

# The influence of nitric oxide on *in vivo* human skeletal muscle properties

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## ABSTRACT

We have investigated the action of exogenous nitric oxide (NO) on the strength and contractile properties of human skeletal muscle working *in vivo*. Maximum isometric voluntary contraction force (MVC) of the quadriceps was measured and superimposed electrical stimulation was used to estimate the level of activation and 'true maximum force' (TMF). Force–frequency relationships were determined to assess changes in contractile properties of the muscle. Subjects in the experimental group (E,  $n = 10$ ) were measured before and during two separate periods of treatment with different doses of glyceryl trinitrate, a NO donor, delivering 100 (GTN100) or 200 (GTN200)  $\mu\text{g h}^{-1}$  as a transdermal patch. A control group (C,  $n = 6$ ) was measured during two similar periods whilst taking an oral placebo. There was a significant increase in strength with GTN200 (MVC: +5.15%; TMF: +3.87%). There was no change in the strength of group C. There was a trend towards reduced forces at submaximal frequencies with GTN administration but the most notable change was a decline in twitch force (approximately 12%,  $P < 0.05$ ) with GTN100 treatment and this remained depressed throughout the study. No changes were seen in the contractile properties of the control group C. The present results show that GTN treatment increased maximum voluntary strength but decreased twitch tension. The time course and dose–response characteristics indicate that these are two separate actions of NO on human muscle working *in vivo*.

**Keywords** force–frequency relationship, glyceryl trinitrate (GTN), maximum voluntary contraction (MVC), nitric oxide (NO), skeletal muscle.

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The largest determinant of muscle strength is its size, specifically the physiological cross-sectional area (CSA), which accounts for about 50% of the variation in isometric strength between individuals (Chapman *et al.* 1984). The remainder of the inter-subject variation has been variously ascribed to morphological and neurological factors such as differences in fibre type composition, angle of fibre pennation or the level of motor unit activation (see Jones *et al.* 1989).

The size of a muscle changes relatively slowly but short-term fluctuations in strength have been observed. Gauthier *et al.* (1996, 1997) found a significant circadian rhythm of elbow flexor strength with an amplitude of 7–8%, which was attributed to variations in central nervous system command and the contractile state of the muscle. Other studies have measured changes during the menstrual cycle and the effect of female reproductive hormones on muscle function with equivocal results. Some investigators have found that the reproductive hormones have no influence on

muscle function (Greeves *et al.* 1997, Gur 1997) but there are other well-documented reports of fluctuations (~10%) in maximum voluntary force during the menstrual cycle (Phillips *et al.* 1996, Sarwar *et al.* 1996). These changes appear too rapid for the synthesis of contractile protein or other morphological changes and suggest that sex hormones may have some inotropic effect resulting in short-term fluctuations in strength.

Since its discovery as a physiologically active substance, nitric oxide (NO) has been found to play a key role in many bioregulatory systems in addition to the control of vascular tone (e.g. Moncada *et al.* 1991, Moncada & Higgs 1993). Nitric oxide has been found to mediate some of the vasodilatory effects of oestrogen on smooth muscle (Darkow *et al.* 1997, Huang *et al.* 1998) and there is evidence that oestrogen increases the expression of calcium-dependent nitric oxide synthase (NOS) in skeletal muscle (Weiner *et al.* 1994).

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The neuronal isoform of nitric oxide synthase (ncNOS) has been found in skeletal muscle cells localized to the sarcolemma (Kobzik *et al.* 1994, Grozdanovic *et al.* 1996). Other investigators have found ncNOS closely associated with mitochondria (Kobzik *et al.* 1995, Wakayama *et al.* 1997) and concentrated around the neuromuscular junction (Kusner & Kaminski 1996, Grozdanovic & Gossrau 1998). Endothelial NOS (ecNOS) appears to be distributed throughout the sarcoplasm of some fibres, irrespective of the fibre type.

There are conflicting reports of the action of NO on the strength and contractile properties of skeletal muscle. There have been recent reports of an increase in maximum isometric force (Reid *et al.* 1996), of no effect (Marechal & Beckers-Bleukx 1996, Albertini *et al.* 1997, Chen *et al.* 1998) or a decrease (Perkins *et al.* 1997, Galler *et al.* 1997). Morrison *et al.* (1996) found no change in isometric force but did find an increase in isokinetic force and power. Murrant & Barclay (1995) found an increase in the sustained force of intermittent tetani (50 Hz) with an *in vitro* preparation, although this was only significant at the slowest repetition rate.

