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Development of a Nitric Oxide-Releasing Analogue of the Muscle Relaxant Guaifenesin for Skeletal Muscle Satellite Cell Myogenesis

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Abstract: Nitric oxide (NO) mediates activation of satellite precursor cells to enter the cell cycle. This provides new precursor cells for skeletal muscle growth and muscle repair from injury or disease. Targeting a new drug that specifically delivers NO to muscle has the potential to promote normal function and treat neuromuscular disease, and would also help to avoid side effects of NO from other treatment modalities. In this research, we examined the effectiveness of the NO donor, iosorbide dinitrate (ISDN), and a muscle relaxant, methocarbamol, in promoting satellite cell activation assayed by muscle cell DNA synthesis in normal adult mice. The work led to the development of guaifenesin dinitrate (GDN) as a new NO donor for delivering nitric oxide to muscle. The results revealed that there was a strong increase in muscle satellite cell activation and proliferation, demonstrated by a significant 38% rise in DNA synthesis after a single transdermal treatment with the new compound for 24 h. Western blot and immunohistochemistry analyses showed that the markers of satellite cell myogenesis, expression of myf5, myogenin, and follistatin, were increased after 24 h oral administration of the compound in adult mice. This research extends our understanding of the outcomes of NO-based treatments aimed at promoting muscle regeneration in normal tissue. The potential use of such treatment for conditions such as muscle atrophy in disuse and aging, and for the promotion of muscle tissue repair as required after injury or in neuromuscular diseases such as muscular dystrophy, is highlighted.

Keywords: Stem cell; NO donor; activation; follistatin; muscle regulatory gene; transdermal

Introduction

Nitric oxide (NO) is an important ubiquitous physiological mediator involved in cellular signaling. Recent research has revealed many unexpected roles for NO ranging from blood vessel relaxation and neurotransmission to pathogen suppression. NO is also believed to play a role in some forms of tissue damage such as ischemia-reperfusion tissue injury and excitatory neuronal death. In the past 10 years, increasing attention has been directed to studies of the function and redox-related signaling of NO in skeletal muscle. These studies provided new insights into the roles of NO-related molecules in skeletal muscle including excitation−contraction coupling, autoregulation of blood flow, myocyte differentiation, respiration, and glucose homeostasis.[1−4]

In 2000, this laboratory reported that NO mediates the rapid activation of satellite precursor cells in skeletal muscle to enter the cell cycle.[5] Such cycling provides new precursor

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cells for skeletal muscle growth and muscle repair from injury or disease.[6−8] Studies of NO-mediated satellite cell activation and regeneration have since led to reports that increased levels of NO in muscle promote regeneration of normal and dystrophic muscle,[5,9,10] confirmed by other in vivo studies of NOS-I overexpression in a transgenic model of mdx dystrophic mice,[11−13] and long-term treatment trials of nitroflurbiprofen on alpha-sarcoglycan-null and mdx mice.[14] As well studies on single muscle fibers in a culture system that models exercise by cyclic mechanical stretching have further established that stretching activates quiescent satellite cells on fibers,[15] and that this effect is regulated by fiber-derived NO.[16,17]

Satellite cells are quiescent precursors in normal skeletal muscle. Satellite cell activation from the normal state of mitotic and metabolic quiescence is a process that occurs very rapidly (within 10 min in vivo)[5] and is the essential first step that initiates muscle growth and repair. Decreased NO production abrogates the early phase of satellite cell activation through an interaction of the c-met proto-oncogene receptor with its ligand, hepatocyte growth factor (HGF), released from the extracellular matrix by NO via MMP-2.[18−22] Reduced NO production, therefore, impairs muscle regeneration in normal muscle and exacerbates mdx mouse muscle dystrophy.[5] NO and HGF are the only two molecules known to activate satellite cells from the quiescent state.[23]

Since musculoskeletal health is exquisitely dependent on growth and repair, developing a system to deliver NO to skeletal muscle and thereby manipulate the regulation of satellite cell activation has the potential to promote normal function in injured muscle tissue and possibly to treat neuromuscular disease. Here, we report the development of an NO-based compound to target NO delivery to skeletal muscle. The new compound is effective in promoting muscle cell proliferation and has potential for skeletal muscle growth and muscle regeneration.

Experimental Section

Materials. All chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless otherwise stated. C57/BL6 mice were used in this study. All mice were maintained with 12-h light/dark cycles with food and water

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ad libitim. All animals received humane care in compliance with institutional guidelines, which are in accordance with criteria set by the Canadian Council on Animal Care.

Muscle cultures. Extensor digitorum longus (EDL) muscles were dissected cleanly and carefully from mice without stretching or trauma that could easily activate satellite cells.[23] Each muscle was aligned radially in one well of a 6-well Flex II culture plate (Flexcell International, NC), and the tendons were pinned so the muscle was at resting length. The muscles were incubated in growth medium (DMEM containing 15% [vol/vol] horse serum [HyClone Laboratories Inc.] and 2% [vol/vol] chick-embryo extract [ICN Biomedicals]) at 37 °C in 5% CO_2, as reported.[24] Cultured muscles were treated with an NO donor or vehicle alone (controls). The nucleotide analogue, [^{3}H]-thymidine (2 μCi/mL of medium), was added to the medium at the beginning of treatment, and cultures were maintained for 24 h to allow incorporation of the isotope into new DNA during the S-phase of one cell cycle. At the end of 24 h, muscles were removed from wells, tendons were trimmed away, and the muscles were frozen at −20 °C for future analysis.

