Short Communication

Aminophylline Pretreatment Modulates the Nitric Oxide System of the Isolated Rat Diaphragm: the Role of Frequency of the Electrical Stimulation

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Abstract. The effect of different frequencies of direct subtetanic electrical stimulation (14, 20, and 30 Hz) and aminophylline (AMPh) pretreatment on the effect of *N*^G-nitro-L-arginine methyl ester (L-NAME) (1 mM) on isolated rat diaphragm was investigated. L-NAME potentiated tension developed (Td) in the diaphragm pretreated with a single concentration of AMPh (1.08 mM) in a frequency-dependent manner. The effect was significantly different in comparison with muscle incubated 30 min with Tyrode solution only. In the muscle pretreated with cumulative concentrations of AMPh (0.36 – 3.60 mM), the frequency-dependent potentiation of Td induced by L-NAME and the difference between L-NAME-treated and untreated muscle were lost.

Keywords: aminophylline, *N*^G-nitro-L-arginine methyl ester, stimulation frequency

After the discovery of the nitric oxide (NO) system in skeletal muscle, the influence of NO and inhibitors of NO synthase (NOS) on muscle contractility were investigated (1, 2). Kobzik et al. (1) have shown that blockade of NOS in the isolated rat diaphragm produced an increase of muscle contractility. They postulated that inhibitors of NO (i.e., 7-nitroindazole, aminoguanidine, and nitro-L-arginine) have a specific, NO-related effect on the muscle contractility. However, some authors observed NO-unrelated effects of NOS inhibitors (3).

NO is synthesized continually at low rates by constitutive isoforms of NOS, the synthesis of NO being increased during repetitive isometric contractions up to 200% (4). NO may modulate skeletal muscle contractility via at least two mechanisms, through the cGMP-NO pathway, and directly, by interacting with calcium release channels of the sarcoplasmic reticulum (5). The effects of NO on the channel activity primarily depend on its concentration, and its interaction with reactive oxygen species (ROS) (2). Reid et al. (6) have shown that superoxide generated by diaphragm muscle bundles increases both twitch and tetanic forces developed

during submaximal stimulation.

N^G-nitro-L-arginine methyl ester (L-NAME) or *N*^G-nitro-L-arginine (L-NNA), a deesterified form of L-NAME, in the concentration range used in our experiments (≥ 1 mM) blocked NOS activity in the skeletal muscle (e.g., rat diaphragm, extensor digitorum longus) (1, 7, 8).

The diaphragm is the primary muscle of inspiration. The relationship between the diaphragm contractile function and NO-synthase activity could have therapeutic significance in neuromuscular diseases associated with changes in muscle fibers and activity of the NO system. Consequently, the effects of various NOS inhibitors on diaphragm contractility have been evaluated during the last decade (1, 2).

The interaction between L-NAME and aminophylline (AMPh) on the contractility of the isolated rat diaphragm stimulated by direct subtetanic electrical stimulation (DSES) and the role of extracellular calcium in such an interaction were already investigated. It was shown that the stimulatory effects of cumulative concentrations of AMPh on the contractility of the isolated rat diaphragm during DSES (14 Hz) were potentiated by L-NAME. In a calcium-free medium, L-NAME completely abolished the effect of AMPh on diaphragm

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0.7688; $P > 0.05$) (Fig. 2B).

In the present experiments, both AMPH pretreatment (single and cumulative concentrations) and frequency of the DSES modulated the effect of L-NAME on Td of the isolated rat hemidiaphragm. We have recently shown that L-NAME (1–10 mM) potentiated Td of the muscle pretreated with cumulative concentrations of AMPH only during DSES at 14 Hz (9, 10). It was presumed that pretreatment with a single concentration of AMPH (1.08 mM) in such an experimental model was probably not a strong enough stimulus for the upregulation of nNOS in the muscle (9). The present study provides the first evidence that such interaction between AMPH and the NO system in the skeletal muscle is changed at higher stimulation frequencies (20 and 30 Hz), that is, the interaction depends on the frequency of electrical stimulation.

The relation between isolated skeletal muscle contractility and frequency of the electrical stimulation was extensively investigated in our laboratory (11), and our model of subtetanic electrical stimulation closely

resembles the physiological conditions.