In addition to any effect on maximum force, endogenous NO may modulate excitation–contraction coupling. Kobzik *et al.* (1994) found that the administration of a NOS inhibitor caused a left shift of the force–frequency curve and argued that NO may, therefore, inhibit (right shift) the force–frequency relationship. Such an action of NO is supported by the findings of Perkins *et al.* (1997) although Reid *et al.* (1996) found a left shift with increased forces at low frequencies of stimulation in response to NO donation.

To date, the role of NO in skeletal muscle has been investigated largely *in vitro* or in animal preparations. The purpose of the present study is to determine whether treatment with a NO donor could affect maximum isometric force and the contractile properties of human skeletal muscle *in vivo*. For this purpose, glyceryl trinitrate (GTN) was employed as it is a well-known organic NO donor that has been used for many years as a vasodilator in the treatment of angina pectoris (Feelisch 1991).

## MATERIALS AND METHODS

### Subjects

Eighteen volunteers, five female and 13 male, were recruited from amongst staff and students at the University of Birmingham. Four of the female subjects were oral contraceptive pill users. All subjects were active recreationally and nine had been involved in systematic strength training for over 6 months.

**Table 1** Physical characteristics of the subjects

| Group        | Male | Female | Age (years) | Height (cm) | Weight (kg) |
|--------------|------|--------|-------------|-------------|-------------|
| Experimental | 8    | 2      | 23 ± 3      | 176 ± 7     | 75 ± 8      |
| Control      | 3    | 3      | 25 ± 2      | 174 ± 8     | 74 ± 18     |

Values are mean ± SD.

Subjects were randomly assigned to either an experimental group (E) or a control group (C). Two male subjects withdrew from the study. Details of the subjects who completed the study are given in Table 1.

The subjects gave their informed consent, completed the standard health questionnaire and were screened for contra-indications to glyceryl trinitrate (GTN) (e.g. low blood pressure, migraine, etc.) before the start of the study. The study was approved by the local Ethics Committee.

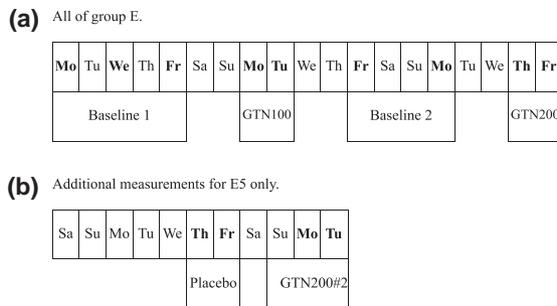
### Experimental protocol

Measurements of maximum quadriceps isometric force and the force–frequency relationship were made on both legs. Each subject was measured at the same time of day throughout the study and the measurements from both legs were averaged for each testing occasion. At the end of the study, subjects were given a questionnaire to rate the consistency of their lifestyle, level of motivation, perception of their strength and any side-effects.

Glyceryl trinitrate was administered via a transdermal patch (Nitro-Dur, Schering-Plough, Ireland) placed on the lateral aspect of the shoulder for 5 h and removed 1 h prior to the strength measurements. The patches released 100 or 200  $\mu\text{g h}^{-1}$  into the bloodstream, so that a total of 500  $\mu\text{g}$  (100GTN) or 1000  $\mu\text{g}$  (200GTN) of GTN was delivered over 5 h.

The side-effects of GTN administration are mild headache and light-headedness, but, consequently, a strict double blind design was not applicable. Subjects in group E were measured on nine occasions over a 3-week period. A diagram of the experimental protocol is shown in Fig. 1. The first three measurements were designated as baseline (B1). The next two measurements were taken on consecutive days after 100  $\mu\text{g h}^{-1}$  of GTN (100GTN) had been administered. Two more measurements were taken during a washout period (second baseline, B2) and finally, on two consecutive days at the end of the third week, two measurements were made after administration of 200  $\mu\text{g h}^{-1}$  of GTN (200GTN).

Five subjects in group E (designated E5) were assessed on four occasions over an additional 2-week period. A placebo (P) consisting of 0.5 g glucose in a single gelatine capsule, taken 6 h prior to measurement



**Figure 1** The experimental protocol for group E. Measurement days are denoted by bold. (a) The measurements for all of group E ( $n = 10$ ). (b) The additional measurements for half of group E ( $n = 5$ ).

was administered before the first two of these additional measurements. The third and fourth additional measurements were made with the subjects receiving  $200 \mu\text{g h}^{-1}$  GTN (200GTN#2).