Drug-Treatment Procedure to Animals. Drug transdermal administration: Male mice (~25 g body weight) were anesthetized with isoflurane. The mid-dorsal skin (on the back) was shaved, washed with soap and warm water, and air-dried. An ointment containing a treatment compound or compounds was formulated such that an NO compound (at 2% by weight) was contained in a cream base (components: sodium lauryl sulfate 1%, propylene glycol 12%, stearyl alcohol 25%, white petrolatum 25%, and purified water 37%) and applied to a sterile bandage (Band-Aid) at the dose of 0.3 g/animal. Animals treated with a bandage containing only base ointment served as controls in each experiment. The animals were fitted with a small plastic neck collar (Elizabethan collar for mice) while anesthetized, to prevent them from chewing on the bandage. Eyes and face of the mice were washed regularly with clean gauze moistened in warm water, since animals were unable to groom well during the period when they were wearing the collar. Animals were housed singly in cages with food and water provided on the cage floor until the end of a 24 h treatment period. Treatment compounds (structures are shown in Figure 2A) included isosorbide dinitrate (ISDN), methocarbamol (MC), MC plus ISDN, or a novel compound, guaifenesin dinitrate (GDN) (as synthesized below). In vivo experiments were conducted in sets such that a control (placebo) animal was always treated in parallel with at least one animal from each treatment group.

For oral administration of GDN, the drug was dissolved in corn oil. A volume of 0.2 mL was delivered to mice (male, 5 months old) by gavage with the dosage of 80 mg/kg body weight, using a 20-gauge bulb-tipped feeding needle (FTP-20-30, Instech Solomon, PA) on a 1 mL syringe. Control mice were administered corn oil only. The animals were sacrificed at 24 h after treatment.

Protein Preparation and Western Blot. Proteins were extracted from 15 μm thick muscle cryosections using a motorized hand-held homogenizer in the protein extraction buffer [125 mM Tris-HCl (pH6.8), 4% SDS, 10% glycerol, 2 M urea, 5% 2-mercaptoethanol, and protease inhibitor cocktail (Roche 04693159001)]. Protein (120 μg) was loaded onto 12% polyacrylamide gels for electrophoresis and blotting. The following antibodies were used for the Western blotting: rabbit antifollistatin polyclonal antibody (10474-1-AP, Protein Tech Group, IL); rabbit anti-Myf-5 polyclonal antibody (sc-302, Santa Cruz Biotechnology, CA); mouse antimyogenin monoclonal antibody (the Developmental Studies Hybridoma Bank, IA). These primary antibody bindings were probed with the corresponding secondary antibodies conjugated with horseradish peroxidase and were visualized by using standard enhanced chemiluminescence (Perkin-Elmer, MA).

Immunohistochemical Analysis. Muscles were dissected and immediately snap frozen in isopentane over dry ice. Sections of 6 μm were collected at −20 °C onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St Louis, MO) coated glass slides using a Leica CM1850 cryostat and stored at −80 °C until use. The slides were first pretreated in PBS for 10 min, and thereafter incubated for 30 min in the blocking solution (20% FBS including 10% goat serum in PBS) at room temperature in a humid chamber. The cryosections were then incubated for 1 h at room temperature with the antibodies directed against Myf-5 (sc-302, Santa Cruz Biotechnology, CA), follistatin (10474-1-AP, Protein Tech Group, IL), dystrophin (VP-D507, Vector Laboratories, U.K.), and myosin heavy chain (MHC) isomer 2b (clone BFF3, DSM Braunschweig, Germany). The secondary antibodies Alexa Fluor 594 goat antirabbit IgG (H+L), and Alexa Fluor 488 goat antimouse IgG (H+L) (Invitrogen, CA) were used to detect binding of the primary antibodies. Images were taken with an inverted fluorescent microscope (Olympus BX51).

Immunofluorescence analysis was conducted on 8 nonoverlapping fields of tissue (400× magnification) using four cross sections from each muscle. Myonuclei (DAPI-stained) were counted from 100 fibers/section that were outlined by dystrophin staining. Nuclei that were stained positive for Myf-5 and were located external to the outline of dystrophin staining were identified as activated/proliferated and differentiated satellite cells.[25] A Myf-5-positive index was calculated as the number of Myf-5-positive satellite-cell nuclei divided by the total number of myonuclei and was used as an index of satellite cell myogenesis.

[^{3}H]-Thymidine Incorporation into DNA and DNA Assay. Two hours before euthanasia, mice were injected with 1 μCi per gram body weight of [^{3}H]-thymidine (intraperi-

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toneal) to label DNA synthesis. Quadratus lumborum (back muscle) and the quadriceps muscle were dissected bilaterally from animals and frozen for future assays. DNA extraction from muscles and the assay for DNA concentration were followed as outlined by Labarca and Paigen.[26] The incorporation of [^{3}H]-thymidine into new DNA was determined by using scintillation counting in duplicate for each sample. The average number of disintegrations per minute (dpm) of each sample was standardized to the total DNA (μg) in each sample.

