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Effect of L-Arginine on Neuromuscular Transmission of the Chick Biventer Cervicis Muscle

S. Asadzadeh Vostakolaei

Abstract**—**In this study, the effect of L-arginine was examined at the neuromuscular junction of the chick biventer cervicis muscle. L-Arginine at 500 µg/ ml, decreased twitch response to electerical stimulation, and produced rightward shift of the dose- response curve for acetylcholine or carbachol. L-Arginine at 1000µg/ ml produced a strong shift to the right of the dose – response curve for acetylcholine or carbachol with a reduction in the efficacy. The inhibitory effect of L-arginine on the twitch response was blocked by caffeine (200µg/ ml). NO levels were also measured in the chick biventer cervicis muscle homogenates, using spectrophotometric method for the direct detection of NO, nitrite and nitrate. Total nitrite (nitrite + nitrate) was measured by a spectrophotometer at 540 nm after the conversion of nitrate to nitrite by copperized cadmium granules. NO levels were found to be significantly increased in concentrations 500 and 1000µg/ ml of L-arginine in comparison with the control group ($p<0.001$). These findings indicate a possible role of increased NO levels in the suppressive action of L-arginine on the twitch response. In addition, the results indicate that the post- junctional antagonistic action of L-arginine is probably the result of impaired sarcoplasmic reticulum (SR) Ca^{+2} releases.

Keywords**—**Chick, L-Arginine, Nitric Oxide, Skeletal muscle.

I. Introduction

L-ARGININE is the principal physiologic precursor of nitric oxide which plays a versatile role in physiology. Nitric oxide is a universal signalling molecule with a key role in an extremely diverse variety of physiological and patho-physiological processes [8]. Endogenous nitric oxide (NO) formation is catalyzed by a family of NO synthase (NOS) enzymes that directly produce NO from L-arginine and NADPH in a calmodulin-dependent reaction that stoichiometrically produces citrulline as a coproduct. Molecular analyses identify three genetic loci for NOS. The corresponding protein products have been named according to their original sites of identification. Hence, neuronal NOS (nNOS or type I) occurs at highest densities in brain; endothelial NOS (eNOS or type III) is prominent in endothelial cells; and inducible NOS (iNOS or type II) expression is dynamically up-regulated in cells following immunological stimulation [10]. Mature skeletal muscle fibres express the three major isoforms of NO synthase with a specific splice variant of the neuronal isoform being predominant [9]. Endogenous NO was originally identified as the endothelial derived factor responsible for smooth muscle relaxation. More recent studies have identified major functions for NO in the development and physiology of mammalian skeletal and cardiac muscle. Contraction of isolated muscle preparations can be affected by exogenous NO and by inhibitors of NO synthesis, and both impairment of endogenous NO supply and overproduction of NO are thought to be involved in some skeletal muscle disorders [6] NO facilitates fusion of cultured myoblasts and mediates retrograde synaptic signaling in myocyte neuronal co-cultures. In mature skeletal muscle, calcium influx associated with muscle depolarization is linked to NO formation which in turn stimulates guanylyl cyclase. This cascade modulates contractile force [13]. NO is formed in skeletal muscle by nNOS that is localized beneath the sarcolemma of fast twitch muscle fibers. Subcellular localization is mediated by association of nNOS with the dystrophin glycoprotein complex. Disruption of the dystrophin complex in Duchenne muscular dystrophy causes a displacement of nNOS from the sarcolemma to the cytosol. This aberrant localization of nNOS may contribute to disease progression in muscular dystrophy [10]. Under normal conditions, SR Ca^{2+} release by the ryanodine receptors is driven by a voltage-induced conformational change in the dihydropyridine receptors present in the t-tubule membrane. Whether or not NO production can modulate this process. Rreactive oxygen intermediates modulate skeletal muscle contraction, but little is known about the role of No. In this study, the effect of L-arginine was examined at the neuromuscular junction of the chick biventer cervicis.

S. Asadzadeh Vostakolaei, P.h.D. is with Animal Science Department, Member of faculty at Islamic Azad University, Gaemshahr Branch, Iran (phone: +98-(123)2275680; e-mail: Asadzadeh80@yahoo.com).

