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Effects of nitric oxide on the contraction of skeletal muscle

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Abstract. A review of the literature suggests that the effects of nitric oxide (NO) on skeletal muscles fibers can be classified in two groups. In the first, the effects of NO are direct, due to nitrosation or metal nitrosylation of target proteins: depression of isometric force, shortening velocity of loaded or unloaded contractions, glycolysis and mitochondrial respiration. The effect on calcium release channels varies, being inhibitory at low and stimulatory at high NO concentrations. The general consequence of the direct effects of NO is to 'brake' the contraction and its associated metabolism. In the second group, the effects of NO are mediated by cGMP: increase of the shortening velocity of loaded or unloaded contractions, maximal mechanical power, initial rate of force development, frequency of tetanic fusion, glucose uptake, glycolysis and mitochondrial respiration; decreases of half relaxation time of tetanus and twitch, twitch time-to-peak, force maintained during unfused tetanus and of stimulus-associated calcium release. There is negligible effect on maximal force of isometric twitch and tetanus. The general consequence of cGMP-mediated effects of NO is to improve mechanical and metabolic muscle power, similar to a transformation of slow-twitch to fast-twitch muscle, an effect that we may summarize as a 'slow-to-fast' shift.

Key words. Nitric oxide; skeletal muscle; tetanus; force-velocity; glycolysis; respiration.

This review reports and discusses the effects of nitric oxide (NO) on the mechanical properties of skeletal muscle and the associated energy transfer controlled by glycolysis and respiration. Other aspects of NO pathophysiology in skeletal muscles have been reviewed or described elsewhere: roles of NO [1], regulation of skeletal muscle blood flow [2–4], interactions with insulin and glucose uptake [5–7], ischemia and reperfusion [8–11], heart failure [12].

Sources of NO in skeletal muscle

NO is synthesized from L-arginine by NO synthase (NOS, E.C 1.14.23) in a Ca^{2+}/calmodulin-dependent manner. The enzyme exists in multiple isoforms, some of which have been purified and characterized: type I from brain, or nNOS; type II from macrophages, or iNOS; type III from endothelial cells, or eNOS [13]. In a study on human brain NOS, unexpectedly, more messenger RNA of nNOS was found in human skeletal muscle than in human brain. Measurements of NOS activity in human muscle homogenates confirmed a high level of nNOS expression in muscle [14]. The presence of NOS was confirmed in rat skeletal muscle [15] and in many different muscles of various animals [16, 17]. Skeletal muscle is one, if not the only, of the few tissues able to express simultaneously all the NOS isozymes.

Normal muscle expresses two NOS isoforms: the neuronal-type nNOS and the endothelial-type, eNOS. There is some uncertainty as to their exact distribution. In the first works on the question [15, 18], immunolabelling demonstrated the presence of nNOS in the sarcolemma of type II muscle fibers [rat extensor digitorum longus (EDL)] and weak expression of eNOS in the cytoplasm of all fibers (rat diaphragm), a distribution confirmed later [19]. In another work [17], nNOS was observed in type I and type II fibers. Neuronal nNOS associates with the dystrophin-α_1-syntrophin complex, which anchors it to the membrane. In dystrophin-deficient *mdx* mice, nNOS was absent from the sarcolemma

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and remained in the sarcoplasm [20–23]. Further varieties of nNOS arise by alternative splicing: the 'μ' isoform, with a 102-bp insert between exons 16 and 17 [24], and the 'penile' isoform, perhaps identical to the μ isoform, with a 102-bp insert transcribed from intron 16 [25]. The endothelial isoform eNOS is more abundant in mitochondria-rich fibers, type I and type IIA, than in type IIB [18]. It is possible that eNOS, or a fraction of it, is attached to mitochondrial membranes [18, 26]. Respiratory muscles display a different pattern of distribution [16]. Neuronal NOS was present in canine diaphragm, but very weak expression was detected in rabbit, rat, and mouse diaphragm; eNOS was detected in rat and mouse diaphragm, but not in canine and rabbit diaphragm. Human muscles are also different in this respect [27]. They expressed two different constitutive isoforms in different cellular compartments. Type I NOS (nNOS) was localized in the sarcolemma and in the cytoplasm of all muscle fibers, but more strongly in type I fibers; type III NOS (eNOS) was observed both in the endothelium of larger vessels and of microvessels. The third isoform, type II or iNOS, plays a major role in immune responses to bacteria and tumors, and iNOS gene transcription is induced by endotoxin and cytokines. Northern blot analyses demonstrated a 4.2-kb iNOS mRNA in skeletal muscle [28]. Anti-NOS II antibody labelled type I muscle fibers [29]. Muscle extracts, total [30] or particulate [29], had some Ca^{2+}-independent NOS activity, an indication of the presence of iNOS. Inducible iNOS is strongly upregulated in skeletal muscle challenged by endotoxin [30].

Little is known about the mechanisms which control NOS expression in skeletal muscle. Type I NOS is upregulated by mechanical loading [31], treadmill training [32], chronic electrical stimulation [33], and in aging rats [34]. It decreases after denervation, but returns back to a normal level after reinnervation [35].

Rat skeletal muscle incubated in vitro at 33 °C produced NO at a rate of 1 pmol min^{-1} mg^{-1} (muscle wet weight). Electrical stimulation increased NO production and blockade of NOS by an analog of arginine ω-monomethyl-L-arginine monoacetate (NMMA) decreased it [36]. Other workers have reported similar rates for NO production in vitro [15, 30, 37].

Effects of NO on muscle mechanics

Isometric contraction

Maximal force of fused tetani. In the experiments reported in this section, skeletal muscles were isolated from an animal, maintained at constant length (isometric) in an oxygenated physiological solution and stimulated with a train of electrical stimuli at a frequency sufficiently high to produce a well-fused isometric tetanus (fig. 1A). Many workers reported that the maximal isometric force, F_o, developed by isolated or blood-perfused skeletal muscles during fused tetani remained unchanged after blockade of NOS or after incubation in the presence of NO donors (fig. 1A) [15, 38–44]. However, in chemically skinned single fibers activated with ATP and 0.1 mM Ca^{2+}, F_o was reduced by 15–35% with 0.1 mM S-nitroso-N-acetylpenicillamine (SNAP) or 5 mM sodium nitroprusside (SNP), but was not changed at the lower dose of 0.5 mM SNP [45, 46]. The effect of SNP was reversed by 10 mM dithiothreitol, a treatment which reduces the contractile proteins oxidized by SNP (see 'mechanism of NO action on muscle mechanics' below).