Chemical Synthesis of GDN (1,2-Di-*O*-nitro-3-(*o*-methoxyphenoxy)propanediol). A schematic of the synthesis of GDN is shown in Figure 2B. The synthesis of the intermediate **3** proceeded as follows. To a stirred suspension of compound **2** (2.00 g, 16.13 mmol) in dry tetrahydrofuran (THF, 50 mL), kept under nitrogen, was added 60% sodium hydride (NaH, 0.675 g, 16.88 mmol). After 15 min, allyl bromide (1.77 mL, 20.44 mmol) was added, and the reaction mixture was heated at 70 °C overnight. Then, an additional amount of 60% NaH (0.169 g) and allyl bromide (0.6 mL) was added, and the heating was continued an additional 8 h. After this time, the reaction mixture was poured into 0.1 N NaOH (50 mL) and extracted with ethyl acetate (EtOAc). The organic layer was washed with brine, dried, and evaporated under reduced pressure. The residue was purified by flash chromatography with 3% ethyl acetate in hexanes to give compound **3** (2.6 g, 15.85 mmol) as a colorless liquid in quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$, rt, TMS): δ = 3.87 (s, 3H), 4.62 (m, 2H), 5.28 (m, 1H), 5.41 (m, 1H), 6.10 (m, 1H), 6.87−6.99 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$, rt): δ = 55.87, 69.85, 112.82, 113.66, 117.82, 120.76, 121.26, 133.47, 148.05, 149.54. MS (EI, $[M]^+$); *m*/*z* calcd for $C_{10}H_{12}O_2$ 164.08, found 164.03.

Synthesis of the dinitrate molecule **1** proceeded as follows, according to a published method.[27] To an ice cooled, stirred solution of intermediate **3** (1.2 g, 7.32 mmol) and $AgNO_3$ (4.98 g, 29.28 mmol) in acetonitrile (50 mL) was added iodine (1.85 g, 7.32 mmol). After the iodine had dissolved, the reaction mixture was refluxed for 12 h, filtered, poured into water, and extracted with ethyl acetate. The organic layer was washed with brine, dried, and evaporated. The oil thus obtained was purified by flash chromatography (eluent from hexanes/EtOAc 20/1 to 8/1 v/v) to afford the compound **1** (1.88 g) as a yellow liquid. Yield 89.5%. ^{1}H NMR: δ = 3.85 (s, 3H), 4.28 (d, 2H, J = 5.2 Hz), 4.81 (dd, 1H, J = 6.6 Hz, J = 12.9 Hz), 4.98 (dd, 1H, J = 3.3 Hz, J = 12.9 Hz), 5.56−5.64 (m, 1H), 6.87−7.08 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$, rt): δ = 55.75, 67.12, 69.11, 77.24, 112.46, 116.83, 120.99, 123.73, 147.14, 150.46. MS (EI, $[M]^+$); *m*/*z* calcd for $C_{10}H_{12}N_2O_8$ 288.06, found 288.00. HPLC analysis shows that the product purity reaches 97.98% (Figure 1 in the Supporting Information).

NO Determination by EPR Spectroscopy. NO release from tissue was determined over time using the NO-trap method.[28] Stock solutions of $FeSO_4 \cdot 7H_2O$ (20 mM), *N*-methyl-D-glucamine dithiocarbamate (MGD) sodium salt (100 mM) were prepared in argon-purged distilled water on the day of the experiment and were used within a few hours. The nitric-oxide trap complex $Fe^{2+}(MGD)_2$ was prepared by mixing equal volumes of the iron and MGD stock solutions just before use. Mice (C57BL6) were euthanized by cervical dislocation under anesthesia. The liver, heart, and back muscle were removed and homogenized in ice-cold sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) in a ratio of 1:1 tissue/buffer (mg/μL). After adding GDN (1 mM in DMSO) and the NO spin-trap $Fe^{2+}(MGD)_2$ to the homogenate, the mixture was incubated at 37 °C for 30 min. Sample aliquots (16 μL) of the tissue mixture were loaded into quartz capillary tubes for EPR analysis. EPR spectra were obtained using a Bruker EMX EPR spectrometer (Bruker Co., Billerica, MA) at room temperature (25 °C).[28] Typical instrument settings were 9.25 GHz microwave frequency; 100 kHz modulation frequency; 20 mW microwave power, 4 G modulation amplitude, 40 ms time constant, 42 s scan time, 100 gauss scan range, and 3410 G center field. An aliquot of the NO-trap and tissue mixture bubbled with NO gas for 10 s served as a positive control, while an aliquot of tissue homogenate plus the NO-trap mixture without the new compound served as a negative control.

Statistical Analysis. Data are presented as mean ± standard error of the mean (SE) for each group, and were compared by *t* test (two groups) or analyses of variance (ANOVAs) (for comparison of more than two groups) followed by post hoc least significant difference (LSD) tests, as appropriate. In all cases, a probability of $p < 0.05$ was used to indicate a significant difference.

Results

Effect of NO Treatment on the Activation of Satellite Cells in Skeletal Muscle. Satellite cell activation and entry into the cell cycle can be assayed directly as an increase in DNA synthesis, measured as increased dpm/μg DNA from incorporation of [^{3}H]-thymidine.