Although there were no placebo patches, subjects in the experimental group E were told that there was a 50% chance of receiving a placebo patch during the 100 and 200GTN periods and subjects in groups E5 and C were told that the placebo capsule was another form of NO donor.

The control group (C) was measured three times a week (Monday, Wednesday and Friday) for 4 weeks. During the third and fourth weeks, these subjects took two separate batches of oral supplements everyday. Both batches of supplements were placebo ( $5 \text{ g day}^{-1}$  glucose in gelatin capsules). Each of the four weeks were treated as an independent testing period designated as baseline 1 (B1), baseline 2 (B2), placebo 1 (P1) and placebo 2 (P2), respectively.

### Materials

Quadriceps strength was measured using a calibrated U-shaped aluminium strain gauge (Jones & Parker 1989) with a linear response up to 1000 N. The strain gauge was interfaced with a computer via an amplifier so the subjects received direct visual feedback.

For electrical stimulation of the quadriceps two large aluminium electrodes ( $\sim 100 \text{ cm}^2$ ) covered in a damp paper were applied proximally and distally to the anterior surface of the thigh. A CED-1401 (Cambridge Electronic Design, England) triggered the electrical stimuli (pulse width  $50 \mu\text{s}$ , up to 200 V, Digitimer DS7, England). The strength of the stimulus was manipulated by changing the current, within a range of 190–380 mA for force–frequency testing and 280–500 mA at 1.25 Hz for twitch superimposition during the MVC.

### Strength testing

The maximum isometric voluntary strength of each quadriceps was measured in a conventional strength-testing chair (Parker *et al.* 1990) at 1.57 rads ( $90^\circ$ ) of flexion. Subjects performed four voluntary maximal knee extensions of at least 2-s duration separated by a minimum of 30 s rest. Subjects received loud verbal encouragement throughout all maximum efforts. The mean of the highest values from each leg was taken as the force of MVC.

A percutaneous twitch superimposition technique (Rutherford *et al.* 1986) was used on the third and fourth maximum contractions of each leg with the subjects in group E. The quadriceps were stimulated just before and during each maximum contraction with a train of single twitches at 1.25 Hz with a current sufficient to generate a twitch force of  $\sim 10\%$  of MVC. The magnitude of twitches before and superimposed during a maximum contraction were compared to estimate the level of activation and ‘true maximum force’ (TMF). The mean of two twitches was used to measure resting and superimposed twitches.

### Force–frequency relationship

A comparison of the forces generated by a range of electrical stimulation frequencies (1, 10, 20, 50, 80 and 100 Hz) was used as an indication of changes in E–C coupling and/or speed of the muscle. Stimulation was with five single twitches at 1 Hz and for 2 s at the other frequencies with 20 s rest between each tetanus. The response to 10 and 20 Hz stimulation had the greatest variability and so these frequencies were measured twice. Maximum tetanic-stimulated force was 25–35% of MVC. The forces were always recorded in the same order to ensure that any effects of fatigue or post-tetanic potentiation remained constant.

### Statistics

A repeated measures ANOVA was used to analyse the changes within groups. The Bonferroni post hoc test was used to identify significant differences between conditions (group E) or periods (group C). A probability level of  $<0.05$  was accepted as the minimum value for statistically significant differences.

## RESULTS

Most of the subjects experienced a mild headache and light-headedness with the administration of GTN200 that resolved in the hour between removing the patch and testing muscle function. However, two male subjects experienced a more severe headache with

|                | B1         | GTN100     | B2         | GTN200     |
|----------------|------------|------------|------------|------------|
| (a)            |            |            |            |            |
| MVC (N)        | 678 ± 34   | 679 ± 35   | 693 ± 35   | 712 ± 37*  |
| TMF (N)        | 704 ± 38   | 709 ± 40   | 712 ± 38   | 731 ± 40*  |
| Activation (%) | 96.4 ± 1.2 | 95.9 ± 1.5 | 97.4 ± 1.1 | 97.5 ± 0.8 |
| (b)            |            |            |            |            |
| MVC (N)        | 565 ± 76   | 561 ± 79   | 561 ± 69   | 559 ± 77   |

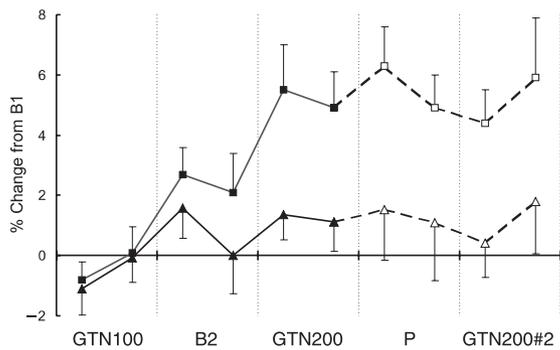
\*Significantly higher ( $P < 0.05$ ) than B1 and B2.

