121,8 ± 6,4#
		CT	0,073 ± 0,005	0,076 ± 0,007	0,071 ± 0,006	0,074 ± 0,004
		RT/2	0,101 ± 0,009	0,116 ± 0,010	0,098 ± 0,008	0,105 ± 0,010
M. EDL						
Normal value	10	CF	100,0 ± 4,5	86,2 ± 3,9*	102,0 ± 6,1	98,7 ± 5,3
		CT	0,057 ± 0,003	0,056 ± 0,005	0,059 ± 0,004	0,058 ± 0,006
		RT/2	0,067 ± 0,005	0,069 ± 0,004	0,065 ± 0,007	0,068 ± 0,005
Spinalization	10	CF	88,7 ± 3,8#	83,1 ± 5,4	85,9 ± 4,8#	83,1 ± 6,7#
		CT	0,068 ± 0,005	0,069 ± 0,006	0,068 ± 0,006	0,067 ± 0,005
		RT/2	0,071 ± 0,006	0,073 ± 0,007	0,070 ± 0,005	0,073 ± 0,004
M. diaphragma						
Normal value	12	CF	100,0 ± 3,7	114,6 ± 5,2*	98,3 ± 4,7	102,9 ± 6,2
		CT	0,065 ± 0,004	0,066 ± 0,003	0,064 ± 0,006	0,064 ± 0,004
		RT/2	0,075 ± 0,006	0,075 ± 0,005	0,074 ± 0,006	0,076 ± 0,004
Spinalization	12	CF	103,2 ± 4,1	112,7 ± 3,9*	102,0 ± 4,9	103,8 ± 7,5
		CT	0,071 ± 0,005	0,070 ± 0,003	0,069 ± 0,004	0,072 ± 0,004
		RT/2	0,074 ± 0,003	0,076 ± 0,006	0,074 ± 0,005	0,075 ± 0,006

Note. *p < 0.05 compared with the control group; #p < 0.05 compared with normal value. CF — contractile force (% from the level in the control group); CT — contractile time, s; RT/2 — half-relaxation time, sec.

The obtained data demonstrate significant differences in the mechanisms of contractility control in fast-twitch and slow-twitch skeletal muscles of warm-blooded animals, which is consistent with our earlier observations in spinal shock models [12]. The suppression of P2-receptors affecting muscle contraction associated with such a striking response to spinalization demonstrates the involvement of the purinergic signaling in the neurotrophic control and functioning of various motor units.

Activation of spinal microglia caused by trauma leads to an increased expression of P2-receptors. For example, P2X4R levels have been shown to increase in association with SCI, while P2X4R inhibition has been shown to reduce neuropathic pain [13]. Another ATP-sensitive purinergic receptor, P2X7, can form a macromolecular pore under repeated or prolonged exposure to high concentrations of ATP [14], which is of paramount importance taking into consideration that ATP release in peritraumatic regions rises massively. The role of this receptor is particularly important in understanding SCI pathophysiology due to its extensive expression in CNS neurons [10]. There are data indicating potential involvement of other receptor subtypes, namely, P2Y6, P2Y13 and P2Y14, in the physiological responses of microglia [15, 16].

Despite the severity of the damage, even with extensive SCI at the level of the thoracic segments, electrical stimulation applied slightly below the level of the injury allows to register stable rhythmic motor activity in the lower limbs, which was demonstrated in a number of animal models [17, 18].

Inhibiting purinergic receptors can improve outcomes in SCI patients. For example, intraspinal injection of a P2X7-receptor antagonist into the peritraumatic region reduced the damage caused by SCI [10]. P2X7R inhibition also reduced motor neuron loss and promoted subsequent functional recovery in injured animals.

On the other hand, axon membrane damage caused by an injury is associated with rapid changes in intracellular ion concentrations. The effects of ATP on spinal cord neurons cause their excitation leading to a persistent irreversible increase in Ca^{2+} levels resulting in a cell death [10].

Moreover, a number of fundamental animal model studies demonstrated pathological changes in skeletal muscles associated with SCI: the massive ATP release from damaged tissues provokes local and generalized inflammatory process with the release of proinflammatory cytokines (in particular, interleukins-1 and -6), which mediates muscle disorders, similar to muscle denervation atrophy [14]. ATP activates ionotropic P2XRs, particularly P2X7, which

mainly leads to an increase in intracellular Ca^{2+} levels and induces cytoskeletal reorganization, inflammation, apoptosis/necrosis, and proliferation, usually in a long-term perspective [19].

Conclusion

Thus, all the data available by now only outline the ways to study the mechanisms of the effects we have described. Further studies of P2-signaling role in post-spinalization processes are required.

Abnormal ATP-mediated modulation of neuromuscular transmission demonstrated in this study indicates axon degeneration and suggests that transsynaptic degeneration of motor neurons may occur below the level of spinal cord injury after the traumas similar to the ones described in the study.

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