Based on previous reports of NO-mediated satellite cell activation, we tested the efficacy of a system that would deliver nitric oxide to skeletal muscle cells in normal adult mice in vivo. As shown in Figure 1, there was an increase in muscle satellite cell activation and proliferation (demonstrated by increased DNA synthesis) induced by 24 h exposure to an ointment-based transdermal delivery of two different compounds, 1% methocarbamol (a muscle relaxant)

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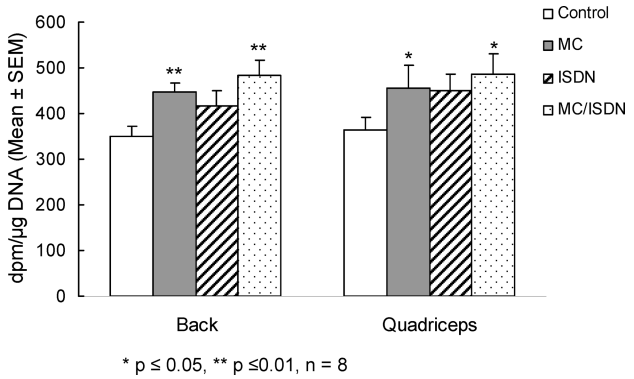

* $p \leq 0.05$, ** $p \leq 0.01$, $n = 8$

Figure 1. Effect of transdermal treatment of NO donor and muscle relaxant on skeletal muscular DNA synthesis in normal mice. Animals were treated for 24 h with transdermal formulations; 2 h before euthanasia, they were injected with [^{3}H]-thymidine to label new DNA synthesis. Back and quadriceps muscles both showed an increase in DNA synthesis after ISDN and muscle relaxant, methocarbamol (MC) treatment. Results demonstrated an additive effect of treatment with methocarbamol in combination with ISDN ($p < 0.01$ and $p < 0.05$, $n = 8$ respectively).

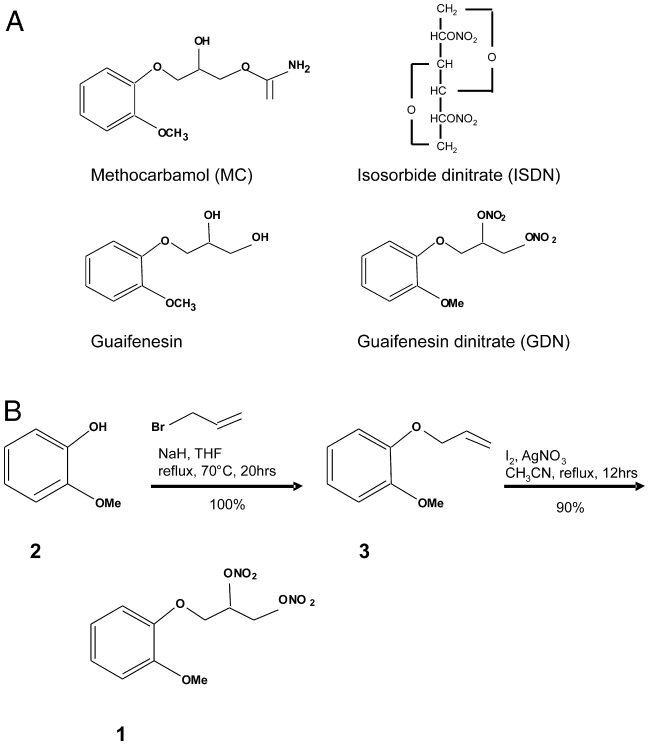

Figure 2. A. Molecular structures of methocarbamol (MC), isosorbide dinitrate (ISDN), guaifenesin, and guaifenesin dinitrate (GDN). B. Schematic showing the main steps in synthesizing GDN (1,2-di-*O*-nitro-3-(*o*-methoxyphenoxy)-propanediol).

and 0.2% ISDN. ISDN promoted a modest trend to increase DNA synthesis by 19% to 24% over control levels in both back muscle and quadriceps muscle. Methocarbamol had similar effects, inducing a significant increase in DNA synthesis by 28% in back muscle and a trend to increase

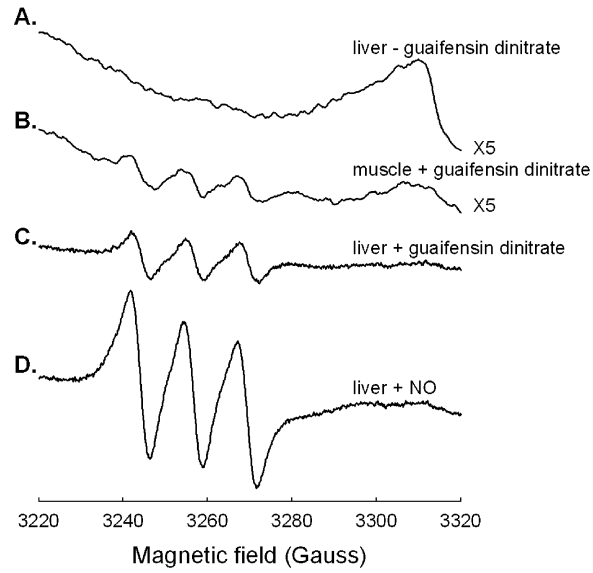

Figure 3. The EPR spectra obtained from experiments with tissue homogenates and NO spin-trap methodology (10 mM MGD and 1 mM $FeSO_4$) showing NO production following the addition of GDN (1 mM). A. Blank or negative control sample of liver homogenate in the presence of the spin-trap agent without GDN. B. Back muscle homogenate plus GDN in the presence of the spin-trapping agent. C. Liver homogenate plus GDN and spin-trap agent. D. Positive control (NO standard: liver homogenate and NO spin-trap compound bubbled with NO gas). In each tissue, the level of NO in the EPR spectrum is stable and shows NO peaks in the range 3225 to 3312.5 gauss. For the samples incubated with GDN, the EPR spectrum (depicted here at 1 h after adding the compound) shows the presence of NO in its unique isotropic triplet signal at gauss = 2.04, as evident by comparison with the NO standard curve D. The spectra in A and B are plotted with the *Y*-axis expanded 5-fold compared to spectra in C and D.