GTN100 and withdrew from the rest of the study. The subjects reported a consistent and maximal level of motivation, a consistent lifestyle and no change in their perceived strength. Mean absolute values for MVC, level of activation and TMF for group E during each condition are shown in Table 2(a). The corresponding values of the four periods of MVC measurement for group C are shown in Table 2(b). There were no interactions between the observed effects of GTN treatment and training status, strength or gender.

#### Maximum voluntary strength

During the GTN200 period, the MVC of group E increased by  $5.15 \pm 1.24\%$  (Mean  $\pm$  SEM, range  $-1.4$  to  $12.0\%$ ) compared with B1. There was no significant change in MVC between GTN100 and B1 ( $0.05 \pm 0.53\%$ , range  $-2.4$  to  $2.5\%$ ). For the five subjects who completed the additional phase of investigation (E5), there were no significant differences in MVC between the first treatment period with GTN200, the placebo (P) or the second GTN200#2 (Fig. 2).

For the control group C, there were no significant differences in MVC between any of the four periods, although in B2, P1 and P2 the MVC was, on average, lower than in B1 (Table 2). The greatest individual changes in this group ranged from  $-5.0$  to  $3.4\%$ .



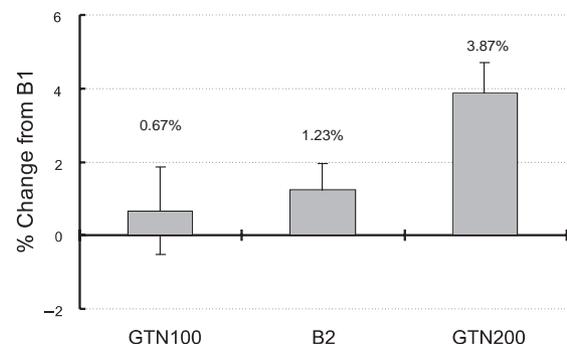
**Figure 2** Mean change in MVC and the level of activation compared with B1. Every measurement is shown, for all of group E ( $n = 10$ ,  $\blacksquare$ – $\blacktriangle$ ) and the additional measurements for E5 ( $n = 5$ ,  $\square$ – $\triangle$ ) (mean  $\pm$  SEM).

#### Level of voluntary activation and true maximum force

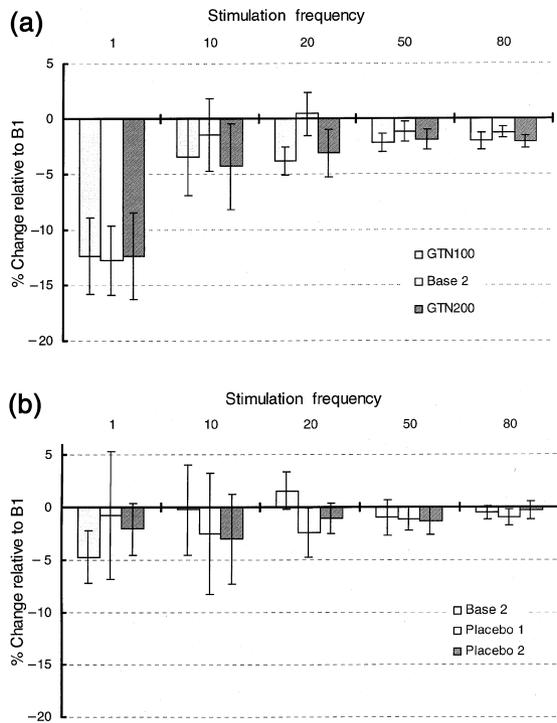
Figure 2 shows the mean individual changes in MVC and the level of activation relative to B1 during the treatment periods for group E and for the additional measurements on group E5. The estimated level of activation of group E did not change significantly during any condition (Table 2). The average level of voluntary activation for all of group E overall conditions was  $96.8 \pm 0.6\%$  (mean  $\pm$  SEM). Correcting the MVC for the level of activation showed that during GTN200 the TMF was significantly higher than during B1 and B2 (Table 2), although the magnitude of this increase was slightly smaller than for the MVC (compare Figs 2 and 3). Overall, the trend of MVC and TMF changes was very similar across all the conditions. There were no differences between TMF measured during GTN100 and B1.