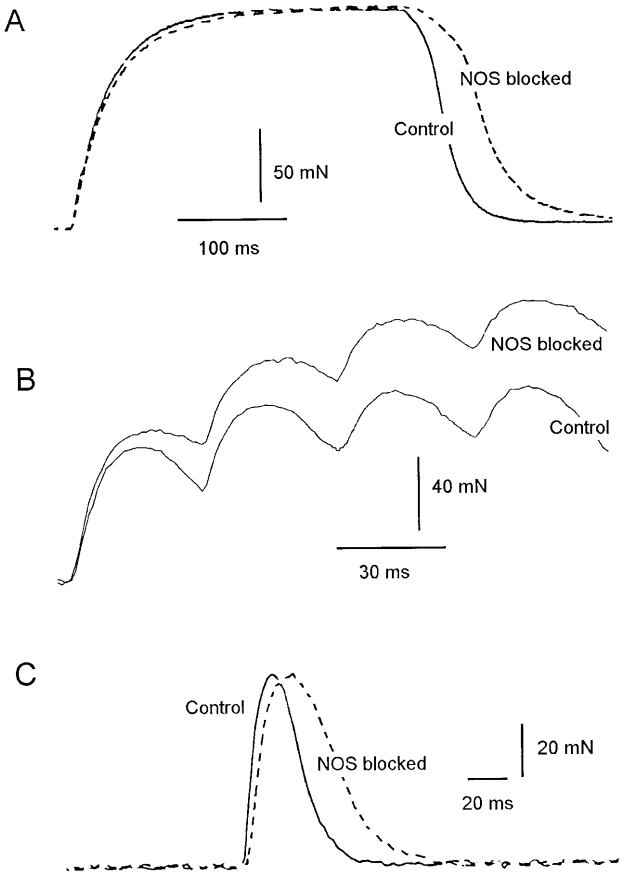

Figure 1. Effects of blockade of NO production on the isometric contractions of mouse EDL muscle (20 °C). The muscle was stimulated in vitro in normal Krebs solution and 30 min after addition of 10 mM ω-nitro-L-arginine (NLA), a NOS blocker (reprinted with permission from Maréchal G. and Beckers-Bleukx G. (1998) Effect of nitric oxide on the maximal velocity of shortening of a mouse skeletal muscle. Pflügers Arch. **436**: 906–913, © 1999 Springer Verlag, Heidelberg). (*A*) Fused isometric tetanus (stimulation frequency: 125 Hz, force measured in milliNewton: mN). (*B*) Unfused isometric tetanus (stimulation frequency: 33 Hz). (*C*) Single twitch.

NOS blockade decreased the initial rate of force development by 14% in the first 25 ms of the tetanus and markedly delayed the relaxation (fig. 1A) [39].

The fatigue effects of repeating maximal tetanic stimulation are complex, because they depend on the type of preparation and the pattern of the stimulation [42]. In mouse soleus, a slow-twitch muscle, and in EDL, a fast-twitch muscle, NO donors slowed the rate of fatigue of the force produced during a series of tetani [47, 48]. On the other hand, in blood-perfused dog gastrocnemius, NO donors had no effect on F_o when the tetani occurred at a rate of 12 contractions min^{-1}, but depressed it when the rate of contractions was increased to 40 contractions min^{-1} [42].

F_o was affected by septic shock, induced in rats by intraperitoneal (i.p.) injection of an endotoxin (*Escherichia coli* lipopolysaccharides, LPS) at a dose which elicited a moderate degree of shock (30% mortality rate over a 24-h period) [30, 49]. The injection caused transient expression of iNOS, which peaked 12 h later, and disappeared within 24 h. Muscle expression of endothelial and neuronal isoforms rose significantly within 12 h and remained above the control values 24 h after LPS injection. F_o of the diaphragm muscle was depressed by 59% and by 30%, 6 and 24 h after LPS injection, respectively; since the muscle content of nitrotyrosine (a peroxynitrite 'footprint') was increased, it was concluded that the force weakness was due to the increased formation of NO. In another series of experiments [50], a very mild peritonitis was induced in rats by i.p. injection of oyster glycogen. The animals appeared to be normal, but increased leukocyte counts in peritoneal fluid demonstrated the existence of peritonitis. There was an increase in inducible NOS, immunohistologically localized in the vascular compartment. No immunolabel was observed within the muscle fibers. F_o was depressed by 10%. The mechanism of muscle weakness appears to be similar to that observed in endotoxemia [30, 49]. iNOS is not calcium regulated; therefore, synthesis of NO by the enzyme is limited only by substrate availability, when cofactors of the enzyme are present. A high rate of unregulated production would favor the formation of highly reactive NO derivatives—peroxynitrite, peroxynitrous acid—exposing the muscle to nitrosative injury [51].

Isometric twitch. All the experiments reported in this section were performed on muscles isolated in vitro and stimulated at constant length by one electrical pulse (isometric twitch, fig. 1C). In mouse muscle—fast-twitch (EDL) [39] and slow-twitch (soleus), [unpublished observations]—, muscle blockade of NOS had little effect on the maximal isometric force (fig. 1C); in rat diaphragm, there was no effect of two NOS inhibitors (ω-nitro-L-arginine, NLA; 7-nitroindazole NIZ), the third (aminoguanidine hemisulfate, AMG) weakly increased the twitch force [37]. In another review [1] it was stated that 'isometric forces produced during the twitch were increased by NOS blockade or by NO scavengers. NO donors reversed the effect.' Unfortunately, no data were given to support the statement, and the paper referred to [15] gave no information on twitches, but only on 'sub-maximal contractions'; this term probably meant unfused tetani. SNAP did not influence the isometric force of soleus muscle stimulated at 2 or 4 contractions min^{-1} [42].

Twitch time to peak (the time necessary for a muscle to reach the peak of twitch contraction) was increased by NOS blockade in mouse EDL (fig. 1C); in rat diaphragm [37], only NIZ was active among the three inhibitors tested. The twitch time of half-relaxation was markedly increased by NOS blockade in a fast-twitch muscle, mouse EDL (fig. 1C), but was not influenced in a mixed muscle, rat diaphragm [37]. In three experiments, rat diaphragm muscles were challenged by altered NOS activity in the whole body. In the first [50], rats were injected with LPS. In the other two [52, 53], LPS-treated rats were injected with a NOS blocker, ω-nitro-L-arginine methyl ester (NAME). Except for a moderate increase in the twitch time of half-relaxation of some LPS-treated rats, the parameters of the twitch were unchanged.

Unfused isometric tetanus. In this type of experiment, muscles are stimulated by supra-maximal electrical shocks at such a low frequency that isometric force oscillates at the frequency of stimulation (unfused tetanus, fig. 1B). The mean value F_u increases as a function of the frequency of stimulation up to a maximal frequency at which the force does not oscillate and is maximal F_o.