DNA synthesis (26%) in quadriceps muscle. However, the combination of treatment with methocarbamol (1%) and ISDN (0.2%) produced an additive effect, resulting in significant increases of 38% in back muscle (compared to 19% in back muscle of control animals, $p \leq 0.01$, $n = 8$) and 35% in quadriceps muscle (compared to 24% in quadriceps of control mice, $p \leq 0.05$, $n = 8$) after in vivo exposure for 24 h (the raw data are shown in Table 1 in the Supporting Information).

Production of Nitric Oxide from GDN in Muscle and Liver Homogenates. Electron paramagnetic resonance (EPR) spectroscopy and NO-trapping methodologies were used to test whether the addition of GDN to tissue homogenates resulted in NO production. Homogenates of liver and back muscle were prepared; addition of the new compound resulted in the rapid production of NO, as assayed using the $Fe^{2+}(MGD)_2$ trap reaction with NO to form a stable $[NO-Fe^{2+}-(MGD)_2]$ complex at room temperature. The unique triplet-NO complex (Figure 3) was characterized by an isotropic triplet EPR signal at gauss = 2.04 and isotropic,

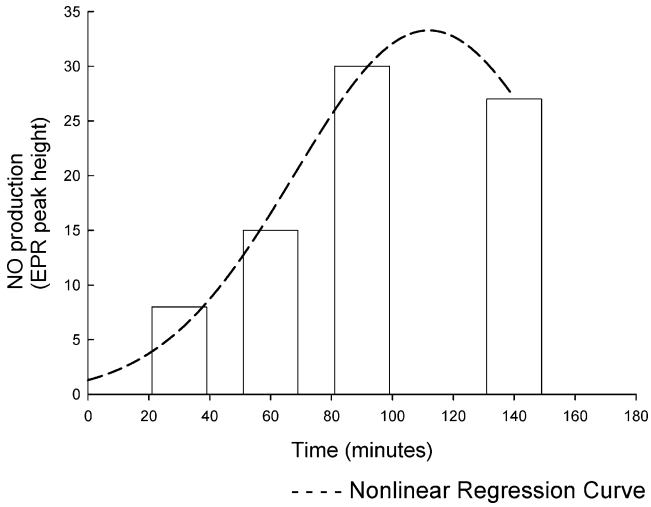

Figure 4. The time-course of NO release, as detected by duplicate longitudinal EPR studies using the NO spin-trap agent, on liver homogenate after a single addition of GDN. The EPR signal (here the peak-to-trough height of the low-field peak in the NO triplet) was plotted over time. The bars are superimposed with a nonlinear regression curve that demonstrates an initial and persistent rise in NO production over time until 1.5 h, after which NO production declined.

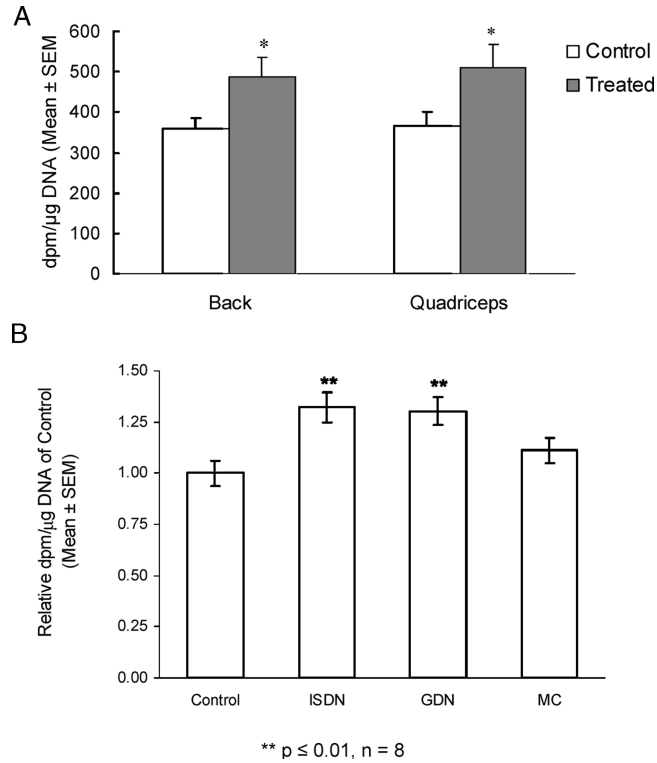

Figure 5. GDN effects on DNA synthesis in vivo and in vitro. A. The effects of treatment with GDN on DNA synthesis in back and quadriceps muscles of normal mice in vivo. Animals were treated for 24 h with transdermally applied GDN in an ointment formulation (dark bars) or base ointment without the drug (white bars); back and quadriceps muscles were collected 2 h following injection of [^{3}H]-thymidine to label new DNA. GDN stimulated a 36−39% increase in cell proliferation (DNA synthesis) in both muscles. B. The effect of NO donor and muscle relaxant on DNA synthesis in whole EDL muscle cultures isolated from normal mice.

hyperfine coupling constant $a_N = 12.5$ G.[28,29] In the control experiment with $Fe^{2+}(MGD)_2$ added to the tissue homogenate alone, no NO-triplet signal was observed (Figure 3, control). However, after incubation with the GDN (1 mM), a characteristic triplet spectrum of the NO complex was observed in both liver and back muscle homogenates. The positive standard (tissue homogenate plus the NO trap, bubbled with nitric oxide gas) showed the characteristic NO-triplet complex at the same frequency as for the test groups with the GDN. These EPR experiments confirmed that GDN induced rapid NO release from tissue homogenates.