#### Force–frequency relationship

The results are expressed as the change in force relative to 100 Hz force (Fig. 4). There was a trend towards a decline in relative force at all the submaximal frequencies for subjects in group E but the fall in relative submaximal forces was especially noticeable at 1-Hz stimulation.



**Figure 3** Mean change in true maximum force (TMF) for group E relative to B1 (mean  $\pm$  SEM,  $n = 10$ ).



**Figure 4** Mean change in force, as a percentage of 100 Hz force, at five frequencies of stimulation (1, 10, 20, 50 and 80 Hz). (a) Group E relative to B1 ( $n = 10$ ), (b) group C relative to week 1 ( $n = 6$ ) (mean  $\pm$  SEM).

In group E, significant differences were found at 1, 20 and 80 Hz (repeated measures ANOVA,  $P < 0.05$ ). Specifically for 1 Hz, during B2 and GTN100 compared with B1 and at 80 Hz between B1 and GTN200 (post hoc Bonferroni test,  $P < 0.05$ ). In group C, no significant differences were found between any of the four periods for any frequency. The absolute force generated by 100-Hz stimulation remained very consistent for both groups (data not shown).

## DISCUSSION

The results presented here indicate that NO administration can modulate skeletal muscle function *in vivo*. The MVC showed a progressive increase of about 5% over a number of days that was significant with GTN200 administration and persisted for at least 7 days. There was a consistent trend towards lower relative forces at submaximal frequencies of electrical stimulation with both doses of GTN and this was most pronounced with the reduced twitch force (about 12%) that was sustained for several days.

There are two features of the increase in strength that require some comment. The first is the small size of the change which raises the question as to whether it represents a 'real' increase in strength. The second

feature is the time course of change that was much slower than had been anticipated.

There are a number of reasons to think that the small increase in strength was not owing to increased muscle activation or experimental bias. An increase in activation, through improved central drive or co-ordination, could facilitate an increase in MVC, but while there continues to be some debate (Hales & Gandevia 1988), many studies have demonstrated that healthy untrained subjects can maximally activate their quadriceps muscle (Belanger & McComas 1981, Rutherford *et al.* 1986). In our present work, the interpolated twitches verified that our subjects were consistently well motivated, with high and stable levels of activation and even after making allowance for any fluctuations in activation, the TMF increased significantly ( $\approx +4\%$ ) after treatment with GTN200. The observation that strength did not increase with the lower dose of GTN (GTN100) suggests that experimental bias was not influencing the results. The design of the trial and side-effects of GTN meant that both subjects and investigators were aware of the treatment, yet this did not appear to influence the motivation or effort put into the contraction by the subjects. The consistent MVC of the control group over the 4 weeks also indicates that there were no significant learning effects or other artefacts influencing the results.

MVC changes of the magnitude we have observed are similar to strength changes reported as a result of the circadian rhythm (Gauthier *et al.* 1996, 1997) or the menstrual cycle (Phillips *et al.* 1996, Sarwar *et al.* 1996). We avoided any diurnal effects by taking measurements at the same time of day for each subject. Group E contained only two females, one was taking the oral contraceptive pill and both showed changes that were typical of their male colleagues for all of the measures examined.

The evidence we have accumulated strongly suggests that the increases in strength with GTN were not owing to increases in muscle activation or to experimental bias and, therefore, represents a true improvement in contractile function.

The increased strength following GTN administration persisted for several days, which suggests a lasting modification of function. The lack of effect during the 100-GTN period may have been a consequence of the low dose but the data in Fig. 2 also suggest a slow onset of the effect as strength continued to rise during the baseline period B2. The prolonged nature of this effect is emphasized by the fact that strength did not return to baseline values during the placebo period that was part of the extended observations made on the group E5 subjects (Fig. 2).