1. *In vitro isolated muscles*. In rat diaphragm the force-frequency relationship was shifted leftwards by NOS blockade [15]. Thus, F_u increased after inhibition of NO synthesis (fig. 1B). In contrast, NO donors reversed the inhibition caused by NOS blockade; used alone, SNP, but not S-nitroso-N-acetylcysteine (SNAC), depressed the unfused tetanic force F_u [15]. Two other works reported that SNP reversed the effect of NLA, although it had no effect when used alone [38, 54]. In experiments with single living fibers of mouse flexor brevis muscle, 0.25 mM SNAC did not influence F_u (fig. 1 in ref. [41]).
2. *In situ blood-perfused muscles*. The two phrenic nerves of pigs were maximally tetanized at various frequencies of stimulation (10–50 Hz) below the frequency of tetanic fusion [44]. The force of pig diaphragm was evaluated as the transdiaphragmatic pressure, P_{dia}, equal to the difference between esophageal and gastric pressures, when the diaphragm contracted under maximal stimulation of

its two phrenic nerves. Animals were first premedicated with indomethacin, a drug which blocks the cyclo-oxygenase pathway and the synthesis of prostaglandins. Bolus administration of NAME or intravenous (i.v.) infusion of SNP had no effect on the P_{dia}-frequency relationship. However, both drugs decreased P_{dia} after stimulation at 10 Hz for 10 s. The fatigue caused by NAME correlated with a decline in blood flow, while the depressing effect of SNP was attributed to a direct action of NO on skeletal muscle.

In experiments with dog gastrocnemius-plantaris muscles pump-perfused with blood in situ at constant pressure [42], SNAP did not change the isometric force developed at 4 Hz, but it significantly increased the rate of fatigue, the force decreasing by -12% when stimulation lasted 3 min, in comparison with -6% for the controls.

3. *Septic shock*. When NO synthesis was increased in diaphragm muscle by i.v. injection of LPS, the force of an unfused tetanus at 50 Hz decreased from 64 to 50% F_o [30]. The effect was already present 6 h after the injection. It was converted to a force increase by simultaneous injection of a NOS blocker [53].

Conclusions: effects of NO on isometric contractions. It has been claimed [1, 15, 37, 52] that NO depresses contractions of skeletal muscle. This conclusion is far too simple to account for all the observations of the works reviewed here. It is likely that the effect of NO may be dependent upon contraction pattern and frequency [42]. It seems to us that another possibility may be interesting to consider. It appears that there is consensus on the effect of NOS blockade on F_o, F_u and the parameters of the twitch. There is more disagreement as to the effects of NO donors. We suggest that in normal muscle there is a given production of NO, which adjusts the contraction parameters to the control level. Let us assume that this level is close to the maximal physiological level. The effect of NOS blockade would consistently lower the local production of NO and, accordingly, would reduce its physiological effects. NO donors increase the existing level of NO. Since the physiological level is, by hypothesis, already close to maximal, NO donors would have small effects; however, they would readily reverse the effects of NO blockade. Cytotoxic levels could be quite close to physiologic ones. It is clear that the high level of NO induced by NOS upregulation in septic shock inflicts damage to the skeletal muscle. It is less clear whether the depressant effects of NO donors on the contraction of skinned fibers should be considered 'physiological' or 'cytotoxic', since NO donors do not depress the maximum tetanic force of intact muscle fibres. The effects of contraction patterns and frequency may in this view change the physiological intracellular level of NO by modulating the activity of NOS.

Shortening

Maximal velocity of shortening. Muscle shortens at maximal velocity under zero load (no external mass to displace). Two methods are currently used to estimate this parameter. The first [55] involves releasing a muscle during a well-fused tetanus at a velocity much higher than the maximal velocity of shortening. The force falls abruptly to zero and rises again after a short delay, needed by the muscle to take up the slack. The maximal velocity V_o is the ratio of the extent of shortening divided by the delay (fig. 2). If the preparation contains several fibers whose V_o are different, as may be the case in bundles or whole muscle, V_o is an estimate pertaining to the fastest fibers of the group [56]. NOS blockade by 10 mM NLA did not change V_o of rat diaphragm [38, 54], but reduced it by -22% (fig. 2A, [39, 57]) to -27% in mouse EDL and soleus [58]. The effect on mouse muscle was reversed by wash out of the drug [39] or by addition of 1 μM adrenalin [59]. NLA was inactive on frog tibialis anterior, but another NOS inhibitor, 1 mM NIZ, reduced V_o by -28% [58]. NO donors had an opposite effect [39]: in mouse EDL, they increased V_o by $+15\%$ (1 mM SNP) or $+8\%$ (1 μM SNAP) and they reversed the inhibition caused by NOS blockade. In contrast to the results observed on living fibers, NO donors were either inactive (isolated fibers of mouse flexor brevis muscle exposed to 0.25 mM SNAC, [41]) or had an inverse effect, depressing V_o, -17% in skinned fibers from rabbit psoas muscle exposed to 1 mM SNP [45], and -35% in rat slow or fast muscle skinned fibers exposed to 100 μM SNAP or 1 mM SNP [46]. In this work [46], using a NO-sensitive electrode, it was established that the NO concentration produced by 0.1 mM SNAP or 1 mM SNP in the bath solution was 80 ± 47 (SD) nM ($n = 5$).

A second method to evaluate the maximal velocity of shortening is to compute it from the parameters of the Hill hyperbolic force-velocity relationship as the ratio of the velocity constant b divided by the force constant a/F_o [60]. We call this estimate V_{max}, to distinguish it from V_o. NOS blockade decreased V_{max} by -11% [54] to -16% [38] in rat diaphragm, -41% in mouse EDL (fig. 2C).

In rat diaphragm, NOS blockade depressed V_{max} but not V_o. One interpretation of this finding was that NO sensitivity differs among fibers: NO would optimize shortening velocity of slower fibers without influencing the fastest fibers [54]. In contrast, NOS blockade depressed both V_{max} and V_o, to approximately the same extent, in three quite different muscles, fast-twitch