Time-Course of Nitric Oxide Production from GDN in Liver Homogenates. The time-course of NO release from the tissue homogenates was examined as a measure of the signal strength and kinetics of the response. In such EPR experiments, the EPR signal strength, measured as the peak-to-trough height of the low-field peak is proportional to the NO concentration in the homogenate mixture. The nonlinear regression curve of the low-field peak height was plotted over time to represent the kinetics of NO release. This regression curve demonstrated that NO production by the liver homogenate persisted for more than 2 h (Figure 4). Very similar results were observed for the back muscle and cardiac muscle samples prepared as homogenates (data not shown). The maximum production of NO by the addition of GDN was between 1 and 2 h after the start of incubation with the liver homogenate. It is important to note that the EPR spectrum of NO also relies on the stability of the spin-trapped adduct. The presence of free radicals including NO in the system shortens the lifetime of $NO-Fe^{2+}(MGD)_2$.[30] For this reason, the decrease in the EPR signal after 1.5 h was probably due to decomposition of the $NO-Fe^{2+}(MGD)_2$ complex.

Transdermal Treatment of GDN Increases DNA Synthesis in Skeletal Muscles in Adult Mice. GDN (2%) ointment was formulated and used to treat adult mice by transdermal delivery on the mid-dorsal (back skin) for 24 h; no obvious side effects were noted. As shown in Figure 5A, the treatment resulted in a significant increase in DNA synthesis in back muscle (489 ± 23 vs 358 ± 13 dpm/μg DNA) and in quadriceps muscle of the same animals (510 ± 28 vs 366 ± 17 dpm/μg DNA) compared to control placebo-treated animals. These 37% to 39% increases of DNA synthesis in back and quadriceps muscle by GDN were

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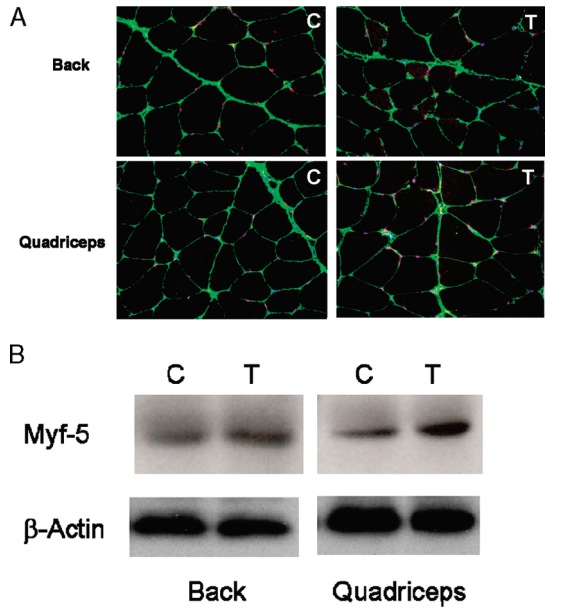

Figure 6. The effects of GDN treatment on skeletal muscle myogenesis in vivo. Mice, 5 months old, were gavaged with GDN (80 mg/kg body weight). Muscles of the back (quadratus lumborum) and quadriceps were collected at 24 h after administration of the compound. Tissue sections were immunostained with anti-Myf-5 (red) and antidystrophin (green), and counterstained with the nuclear dye, DAPI (blue). A. Representative images of Myf-5-positive nuclei in back and quadriceps muscle. "C" and "T" denote the samples from control and treated animals, respectively. B. Expression of Myf-5 and β-actin proteins were determined by Western blotting; this panel shows blots from a pair of one control (C) and one treated (T) animal that are highly representative of findings from four independent, paired experiments.

higher than those by ISDN (Figure 1). However, this additional effect was not observed in the in vitro study of EDL muscle cultures (Figure 5B, the raw data are shown in Table 2 in the Supporting Information), which suggests that muscle proliferation, as part of early muscle regeneration (measured by DNA synthesis), mainly occurred during contraction−relaxation cycles, or have required some systemic influence to mediate the change. Furthermore, since the GDN ointment was applied directly on the dorsal skin of mice, overlying the back muscle, and the effects of treatment were the same for back and leg muscle, these results suggest that the effect of GDN on muscle may be through a systemic action.

Effect of GDN on Myogenesis of Satellite Cells in Vivo. Mice (5 months old) were treated with GDN by oral administration, without obvious side effects. There was no significant change in serum levels of alanine transaminase, alkaline phosphatase, total bilirubin, blood urea nitrogen, gamma-glutamyl transferase, and creatinine (data not shown). Two molecular markers of satellite cells were assayed in back muscle and quadriceps. As shown in Figure 6A, the Myf-5-positive index was increased in back muscle by 40% (0.25 $\pm$ 0.07 vs 0.35 $\pm$ 0.06) and in quadriceps muscle by 55%

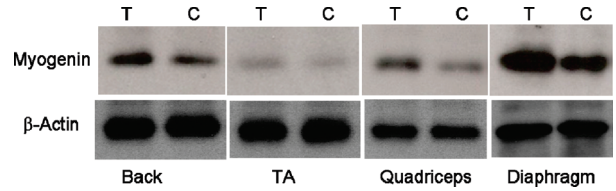

Figure 7. GDN up-regulates myogenin expression in four different muscles: back, quadriceps, diaphragm and tibialis anterior (TA). Muscles were collected from 5-month-old mice at 24 h after oral administration of GDN (80 mg/kg). The level of expression of myogenin and β-actin (the loading control) was determined by Western blotting. The images are representative of four repeat experiments. C and T represent the samples of control and treated animals respectively.