Abbate et al. (12) have shown that in intact single muscle fibers of the mouse, higher stimulation frequency produced an increase in intracellular calcium level and the myofibrillar calcium sensitivity. Also, an increasing frequency of stimulation enhanced NO production in the skeletal muscle (13). The present experiments show that L-NAME potentiated Td of the hemidiaphragm pretreated with a single concentration of AMPH (1.08 mM) in a frequency-dependent manner (Fig. 2A, L-NAME-30). Simultaneously, Td of the muscle incubated with Tyrode solution only was not changed when the stimulation frequencies were increased from 14 to 20 or 30 Hz (Fig. 2A, C-30). Skeletal muscle's nNOS is a membrane bound, calcium-dependent constitutive isoform. Accordingly, the presumed frequency-dependent stimulation of nNOS could be due to a higher influx of extracellular calcium. However, the higher NO concentration in the muscle originated by nNOS could explain, at least partially, the lack of expected frequency-dependent (i.e., calcium-dependent) increase in the muscle contractility

Fig. 2. The effect of different frequency of subtetanic electrical stimulation ($F = 14, 20$, and 30 Hz) on the effects of L-NAME (1 mM). A: Change of Td of the rat hemidiaphragm pretreated with a single concentration of aminophylline (AMPH) (1.08 mM) after 30 min of incubation without (Tyrode solution only: C-30, empty bars) or with L-NAME (L-NAME-30, shaded bars) (Experimental design 1). $P < 0.05$: significantly different in comparison with values obtained after 30 min of incubation with Tyrode solution only (C-30) (Student's t -test – unpaired data). B: Change of Td of the rat hemidiaphragm pretreated with cumulative concentrations of AMPH (0.36–3.60 mM) after 30 min of incubation without (Tyrode solution only: C-30, empty bars) or with L-NAME (L-NAME-30, shaded bars) (Experimental design 2). Abscissa: frequency of the electrical stimulation (Hz). Ordinate: ΔTd (%): the mean percentage change of Td from the corresponding control (C) before the addition of L-NAME. Each vertical bar represents the mean $\pm$ S.E.M. from 4–7 separate experiments. Inserted graphs represent the frequency-response curves for the effect of L-NAME-treated (L-NAME-30, closed circle) or L-NAME-untreated (C-30, open circle) muscle on Td. Each point represents the mean $\pm$ S.E.M. from 4–7 separate experiments. * $P < 0.05$: significantly different in comparison with C-30 (Student's t -test – unpaired data).

(Fig. 2A, C-30). Therefore, the observed frequency-dependent L-NAME potentiation of Td (Fig. 2A, L-NAME-30) is most likely due to blockade of NOS (i.e., the loss of the inhibitory effect of NO on muscle contraction).

Also, it was noticed that L-NAME significantly decreased Td in comparison with the corresponding control (L-NAME-30 vs C-30) at 14 Hz only (Fig. 2A). The observed effect of L-NAME is probably not due to inhibition of nNOS, because the enzyme is not fully activated at the lower stimulation frequency of 14 Hz (9). In addition, even if production of NO by nNOS was high enough, a potentiation of Td by L-NAME would be expected instead of a depression. The decrease in Td by L-NAME is probably a consequence of its nonspecific action, but the precise mechanism(s) of this depressant effect at 14 Hz is unknown so far.

In muscle pretreated with cumulative instead of a single concentration of AMPh, the relationship between stimulation frequency and muscle contractility is changed, that is, the significant linear increase of Td was observed in muscle incubated with Tyrode solution only (Fig. 2B, C-30). NO originated by muscle's nNOS was probably not involved in such effects because L-NAME (1 mM) has not significantly potentiated Td in comparison with the control values at 14, 20, and 30 Hz. In addition, there was no linear relationship between Td and frequency of stimulation in muscle treated with L-NAME (Fig. 2B, L-NAME-30). It is possible that under our in vitro experimental conditions (muscle pretreated with cumulative AMPh, frequency of 30 Hz; experimental design 2, Fig. 1), intracellular L-arginine content is insufficient for the increased activity of nNOS during long-lasting isometric muscle contraction. It was shown that when local L-arginine supply is low, NOS could produce ROS instead of NO (14). Also, it was reported that ROS potentiate the rat diaphragm contractility during submaximal electrical stimulation (6). Therefore, it could be presumed that the linear frequency-dependent increase in control Td values of the muscle pretreated with cumulative concentrations of AMPh was a consequence of an increased production of ROS by nNOS. It is likely that nNOS generates ROS more than NO at 30 Hz, due to local depletion of L-arginine. L-NAME blocks both NO synthesis and ROS formation (15) and difference between Td values at 30 Hz seen at Fig. 2B could be explained by L-NAME-induced inhibition of ROS formation by nNOS.

In conclusion, this study provides the very first evidence that frequency-related change of inhibitory action of nitric oxide on diaphragm contractility depends on the type of AMPh pretreatment. The NO-induced inhibition of muscle contractility is lost after pretreat-

ment with cumulative AMPh, but not with single AMPh when stimulation frequency is increased. The obtained results may be of a great clinical importance for the treatment of respiratory insufficiency.

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