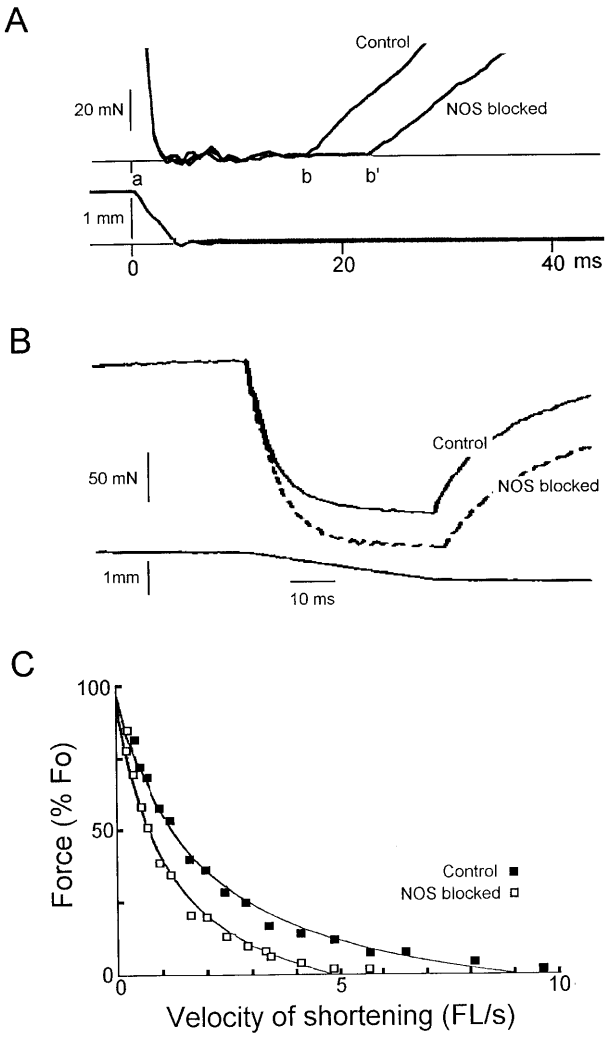

Figure 2. Effects of blockade of NO production on the shortening velocity during a fused tetanus of mouse EDL muscle (20 °C) in a normal Krebs solution or after addition of 10 mM NLA, a NOS blocker. (*A*) Unloaded shortening: note that the delay needed by the muscle to take up the slack after the fast release (time interval between a and b or b′) is increased by NOS blockade (reprinted with permission from Maréchal G. and Beckers-Bleukx G. (1998) Effect of nitric oxide on the maximal velocity of shortening of a mouse skeletal muscle. Pflügers Arch. **436**: 906–913, © 1999 Springer Verlag, Heidelberg). (*B*) Force maintained during slow releases at a controlled shortening velocity of 27 mm s^{-1} (from ref. [57]). (*C*) Force-velocity relationship in (*B*).

mouse EDL, slow-twitch mouse soleus, and frog tibialis anterior muscle. These findings suggest that maximal velocity of shortening increases with the local NO concentration in all types of muscle fibers, from mammals to amphibians [39, 58].

Force-velocity relationship. NOS blockade by 10 mM NLA or 1 mM NIZ slows the velocity of shortening against a constant load in rat diaphragm muscle [38, 54], or decreases the force maintained during shortening at constant velocity (fig. 2B,C) in mouse EDL [61]. The data were fitted to Hill's hyperbolic force-velocity relationship. NOS blockade respected the general shape of the hyperbolic relationship but it modified its parameters (fig. 2C). In one study [61], it was found that NOS blockade increased the force constant a/F_o by $+17\%$, while in the other study [54], no effect on a/F_o was observed. In both studies, NOS blockade depressed the velocity constant b by -18% [61] and -17% (recalculated from the data in ref. [54]). Both studies also agreed that NOS blockade impairs maximal mechanical power by about 20%. Delivery of NO with 1 mM SNP did not change the force-velocity relationship, but it prevented the decrease in shortening velocity caused by NOS blockade with NLA [54].

Stretch-response. Tetanized muscle resists a stretch with a force larger than isometric. At high stretch rates, the mechanical energy spent on the muscle is degraded into heat, causing severe damage. At low stretch rates, a sizeable fraction of the work imposed on the muscle is absorbed and recovered as spared chemical energy (for a review see ref. [60]). Muscle is able to sustain many tetani with slow stretches without apparent mechanical or histological damage [62]. In contrast, dystrophin-deficient muscles are very fragile: EDL muscle from *mdx* mice, a dystrophin-deficient strain, suffered severe histological lesions proportional to the mechanical decay measured by the fall of isometric force in a series of tetani with stretches [62]. In these *mdx* muscles, not only was dystrophin absent from sarcolemma, but nNOS was absent as well [20–22]. Genetically 'repaired' *mdx* muscles reincorporated nNOS in their sarcolemma [63], and also markedly improved their resistance to stretch [64, 65]. It is believed that the dystrophin complex transmits force generated in the sarcomeres via the subsarcolemmal actin network through the sarcolemma to perimysium. It presumably protects and stabilizes the fragile sarcolemma against the strong shearing forces created during a tetanus. A recent series of experiments [31] may help to unravel the mechanism of the protection: rat muscle stretched by 20% in vitro increased NO production by 20%. This effect was dependent on the presence of extracellular calcium. These results indicate that stretch could allow calcium influx and activate sarcolemmal-bound nNOS. There is a potential for a mechanism which could stabilize the shearing forces along a muscle fiber. If a weak portion of a muscle fiber is stretched by stronger vicinal portions, the local calcium-activated NOS could induce a local compensatory increase in mechanical power. Inactivation of this mechanism in dystrophin NOS-deficient mice would permit shearing forces to locally tear the sarcolemma and cause muscle necrosis.

Mechanism of NO action on muscle mechanics

Excitation-contraction coupling. The effects of NOS blockade or NO donors on the force developed during unfused tetani, twitch time to peak and on the rate of tetanic or twitch relaxation strongly suggest that NO inhibits one or several steps of the processes which control the signal pathway between muscle action potential and calcium activation of actomyosin.

No effect of NO has been reported so far on muscle membrane potential either resting or active. However, it cannot be excluded, since it has been shown that NO hyperpolarizes vascular smooth muscle [66].

In skeletal muscle, there is a direct relationship between the extent of contraction and the amount of calcium released from the sarcoplasmic reticulum (SR) through the ryanodine receptor Ca^{2+}-release channels RyR1. The NOS inhibitor NAME increased Ca^{2+}-release activity in muscle homogenates and a NO donor, 10 μM SNAP, inhibited Ca^{2+} release from isolated SR vesicles [67, 68]: NO donated by SNAP caused a significant (-60%) decrease in the overall open probability of single RyR1 channels isolated in a lipid bilayer. However, in similar experiments reported by another group [69], 1 mM SNAP increased the open probability of RyR1 channels and induced a release of calcium from SR vesicles, an effect reversed by dithiothreitol. The contradiction is perhaps explained by the finding that the effect of NO is dose-dependent [70]. At low concentration, NO donors would prevent channel opening whereas at high concentration, they would favor it [70].