II. Materials and Methods

A. Organ-bath Experiments

Biventer cervices muscle preparations from chicks aged between 5 to 10 days were set up as described by Ginsborg and Warriner (1960), except that the physiological solution was Tyrode. The isolated chick biventer cervicis muscle was put in the organ bath. The organ bath had a vessel with volume of about 70 ml; it contains Tyrode solution aerated with oxygen and is kept at 37º C. The nerve supplying the

twitch-fibres was located in the tendon against which the electrodes were placed. It was stimulated, usually at a frequency of 0.1 HZ, duration of 0.5 msec. and voltage of 5 volt and twitch response was recorded by a polygraph apparatus after transfering through a transducer. Contractures of muscles were recorded isotonically on curvilinear chart paper. The resting tension on the muscle was 1 g, and the magnification of the lever was 8X. Log dose-response curves for acetylcholine and carbachol were constructed by increasing successive doses of agonists until the respective maximal responses were obtained. The agonists were added to the bath at 5 min intervals after complete relaxation and were allowed to remain in contact with the tissue for 30 sec before being washed out of the bath by overflow for 30 sec. Each position on the log dose-response curves represents the mean (±S.E.M.) of 4 separate experiments.

B. Performing the Assay

Homogenates of chick biventer cervicis muscles (1 g of tissue per 50 ml of 0.1 phosphate buffer, pH 7.4) were prepared and centrifuged at 1000 x g for 15 min at room temperature to remove cell and large particles. All subsequent incubations were performed in red polypropylene tubes to protect the light-sensitive nitrate reductase. Nitrate was stochiometrically reduced to nitrite by incubating sample aliquots (up to 150 µl) for 15 min at 37 ºC in the presence of 0.1 unit/ml nitrate reductase (from Aspergillus species; Boehringer Mannheim), 50 µM NADPH, and 5 µM FAD in a final volume of 160 µl. When nitrate reduction was complete, NADPH (up to 0.3 mM) was oxidized to avoid interference with the following nitrite determination. For this purpose, samples were incubated with 10 unit/ml lactate dehydrogenase (from rabbit muscle; Boehringer Mannheim) and 10 mM sodium pyruvate for 5 min at 37 ºC in a final volume of 170 µl. Total nitrite was then determined spectrophotometrically by using the Griess reaction (Griess 1864).

III. Results

L-Arginine at 500 µg/ ml, decreased twitch response to electerical stimulation, and produced rightward shift of the dose- response curve for acetylcholine or carbachol. L-Arginine at 1000µg/ ml produced a strong shift to the right of the dose – response curve for acetylcholine or carbachol with a reduction in the efficacy. The inhibitory effect of L-arginine on the twitch response was blocked by caffeine (200µg/ ml). These findings indicate a possible role of increased NO levels in the suppressive action of L-arginine on the twitch response. In addition, the results indicate that the post- junctional antagonistic action of L-arginine is probably the result of impaired sarcoplasmic reticulum (SR) Ca^{+2} releases.

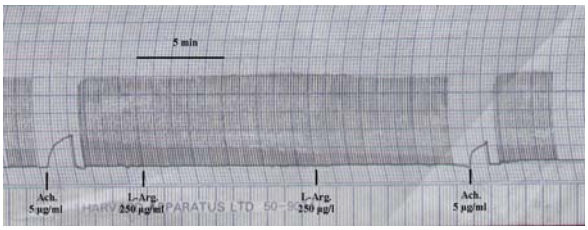

Fig. 1 Effects of L-Arginine (500µg/ml) & acetylcholine on twitch and contracture response

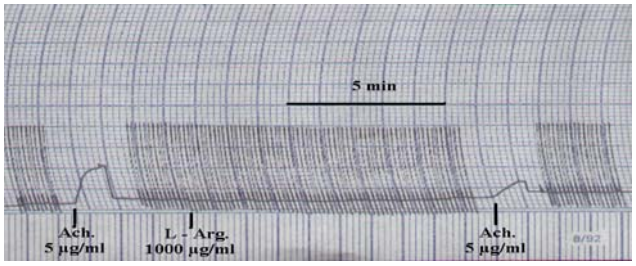

Fig. 2 Effects of L-Arginine (1000µg/ml) & acetylcholine on twitch and contracture response