(0.22 $\pm$ 0.09 vs 0.34 $\pm$ 0.05) compared to the respective muscles from control animals ($p < 0.05$, $n = 4$). Western blot analysis of Myf-5 expression in the muscles was consistent with the increased Myf-5 index from both muscles (Figure 6B). Figure 7 shows that myogenin expression in the muscles (back muscle, quadriceps, diaphragm, and tibialis anterior) was consistently up-regulated in the treated animals compared to controls. These results suggest that satellite cells were activated by GDN after 24 h, and had started to produce satellite cell-derived myoblasts that were committed to myogenic differentiation.

GDN Up-Regulates Follistatin Expression in Slow-Twitch Muscle Fibers in Vivo. NO-mediated up-regulation of follistatin in myoblasts plays a pivotal role in promoting myoblast fusion during muscle development.[31] Guaifenesin dinitrate was administrated orally to 5-month-old mice. As shown in Figure 8A, follistatin levels in the back and quadriceps muscles were increased in the treated animals compared to controls. Using antibodies against the MHC isomer 2b and follistatin, double-staining immunohistochemical studies (Figure 8B) showed that follistatin expression and its up-regulation after GDN treatment were present only in type I (slow-twitch) muscle fibers. Follistatin was absent from type II (fast-twitch) muscle fibers.

Discussion

The significance of nitric oxide (NO) as a signaling molecule in numerous biological systems has stimulated researchers to search for new NO-releasing compounds. Indeed, NO donors have become an important class of drugs that have proven useful as treatment for a number of pathological conditions that are associated with NO deficiency and regulation. These conditions include the treatment of nervous, sexual, respiratory, muscular and gastrointestinal disorders, enhancing immunological response, and regulating

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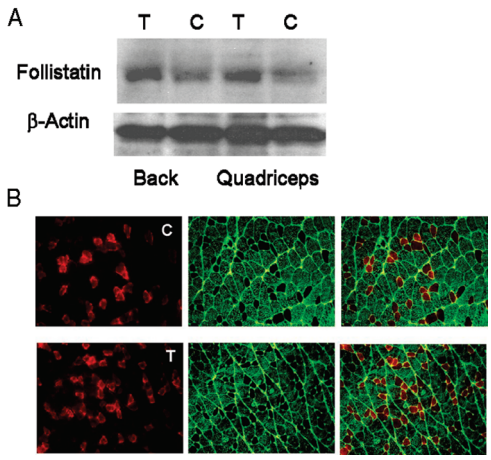

Figure 8. GDN up-regulates follistatin expression in slow-twitch muscle fibers. Back and quadriceps muscles were collected from 5-month-old mice at 24 h after oral administration of GDN. A. Follistatin and β-actin expression in the muscles were determined by Western blotting. B. Double immunostaining for follistatin (red) and the myosin heavy chain isomer 2b (green) which recognizes the fast-fiber type IIB. C and T represent the samples of control and treated animals respectively. Results are representative of four repeat experiments.

tumor growth.[32−37] In the present experiments, results demonstrated that a new NO-donor formulation, GDN, stimulated a 37−39% increase in DNA synthesis in two skeletal muscles after 24 h transdermal treatment in mice, and a 40−55% increase in the number of activated myogenic satellite cells 24 h after oral treatment. Given the key role of NO in mediating satellite cell activation, the essential nature of activating steps in successful muscle regeneration, and the effect of GDN to release NO from tissue homogenates over a 2 h period, we propose that this new class of NO-donor molecules has significant potential to promote muscle regeneration after traumatic injury and in muscle disease, and to stimulate muscle growth in age-related atrophy.

We initially reported that NO mediates activation of satellite precursor cells to enter the cell cycle,[5] according to results of three independent tests that showed NO was critical for the rapid transduction of an injury signal to activate satellite cells in vivo. These tests included the absence of early activation in nNOS knockout mice due to loss of NO release from neuronal nitric oxide synthase (nNOS); down-regulation of early activation in muscle where nNOS expression was reduced secondary to dystrophin deficiency; and the inhibition of activation after NOS blockade using L-NAME (L-nitroarginine methyl ester) in vivo during a muscle injury.[5] We and other investigators have demonstrated that NO mediates activation of quiescent satellite cells in cell culture via HGF release [19−21,38] and confirmed that NO is released by stretching,[17] regulates satellite-cell quiescence[16] and plays an important role in signaling muscle precursor fusion into myofibers and myogenic differentiation and growth.[31,39] While the role(s) of NO in signaling pathways of muscle satellite cell activation are still under investigation, the critical role of satellite cells in muscle repair is demonstrated by many reports, as reviewed elsewhere.[8,40] NO also works as a cell-signaling molecule responsible for regulating blood flow, oxygen delivery, glucose uptake, the velocity of contraction, and muscle power output, and therefore has counterpart physiological and cellular effects that combine to enhance muscle growth and repair. The results in this study show a substantial increase in DNA synthesis, and expression of Myf-5, myogenin, and follistatin proteins in normal skeletal muscles following GDN administration transdermally and orally. Myf-5 and myogenin are members of the well-characterized family of myogenic helix−loop−helix proteins that play an important role in muscle development. When satellite cells are activated and become proliferative, Myf-5 expression is increased. Myogenin expression then marks the subsequent myogenic differentiation processes.[25] Follistatin can inhibit myostatin, a protein that inhibits satellite-cell activation, and lead to activation, myogenic differentiation and muscle growth.[41] Overall, our study suggests that the new compound, GDN, functions to activate satellite cells and then promote myogenesis. Since skeletal-muscle satellite-cell activation and proliferation typically demonstrate a decline in older adult

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animals,[42,43] the substantial increase in activation and myogenesis in adult muscle in 5-month-old mice following 24 h of exposure to GDN in this study indicates, therefore, that GDN is very promising as a potential treatment to stimulate proliferation and alleviate age-related atrophy. Moreover, the complexity of NO effects in muscle results in improvement of muscle health in mdx mouse muscular dystrophy.[9−11,35] These contexts for the current experiments suggest the new NO compound would be expected to be beneficial to dystrophic muscle.