Free Ca^{2+} was measured in mouse single fiber with Indo-1, an intracellular fluorescent indicator of Ca^{2+} concentration [41]. NO donors, 0.25–1 mM SNAC or 1–5 mM SNP, increased Ca^{2+} transients during unfused tetani, without changing the oscillating force F_u: it may be that NO donors were used at doses high enough to activate calcium release, while other workers [15, 39, 45]) used lower doses of NO donors which would have inhibited the release of Ca^{2+}. The authors favor another interpretation [41]. Their results would indicate that NO donors have no effect on the passive Ca^{2+}-release leak from SR, which would be the equivalent to the open probability measured in isolated RyR1. The increase in tetanic $[Ca^{2+}]_i$ would be partially due to a small increase in voltage-activated Ca^{2+} release. In the same experiments, 0.25 mM SNAC shifted the force-$[Ca^{2+}]_i$ to the right: NO impairs Ca^{2+} activation of actin filaments. Since NO at the same time increased $[Ca^{2+}]_i$ during unfused tetani, force production would depend on a delicate balance between two opposing effects. The $[Ca^{2+}]_i$ of a fiber at rest was increased $+10\%$ by SNAC [41]. This was explained not by an increase in Ca^{2+} leak from the SR but by a -33% decrease of the activity of the sarcoplasmic calcium pump.

NO-cGMP pathway. NO is the most potent and effective activator of soluble guanylate cyclase (sGC). NO binds to the heme moiety of sGC and by dislocating the heme iron induces a conformational change in sGC. This either desinhibits or activates the catalytic site of sGC [71]. cGMP in turn can activate a large number of physiological processes in almost all cells [72]: cGMP-gated ion channels, inhibition or stimulation of cGMP-dependent AMP phosphodiesterases leading to increased or decreased response to cAMP, and phosphorylation of proteins, via cGMP-dependent kinases (e.g., protein kinase G, PKG). sGC was discovered in muscle over 20 years ago [73]. Immunostaining demonstrated the presence of cGMP in most fibers of rat diaphragm; its concentration in the whole muscle was 0.25 pmol mg^{-1} [74]. Resting EDL muscle of mouse contained less than one-tenth, 2 fmol mg^{-1} [75]; when stimulated (fairly long unfused tetanus of 30 s at 30 Hz, 30 °C), cGMP increased to 7 fmol mg^{-1}. An intermediate value, 3.5 fmol mg^{-1}, was observed in mouse tibialis anterior stimulated less vigorously (four fused tetani of 0.5 s at 125 Hz, 20 °C [39]). Blockade of NOS by NLA had no effect on the cGMP content of a resting muscle, but it inhibited its stimulation-induced increase [75]. NLA or NIZ decreased by -27% or -12% the cGMP content of mouse EDL tetanized for 2 s [39]. NO donors, SNP or SNAC, doubled the content of cGMP [74, 75]; simultaneous treatment with NLA did not inhibit this effect [75]. In high concentrations of SNP (10–15 mM), cGMP increased 10- to 80-fold [4, 76]. All these experiments point to the same conclusion: NOS is activated during muscle contraction and the resulting NO activates sGC, which in turn increases the level of cGMP.

Pharmacological probes have been used to decide whether cGMP acts as a second messenger between NO and muscle contraction. Blockade of cGC by LY83583 (6-anilino-5,8-quinolinequinone) potentiated the NLA-induced increase of F_u [15, 74], the force maintained during an unfused tetanus, and depressed by -18% V_o, the unloaded shortening velocity [39]. LY83583 also increased the calcium released during stimulation of living single fibers [41]. An increase in cGMP has been obtained by inhibition of phosphodiesterases (dipyridamole, 3-isobutyl-1-methylxanthine) or by a cell-permeable analog of cGMP, 8-bromo-cGMP: the treatments reversed the NLA-induced increase of F_u [15, 74] and increased V_o by $+8\%$ to $+17\%$ [39]. None of the pharmacological probes affected F_o, the maximal force of fused tetanus. Thus blockade of sGC exerts effects similar to blockade of NOS whereas increase of cGMP imitates the effects of NO donors.

Direct effects of NO. Some physiological effects of NO do not seem to be mediated by cGMP, but have been ascribed to direct nitrosation or oxidation of sulfhydryl

bonds by NO (the biochemistry of NO is reviewed in refs [77, 78]). In skinned, Triton-treated muscles, mitochondrial and SR membranes are destroyed, and the sarcoplasmic fluid is washed away with its content of soluble metabolites and enzymes. cGMP, cGC, and NOS are presumably eliminated. NO donors depressed isometric force [45, 46], actomyosin ATPase [45, 46], and the velocity of shortening [45]. The authors postulated nitrosation of actomyosin to account for the effects of NO. Since NO donors did not depress the isometric force F_o of living muscle, and accelerated the velocity of shortening V_o (see above), we are of the opinion that, in living muscle, actomyosin is protected from deleterious contact with NO, perhaps because the concentration of NO needed to activate sGC is far lower than that needed to nitrosate actomyosin, or because NO is destroyed so fast that it does not exist long enough to diffuse and reach the contractile proteins, or because NO is counteracted by cellular antioxidants. There is some support for the latter view, since dithiothreitol reversed NO effects on F_o and actomyosin ATP-ase [45], on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity [79] (see below) and calcium release from SR vesicles [69].

NO directly affects the SR calcium release machinery, an effect reversed by reducing agents, supporting the notion that NO nitrosates or/and oxidizes critical regulatory thiol groups of RyR1 [67, 69, 70]. Oxidation of regulatory sulfhydryls was detected by the doubling of molecular weight due to cross-linkage of RyR1 subunits by resulting disulfide bonds. At high dosage, NO cross-linked the subunits and activated the calcium channels, while at low dosage, NO prevented cross-linking and activation by diamide, a disulfide-inducing agent. These findings [70] help to explain the contradictory effects of NO on the activity of isolated calcium-release channels [67–70]. Their impact on the observations made on living muscle is still unclear.

The NO-induced acceleration of relaxation (fig. 1) [15] could conceivably be due to an acceleration of SR calcium-ATPase. There is no support for this view. NO had no effect on calcium-ATPase [69, 80] as long as the ATP concentration was higher than 2 mM. Since soluble [80–82] and SR-bound [80] creatine kinase was inhibited by nitrosation of critical thiol groups, circumstances could arise in which NO could slow relaxation by inhibition of the SR Ca-ATPase, as for instance in extreme fatigue, when nearly all creatine phosphate is hydrolyzed and ATP content is lowered under 2 mM.

Interplay with reactive oxygen intermediates. Production of NO increased during muscle contraction, but there was an even greater production of reactive oxygen intermediates, superoxide and other oxygen free radicals [15]. It is well known that NO binds to superoxide, thus inactivating it. In this way, NO may prevent oxidation of critical regulatory thiols [15, 70]. On the other hand, the combination of NO and superoxide can form peroxynitrite, a very reactive compound. Nitrotyrosine accumulates with age in an isoform of the Ca-ATPase SERCA2a [83] or in the muscle proteins during septic shock, when NOS isoforms are strongly upregulated [30]. One can speculate that NO modulates the effects of oxygen free radicals formed during muscle contraction, though whether NO diminishes or enhances these effects is unclear.