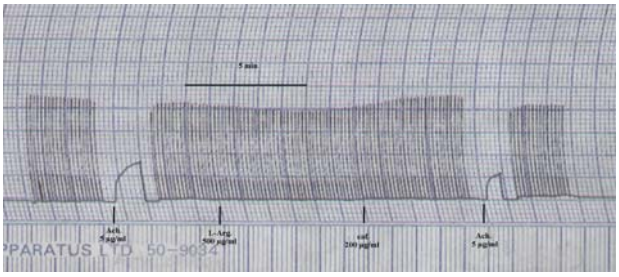

Fig. 3 Effects of caffeine on inhibitor response of L- Arginine

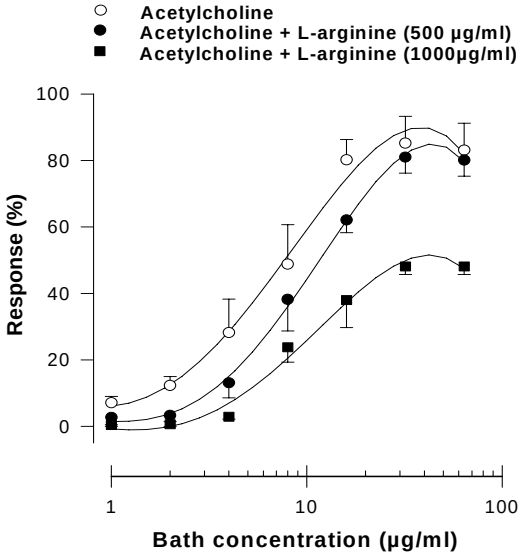

Fig. 4 Dose – Response curve of acetylcholine & l- arginine

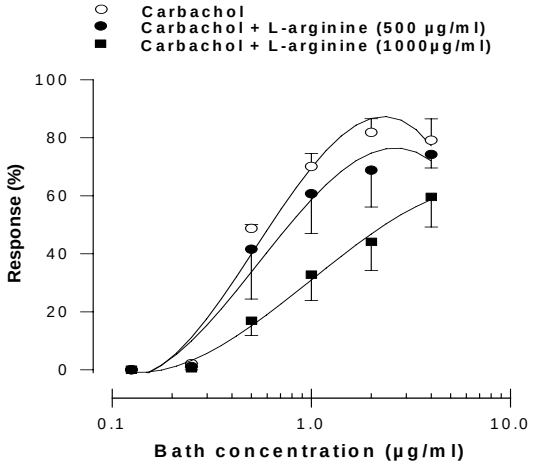

Fig. 5 Dose – Response curve of carbachol & l- arginine

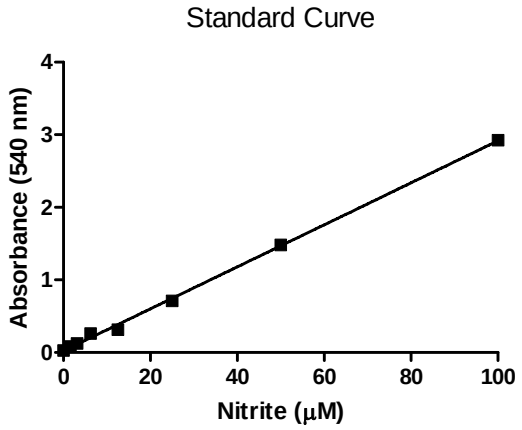

Fig. 6 Standard curve of nitrite

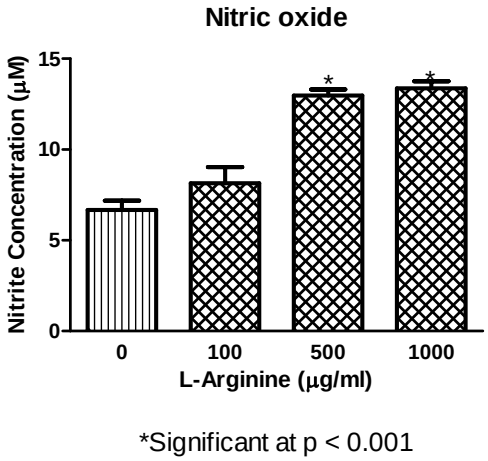

Fig. 7 Nitrite concentration at different concentration of l-arginine

IV. Discussion

There is accumulating evidence that NO participates in skeletal muscle in many aspects of excitation–contraction coupling, including receptor signal transduction, L-type Ca-channel activity, sarcoplasmic reticulum (SR) Ca2+ release through the ryanodine receptor, and mithocondrial respiration [3]. The NOS inhibitor L-NAME increases Ca2+ release activity in muscle homogeates, while SNAP, a NO donor, inhibits Ca2+ release from isolated SR vesicles [7]. It has, therefore, been claimed that NO depresses skeletal muscle contractions and hence, inhibits force output by altering excitation–contraction coupling. In addition, NO donors and cGMP depress contractile function, while NOS inhibition, extra-cellular NO chelation and guanylyl cyclase inhibition increase contractile functions [4]. In this way, muscular increase of NO would reduce physical performance. Different mechanisms have been proposed to explain the role carried out by NOS in skeletal muscle. NOS increased glucose transport in skeletal muscle enhancing muscular metabolism; in addition, NO regulated intracellular Ca2+ concentration modulating Ca2+ release from SR[2] .

Meszaros et al. showed that S-nitroso-N-acetyl-penicillamine, a NO donor, reduced caffeine-induced Ca2+ release in isolated skeletal muscle SR vesicles suggesting that caffeine could interfere with NO production [7]. Our results are consistent with these data and demonstrate that caffeine administration inhibits NOS expression and so would interfere with NO production inducing increased muscle contraction. Moreover, since NOS activity has been shown to inhibit mitochondrial respiration in skeletal muscle [5], we suppose that caffeine administration, by reducing NOS, could also improve the mitochondria metabolism. The normal muscle mainly expresses nNOS and eNOS, while iNOS plays a major role in immune responses, however their exact localization is still debated [10].