In synthesizing a new NO-donor compound (Figures 2A and 2B), guaifenesin was selected as the base compound. Guaifenesin is the active metabolite of methocarbamol,[44] and has the same pharmacological function.[45] Methocarbamol, a carbamate structural analogue of guaifenesin, is commonly used as a muscle relaxant for the symptomatic treatment of skeletal muscle disorders. This medication is sedating in larger doses, and is believed to work through the nervous system by decreasing nerve impulses from the brain and spinal cord that lead to tensing or tightening of muscle fibers. In this study, we showed that methocarbamol increased DNA synthesis in skeletal muscle in vivo, and also was additive to the significant effects of NO (released from the NO donor, ISDN) on the muscle in whole-animal experiments. Interestingly, there was no significant effect of methocarbamol on satellite-cell activation in EDL-muscle cultures (Figure 5B), even though methocarbamol treatment of dispersed cultures of single muscle cells induced an increase DNA synthesis (data not shown). This observation is consistent with the idea that neuronal regulation and muscle activity are likely involved in later aspects of skeletal muscle regeneration, and that there are distinct differences in modeling myogenesis in cells, single fibers, whole muscle cultures and in vivo, as reviewed elsewhere.[23,46] Muscle relaxation by botulinum toxin is shown to improve rehabilitation in muscle-tendon repair.[47] At this time, therefore, it is not clear whether the mechanism of methocarbamol action can be attributed to vascular effects on perfusion or to a direct action on motor and/or sensory nerve axons exposed to methocarbamol. However, it seems probable that GDN could be expected to perform multiple pharmacological functions for skeletal muscle regeneration by controlling pain, relaxing muscle, and by providing NO.

As an organic nitrate like nitroglycerin and isosorbide dinitrate, GDN likely produces nitric oxide by both nonenzymatic and enzymatic pathways.[48] Although nitric oxide can be generated by direct chemical interaction between organic nitrates and thiols, specifically cysteine,[49,50] the current consensus favors enzymatic transformation of organic nitrate to nitric oxide as the mechanism producing NO in vivo. A number of enzymes, including glutathione-S-transferase,[51] cytochrome P-450-systems,[52] xanthine oxidoreductase,[53] and mitochondrial aldehyde dehydrogenase,[54] have been shown to catalyze the conversion of organic nitrates to nitrite and nitric oxide. The abundance of these enzymes in back muscle is likely lower than in liver, since Figure 4 shows that the same concentration of GDN generated a weaker NO-triplet signal by EPR in homogenates of back muscle than in liver.

To achieve optimum therapeutic outcome, proper drug selection and effective drug delivery are required. Transdermal drug delivery could be an important preferred route of administration, for example in the elderly. Since NO plays widespread pharmacological roles in biological systems and also has the potential to induce significant cytotoxicity at high levels of exposure, targeting delivery of NO is one of the major challenges in formulating NO-donor compounds. Transdermal drug delivery would seem to be preferable over oral or other routes of administration, given the large area available for drug application, the ability to regulate dosage levels to skin, and the ease by which the source of transdermal treatment could be removed. Nitrate esters such

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as nitroglycerin are well established as being systemically vasoactive on transdermal application, as, for example, in the application of the nitroglycerin patch. In this study, we applied GDN ointment on the dorsal skin of mice with the aim of delivering a new NO-donor compound directly to the underlying muscle. However, the same increase in DNA synthesis was demonstrated in both underlying back and more distant leg muscles, suggesting that the effect of GDN on DNA synthesis in skeletal muscle was mainly through the systemic circulation, possibly in combination with HGF release from skeletal muscle by NO and subsequent release of HGF to the systemic circulation, as well as with effects mediated by the nervous system. The development of a more specific formulation to target NO donors directly to muscle, by designing diazeniumdiolates or S-nitrosothiol molecules, should be much better able to limit the action of the NO donors to the site of application, even in a controlled-release formulation. These would be valuable areas for future study.

In conclusion, this initial study adds to our knowledge on the action of nitric oxide donors and skeletal-muscle relaxants. Importantly, it opens a new category of compounds that combine NO-donor and muscle-relaxant functions. This new muscle relaxant promotes muscle-cell proliferation and also enhances the effect of NO in promoting satellite-cell activation and proliferation for growth or regeneration. This finding has significant implications on the potential to alleviate the impact of neuromuscular interactions in muscle growth, aging and rehabilitation from trauma, including age-related osteoporosis linked with muscle atrophy and disuse, to regulate sarcolemmal damage in muscle with aberrantly localized nNOS such as in muscular dystrophy, and for other therapeutic applications,[55,56] once targeting is further refined.

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Supporting Information Available: HPLC analysis of GDN and tables of raw data as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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