Effects of NO on muscle metabolism

Glucose uptake

Glucose uptake and utilization in skeletal muscle appears to be controlled essentially by insulin stimulation and contraction (or hypoxia) [84]. Different observations suggest that insulin and contraction stimuli exert their action through (at least partially) distinct pathways [85]. Maximal insulin stimulation and contraction have additive effects on glucose uptake [86]; wortmannin, a phosphatidylinositol 3-kinase inhibitor, blocks the insulin-induced uptake of glucose but has no effect on the contraction pathway [76, 87]. Conversely, the NOS inhibitor, NAME, inhibits the contraction-induced uptake of glucose and the translocation of glucose transporters (GLUT-4) to the plasma membrane [88]; insulin and contraction induce translocation of different specific pools of glucose transporters (GLUT-4) to plasma and T-tubule membranes [89, 90]. Both insulin and contraction have a direct effect on skeletal muscle fibers and an indirect effect, mediated by vasodilation of muscle vessels.

Role of muscle blood flow in glucose uptake

1. *Role of insulin-induced vasodilation.* Physiological concentrations of insulin increased blood flow to skeletal muscle, and hence, insulin and glucose delivery [91]. During euglycemic hyperinsulinemia of healthy volunteers [92, 93], leg blood flow (LBF) increased about twofold whereas leg vascular resistance (LVR) decreased consistently. The increase of muscle perfusion could account for 30% of the increase in glucose uptake induced by insulin ([94]; reviewed in ref. [6]). Thus, insulin-mediated vasodilation augments the delivery of glucose and insulin and therefore increases the direct effect of insulin on skeletal muscle fiber (detailed below). This mechanism probably also involves capillary recruitment and redistribution of flow through nutritive vessels at the expense of non-nutritive ones [6]. As NO modulates the tone of skeletal muscle resistance ves-

sels [95–97], its role in the control of basal and insulin-stimulated blood flow in humans was tested by infusion of NMMA into the femoral artery: basal LBF decreased by about 20%. Arterial infusion of NMMA caused a 50% fall in basal blood flow, and attenuated the dilator response of acetylcholine (an endothelium-dependent vasodilator) but not that to glyceryl trinitrate, showing that endothelium-derived NO contributes to basal and stimulated regional blood flow in humans [98]. Infusion of NMMA also completely abolished the euglycemic hyperinsulinemia-induced increase of LBF and decrease of LVR, suggesting that insulin-mediated vasodilation is NO dependent [93]. In a further study [99] on arterioles isolated from rat cremaster muscle, insulin significantly dilated arteries maintained at constant pressure; endothelium removal or incubation with NLA abolished this response, demonstrating that the effect of insulin is endothelium dependent and mediated by NO (reviewed in ref. [100]). In humans, induced acute hyperinsulinemia caused a significant increase in urinary excretion of NO_2^-/NO_3^- together with a significant decrease in blood pressure, again supporting the concept that NO may mediate the vasodilatory action of insulin in humans [101].

2. *Role of exercise-induced vasodilation*. Exercise-induced hyperemia seems to be determined by metabolic vasodilation and increased vascular conductance via the muscle pump [2, 102]. The role of endothelium-derived NO in the vasodilation induced by exercise is more controversial [1, 103, 104]. Acute exercise is associated with an increase in the plasma catecholamine level that may stimulate endothelium α_2-receptors and induce the release of NO. The vasodilation produced might be compensated by a direct action of catecholamines on α-receptors on smooth muscle cells; however, this effect could be inhibited by metabolites diffusing from the contracting muscle [105]. Another potential stimulus for NO release during exercise is increased shear stress [106–108]. However, results reported so far are contradictory. On isolated muscles, some authors showed that NOS blockade inhibited the vasodilatory response to muscle contraction [109] whereas others showed that NOS inhibition reduced the blood flow at rest but not that in response to muscle contractions [110, 111]. Studies performed on human forearm blood flow present the same controversy [112, 113].

Isolated EDL muscle releases NO at rest; this release is increased during electrical stimulation and diminished by about 70% after NOS blockade [36]. Electrical stimulation of mouse EDL inhibited phosphorylation of the myosin regulatory light chain of smooth muscle and arteriolar dilation [75]. Therefore, it is likely that NO diffuses out of skeletal muscle fibers and mediates vasodilation.

Direct action of NO on skeletal muscle cell. Glucose transport was assessed by measuring the accumulation of a labelled analog (3-O-methyl-D-^{3}H-glucose or 2-deoxy-1,2-^{3}H-glucose) in isolated rat soleus, epitrochlearis and EDL muscles [4, 19, 32, 76, 114, 115]. SNP [4, 32, 76] but not [1,2,3,4-oxatriazolium, 5-amino-3-(3-chloro-2-methylphenyl)-chloride (GEA 5024)] [19] induced a large increase in glucose transport. Insulin also induced an uptake of glucose that, in contrast to the effect of SNP, was inhibited by wortmannin. The effect of NO donors on the insulin-induced uptake of glucose is controversial: 1–25 mM SNP [32, 76] potentiated the uptake induced by submaximal concentrations of insulin whereas 10–100 μM GEA5024 or 0.1–1 mM SNP had an opposite effect [19]. Insulin-induced uptake of glucose was not affected by NOS blockade [4, 19, 32, 115], an indication that NO and insulin exert their effects through different pathways.

To assess the possible role of NO in mediating the exercise-induced uptake of glucose, isolated rat epitrochlearis muscle was stimulated electrically (2×10 min separated by a 1-min rest, 200-ms trains of 100 Hz, two trains s^{-1}). This protocol induced a 10 to 12-fold increase in glucose uptake that was inhibited neither by wortmannin nor by NMMA [4]. This contrasts with the observation that stimulation in situ (electrical stimulation of the sciatic nerve, 2×5 min separated by 1 min rest, one 500-ms train s^{-1} of 100 Hz) induced an increase in glucose uptake (measured in vitro on EDL isolated just after the exercise) that was almost completely abolished by NMMA [32].

Glucose transport in response to SNP, as in the response to insulin or exercise, seems mediated by translocation of glucose transporters GLUT-4 to the plasma membrane [4, 84, 90]. In vitro contraction of rat soleus stimulates translocation of GLUT-4 through a mechanism distinct from that of insulin [85]. Moreover, it appears that two pools of intracellular GLUT-4 respond specifically to insulin and contraction [89, 90].