Previous work showed that eNOS levels are associated with oxidative muscles fibres and that eNOS co-localizes with mitochondria markers in a subset of fibres within numerous rat muscles suggesting a functional role in oxidative, mitochondria-rich fibres [5]. Those data had showed that eNOS was moderately and constitutionally expressed in muscle fibres of quadriceps, but was inhibited by caffeine administration. However, eNOS expression was the only NOS isoform that moderately increased in SSl 2 h after treatment. These findings would indicate the more important role of eNOS in muscular fibres physiology; eNOS increase could help blood perfusion of the muscle, thus improving its activity [5]

iNOS is a high-output, Ca2+-independent enzyme whose activity in skeletal muscles is variable. Reports on iNOS expression in muscle are controversial. Immunoblot and immunocytochemical analyses show no or minimally detectable iNOS immuno-staining in various skeletal muscles of rat [10]. While Thompson et al. showed that iNOS is induced in muscle under stress [11], Gath et al. found that iNOS is expressed in gastrocnemius of guinea pig not stimulated in type I fibres only[2]. NO produced by iNOS modulates contractile response: an enhanced expression of iNOS decreases whereas inhibition of NOS activity increases contractile force [2]. Waring et al, in rat quadriceps and gastrocnemius muscles, demonstrated that chronic inhibition of NOS with L-NAME was associated with decreased muscle mass, in muscle fibre cross sectional areas. Moreover, walking

speed appeared to decrease from day 11 to 22 after L-NAME administration [12]. In our experiments, NOS-inhibition was induced by single acute caffeine-administration but its effect was short and so we did not observe any muscular loss. NO levels were also measured in the chick biventer cervicis muscle homogenates, using spectrophotometric method for the direct detection of NO, nitrite and nitrate. Total nitrite (nitrite + nitrate) was measured by a spectrophotometer at 540 nm after the conversion of nitrate to nitrite by copperized cadmium granules. NO levels were found to be significantly increased in concentrations 500 and 1000µg/ ml of L- arginine in comparison with the control group ($p<0.001$). These findings indicate a possible role of increased NO levels in the suppressive action of L-arginine on the twitch response. In addition, the results indicate that the post-junctional antagonistic action of L-arginine is probably the result of impaired sarcoplasmic reticulum (SR) Ca^{+2} releases.

Our experiments showed that in skeletal muscles, caffeine-induced NOS inhibition was very fast and spontaneously reversible, so we suppose that caffeine administration may improve muscular respiration and so temporarily increasing muscle force. NO inhibition could be one of the mechanisms involved in the increase of athletic performance observed after caffeine ingestion and would partially explain the reduction of ratings during exercise in humans reported by Doherty and Smith [1].

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