The changes in MVC seen here are similar in magnitude and, possibly, time course to changes

during the menstrual cycle (Phillips *et al.* 1996) and reproductive hormones are known to influence NOS in skeletal muscle (Weiner *et al.* 1994). Intuitively, the possibility of significant growth or other morphological changes within a few days seems unlikely. There is, however, evidence that NO is involved in the control of protein synthesis in a variety of tissues including skeletal muscle (Fryburg 1996). During repetitive isometric contraction, NO release from skeletal muscle increases by ~150% (Balon & Nadler 1994) and it is possible that NO is a link between high resistance work and gains in strength (including muscle hypertrophy).

There are no studies pertaining to NO effects on skeletal muscle properties *in vivo*, although there are a number of *in vitro* studies. The increase in maximum isometric force with a NO donor that we have found *in vivo* is in agreement with only one *in vitro* study (Reid *et al.* 1996). In contrast, there are a number of other studies that have reported no change or a decrease in strength (Marechal & Beckers-Bleux 1996, Morrison *et al.* 1996, Galler *et al.* 1997, Perkins *et al.* 1997, Chen *et al.* 1998, Albertini *et al.* 1998). It is notable that the increase in strength we have observed took place over several days, a longer time scale than any of the *in vitro* studies. Reid *et al.* (1996) found exogenous NO to increase  $\text{Ca}^{2+}$  transients and force and there are a number of reports that NO donors can affect the  $\text{Ca}^{2+}$ -release channels of the sarcoplasmic reticulum (Mészáros *et al.* 1996, Stoyanovsky *et al.* 1996, 1997, Aghdasi *et al.* 1997). It is possible that NO could augment force by increasing  $\text{Ca}^{2+}$  transients. This increase would be expected to move the entire force–frequency relationship to the left and Reid *et al.* (1996) have reported such a shift corresponding with an increase in maximum force. In contrast, our observation of smaller twitches and a general shift of the force–frequency relationship to the right does not provide a ready explanation for the increase in maximal force.

The force–frequency relationship was assessed by the more objective measurement of electrically stimulated forces and is, therefore, unlikely to have been confounded by subject or observer bias and there was no consistent or significant change in the control group over the four weeks of observation. The consistent and significant (at some frequencies) changes in group E indicate that GTN administration inhibited the force–frequency relationship *in vivo*, causing a slight shift of the relationship to the right.

The effect of GTN100 on the force–frequency relationship was more immediate and just as strong as GTN200, demonstrating an independent time course and dose response to that of MVC. The change in the force–frequency relationship was most noticeable in the sustained drop of twitch force after the first GTN

treatment. In canine skeletal muscle *in situ*, Murrant *et al.* (1997) also found a greater reduction in twitch than tetanic force following infusion of a NO donor. Interestingly, L-arginine administration has been found to be a potent modulator of twitch tension (Ambiel & Alves-Do-Prado 1997), presumably because it facilitates endogenous NO production.

The left shift of the force–frequency relationship with NO donation reported by Reid *et al.* (1996) is contrary to other *in vitro* evidence. Administration of a NOS inhibitor has been found to cause a left shift of the force–frequency curve, suggesting that endogenous NOS activity depresses the force–frequency relationship (Kobzik *et al.* 1994). In support of this, Perkins *et al.* (1997) found exogenous NO to depress the force–frequency relationship possibly by reducing  $\text{Ca}^{2+}$  sensitivity. Andrade *et al.* (1998) found NO and  $\text{NO}_2^-$  exposure to increase  $\text{Ca}^{2+}$  transients, reduce  $\text{Ca}^{2+}$  sensitivity and thus to have no overall effect upon the force of submaximal tetani. Other investigators have reported that NO can influence actomyosin ATPase activity (Galler *et al.* 1997) and cross-bridge kinetics (Marechal & Beckers-Bleux 1996, Morrison *et al.* 1996, Galler *et al.* 1997). Clearly, there is no consensus on the effects of NO on excitation–contraction coupling. Andrade *et al.* (1998) concluded that NO has a multifactorial effect on contractile function that is dependant upon the dose and the precise *in vitro* preparation. However, the physiological significance of each of these effects remains to be elucidated.

In conclusion, NO appears to play a role in skeletal muscle function *in vivo* and GTN administration is an effective means of influencing NO levels in skeletal muscle. The time course and dose–response relationship of exogenous NO on contractile properties *in vivo* requires further elucidation, particularly the surprisingly slow time course of NO effects upon maximal strength and how this relates to changes in the force–frequency relationship. As NO is administered systemically, its effects should be ubiquitous with the potential to modulate all manner of muscular actions in sporting and everyday tasks.

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