Glycolysis

Glycolysis and glycogenolysis were studied by measuring net lactate release and ^{14}C-lactate release in the presence of ^{14}C-glucose [76]. Net lactate release, determined spectrophotometrically, represented the lactate generated potentially from both intracellular glycogen and extracellular glucose, whereas the ^{14}C-lactate release derived only from extracellular ^{14}C-glucose. In isolated soleus muscle, 1–25 mM SNP significantly stimulated the rates of both net lactate and ^{14}C-lactate release. At 25 mM, there was a marked difference between the two parameters, an indication that high

concentrations of SNP stimulate glycogenolysis. The effect of SNP on the rate of lactate release was observed in the presence or absence of insulin. The release of ^{14}C-lactate was directly proportional to glucose transport [116]; together, these results corroborate an independent action of NO and insulin on glucose transport [4, 19, 32, 115].

In contrast, NO inhibited the activity of GAPDH by S-nitrosylation of cysteine 149, NADH attachment, and/or ADP-ribosylation [79, 117]. The same opposite effects were observed in macrophages [118]. Addition of SNAP to the macrophage cultures increased glycolysis, except at the highest dose tested, 1 mM. Only at high concentrations of SNAP (above 500 μM) was the GAPDH activity inhibited, explaining the absence of effect of 1 mM SNAP on glycolysis. Thus, it seems that a modest inhibition of GAPDH by a low concentration of NO is not rate limiting for glycolysis which is therefore accelerated by low concentrations of NO.

Mechanisms of NO action on glucose uptake and glycolysis

Incubation of epitrochlearis or soleus muscles with NO donors (10–15 mM SNP) increased the level of cGMP by 10- to 80-fold [4, 76] and this level remained elevated for 60 min [76]. This was associated with increased glucose uptake and utilization [4, 76]. Contraction clearly induced a production of NO [35] and a small increase in cGMP content in the muscle that was diminished by NOS blockade [39, 75]. Incubation of soleus muscle with LY83583 or methylene blue inhibited the rise of cGMP caused by SNP and reduced both glucose transport and ^{14}C-lactate release [84]. Moreover, the cGMP analogs 8-bromo-cGMP and dibutyryl cGMP significantly increased the rates of glucose uptake and net lactate release in rat soleus, epitrochlearis, and diaphragm muscles [4, 76, 114]. Similar results were obtained by increasing the level of cytosolic cGMP with the cGMP phosphosdiesterase inhibitor, zaprinast [119]. Taken together, these results indicate that contraction-induced glucose uptake and lactate release is controlled by the NO/cGMP pathway. However, it is important to mention that the role of NO itself in exercise-induced uptake of glucose has been recently challenged [4] (see above) and that the effects of sGC inhibitors on the modifications of glucose metabolism induced by contraction have not been tested.

Oxygen consumption

Direct effects of NO. When NO synthesis was blocked by systemic injection of NLA in the resting dog, there was an elevation in total body oxygen consumption accompanied by an elevation in body temperature [120]. The contribution of skeletal muscles could be estimated in dogs whose paw was excluded from circulation and perfused by pumping blood into the femoral artery at a pressure near the auto-perfused level. Oxygen consumption increased by $+42\%$ after injection of a NOS blocker [121]. In dogs whose hindlimb was not excluded, O_2 consumption of hindlimb skeletal muscles increased by $+55\%$ [122, 123]. In contrast, NOS inhibition did not change O_2 consumption in rat diaphragm at rest but attenuated it during exercise [124, 125]. This paradoxical effect was attributed to a decrease in blood flow.

Tissue O_2 consumption was measured with a Clark-type electrode at 37 °C in isolated bundles of canine muscles, either the accessory head of biceps brachii, a predominantly slow-twitch muscle [122], or extensor carpi radialis, a predominantly fast-twitch muscle [123]. A NO donor SNAP decreased O_2 consumption by -12% (10^{-7} M SNAP) to -55% (10^{-3} M SNAP). The effects were similar in both slow- and fast-twitch muscles [123].

Mitochondria from rat skeletal muscle incubated with a NO donor, S-nitrosoglutathione, reversibly decreased oxygen utilization when using glutamate or succinate as substrate [126]. Analysis of the respiratory chain indicated an inhibition of cytochrome a_3, a component of the oxygen-binding site of cytochrome c oxidase. More prolonged exposure may involve permanent inhibition of two other complexes of the respiratory chain, complex I (NADH-CoQ reductase) and complex II (succinate-CoQ reductase) [127, 128]. Using synaptosomes [129], it was possible to evaluate the level of NO needed to reversibly inhibit respiration. Addition of 3 mM SNP in the dark did not liberate any NO, as measured with a sensitive NO electrode. When illuminated by a 150-W domestic light bulb, SNP liberated NO and increased its concentration to 100–200 nM in a few minutes. At the same time, respiration of synaptosomes was inhibited. At light extinction, the concentration of NO returned to zero in a few minutes, while respiration resumed its previous rate. Respiration was half-inhibited at 30 nM NO, when O_2 concentration was 30 μM, a physiological concentration in tissues. When the O_2 concentration was raised to 145 μM, a higher concentration of NO, 270 nM, was required to inhibit respiration. Thus NO raises the apparent K_m for oxygen. Since NO inhibition of respiration is completely reversible at concentrations known to be well within the range of many physiologically normal organs [129], NO could be the first physiological regulator recognized as acting directly on the mitochondrial respiratory chain [130]. Cytotoxic concentrations of NO which irreversibly damage respiration are about 1000-fold higher [131, 132]. In another series of experiments [82], rat soleus muscles were incu-

bated in 1 mM SNP, homogenized, and tested for activities of three enzymes of the tricarboxylic cycle. Maximal activity of aconitase was decreased by − 50%, but activities of citrate synthase and oxoglutarate synthase did not change. Lactate production by the muscle was increased by 50%. Presumably, the increase in glycolysis compensated for the decrease in oxidations.

NO acting on mitochondria may come from three sources. First, from endothelium of the muscle vessels, a well-known source of NO contributing to the control of vasomotion. In canine skeletal muscles, NO-dependent inhibition of respiration was elicited by activation of cholinergic muscarinic M_2 and bradykinin BK_2 receptors, which are thought to be localized on this cell type [122, 133]. Second, NO may be generated by NOS located in the muscle fibers in which the enzyme is very active and abundant. Third, NOS may be associated with mitochondria. Histochemical analyses of serial sections showed marked correlation of eNOS with succinate dehydrogenase, a mitochondrial enzyme [18]. Rat diaphragm mitochondria had an enriched calcium-dependent NOS activity relative to that measured in the total particulate fraction. Addition of arginine, a NOS substrate, inhibited mitochondrial respiration by − 50% at a concentration of 100 μM, a physiological concentration [18]. Silver-enhanced gold immunolabelling of eNOS was seen in 70–80% of mitochondria prepared from heart, skeletal muscle, and kidney [26].

The experiments reported above lead to the conclusion that NO inhibits O_2 consumption of skeletal muscle, in keeping with the well-established notion that NO inhibits mitochondrial respiration by inhibiting cytochrome c oxidase and aconitase (reviewed in ref. [133]). Inhibited O_2 consumption could be accounted for by a direct effect of NO on the mitochondrial enzymes.

NO/cGMP pathway. We come now to another series of experiments which leads to the exactly opposite conclusion, that NO stimulates respiration. Oxidation of ^{14}C-labelled substrates was measured by the release of labelled $^{14}CO_2$. Substrates were added to the incubation medium bathing isolated rat soleus muscles. Addition of 15 mM SNP increased oxidation rates of all the substrates tested: pyruvate by + 31%; palmitate by + 45%, leucine by + 36% [134]. Oxidation of glucose was even more stimulated: + 234% (23) and + 300% [76, 135], and was dependent on SNP concentration [76].

An explanation of this effect might be that NO stimulates respiration via the cGMP pathway. There is good support for this view. Blockade of sGC by LY83583 or methylene blue inhibited oxidation of palmitate induced by SNP in isolated rat soleus muscle [134]. Zaprinast increases cGMP in rat soleus muscle by inhibition of the enzyme which catalyses its destruction, cGMP phosphodiesterase. Zaprinast also stimulated oxidation of glucose, detected by the production of labelled $^{14}CO_2$ [119, 135]. Activities of a cGMP-dependent protein kinase, PKG, in rat soleus muscle increased with increasing doses of SNP: this effect was blocked by LY83583 [134]. A possible target for PKG is the mitochondrially located uncoupling protein-3, UCP-3, a protein able to uncouple respiration, and which is highly expressed in skeletal muscle [134, 136, 137].

However, there is a contradictory report: a cell-permeant analog of cGMP, 8-bromo-cGMP, decreased oxygen consumption of bundles isolated from dog muscles, in vitro [122].

Muscle efficiency. The most important function of muscle is to transform chemical energy released by respiration and glycolysis into useful mechanical work. Efficiency is the proportion of chemical energy effectively transformed. Does NO change muscle efficiency? The problem has not been studied in skeletal muscle, but it has been studied in heart [138]. Conscious dogs were instrumented to measure heart oxygen consumption at three running speeds for 5 min. Heart work was calculated from the pressure-volume loop area. Heart production of NO increased with running speed. When NO production was blocked with NLA, O_2 consumption increased at constant work load. This result could mean that NO improves heart efficiency. However, consumption of free fatty acids decreased, while there was little change in the utilization of glucose and lactate. Changes in O_2 and substrate consumption moved in opposite directions: in heart, NO inhibits respiration and increases substrate oxidation. It is even more surprising to recall that we described here a similar situation in skeletal muscle: NO inhibits energy usage evaluated by O_2 consumption and increases substrate oxidation evaluated by $^{14}CO_2$ production; the former appears to be a direct effect of NO on mitochondria, the latter to be mediated by cGMP. Obviously, we need a new careful study of the effects of NO on the efficiency of skeletal muscle.

Conclusions

We have classified in table 1 the main effects of NO observed on the contraction of skeletal muscle in two series, according to the mechanism proposed to explain them. In the first series, the effect of NO is due to direct nitrosation or metal nitrosylation of target proteins. Direct effects inhibit contraction: they result in lower force, slower velocity of shortening, and depressed glycolysis and respiration. Production of NO appears to act as a 'brake', perhaps to protect muscle from fatigue or mechanical damage. In the second series, the mechanism is mediated by the cGMP pathway, but the eventual changes produced in the targets are unknown. The

Table 1. Effects of NO on the contraction of skeletal muscle.

Variable	Slow-to-fast shift mediated by cGMP/NO	Brake action: direct effect of NO
Fused tetanus		
F_o	no effect	↘
F_V	↗	ND
V_{max}	↗	ND
P_{max}	↗	ND
V_o	↗	↘
dF/dt_i	↗	↘
Tetanic *HRt*	↘	ND
Ca^{2+} *release*	↘	↘ or ↗
Unfused tetanus		
F_u	↘	ND
v_f	↗	ND
Isometric twitch		
t_p	↘	ND
Twitch *HRt*	↘	ND
Metabolism		
Glucose uptake	↗	ND
Glycolysis	↗	↘
Mitochondrial respiration	↗	↘

Variables: F_o: maximum isometric tetanic force; F_V: load lifted at constant velocity of shortening; V_{max}: Hill's maximum velocity of shortening; P_{max}: maximum tetanic power; V_o: maximum velocity of unloaded tetanus; dF/dt_i: initial rate of force increase during fused isometric tetanus; Tetanic *HRt*: time of half-relaxation for tetanus; F_u: force during unfused tetanus; v_f: frequency of tetanic fusion; t_p: twitch time to peak; twitch *HRt*: twitch time of half-relaxation; ND: not determined.

fundamental influence of NO is to enhance the velocity of shortening, perhaps by cGMP/PKG-mediated myosin phosphorylation, without change of the isometric force. In terms of cross-bridges, it could mean that the rate-limiting steps of the cross-bridge cycling are proportionally accelerated while the relative distribution of the various states of the cross-bridges is conserved. This increases mechanical power, fed by an increase in ATP breakdown and the higher metabolic requirements of the muscle, both glycolytic and oxidative. The increased velocity of shortening accounts, at least in part, for the shortened twitch time-to-peak and the increased rate of force development early in the fused tetanus. As a rule [139], more powerful fast muscle relaxes more rapidly than less powerful slower muscle. This physiological adaptation is also observed here: relaxation is accelerated in twitch and in fused tetanus. Shortened time-to-peak and accelerated relaxation combine to depress the force maintained during an unfused tetanus and increase the frequency of fusion. It is important to point out that in our view, inhibition of unfused contraction and enhancement of mechanical power are two effects of NO which far from being contradictory are complementary and physiologically meaningful.

The changes operated by NO/cGMP are similar to several of those observed in the transformation of a slow-twitch muscle into a fast-twitch muscle. Many factors are able to induce such a 'slow-to-fast' shift: e.g., chronic stimulation, training and hormones (reviewed in refs [140, 141]). In all cases reported up to now, transformation was due to a change in the expression of myosin isoforms and associated muscle proteins. Since myosin turnover is slow (half-time: 12 days), complete transformation requires several weeks. The NO/cGMP system may possibly be the first physiological factor able to induce reversibly a 'slow-to-fast'shift on a very short time scale, a few seconds or even less. Its physiological role would be the same as that proposed for the genetically controlled mechanism: to match the mechanical power produced by the contracting muscle to the mechanical power needed to move the load, in order to obtain the best possible efficiency of power transfer [39, 142].

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