SHORT COMMUNICATION

Osteoprotegerin and β2-Agonists Mitigate Muscular Dystrophy in Slow- and Fast-Twitch Skeletal Muscles

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Our recent work showed that daily injections of osteoprotegerin (OPG)—immunoglobulin fragment complex (OPG-Fc) completely restore the function of fast-twitch extensor digitorum longus muscles in dystrophic mdx mice, a murine model of Duchenne muscular dystrophy. However, despite marked improvements, OPG-Fc was not as effective in preventing the loss of function of slow-twitch soleus and diaphragm muscles. Because β2-agonists enhance the function of slow- and fast-twitch dystrophic muscles and because their use is limited by their adverse effects on bone and cardiac tissues, we hypothesized that OPG-Fc, a bone and skeletal muscle protector, acts synergistically with β2-agonists and potentiates their positive effects on skeletal muscles. We observed that the content of β2-adrenergic receptors, which are mainly expressed in skeletal muscle, is significantly reduced in dystrophic muscles but is rescued by the injection of OPG-Fc. Most important, OPG-Fc combined with a low dose of formoterol, a member of a new generation of β2-agonists, histologically and functionally rescued slow-twitch dystrophic muscles. This combination of therapeutic agents, which have already been tested and approved for human use, may open up new therapeutic avenues for Duchenne muscular dystrophy and possibly other neuromuscular diseases. (Am J Pathol 2017, 187: 498–504; http://dx.doi.org/10.1016/j.ajpath.2016.11.006)

Duchenne muscular dystrophy (DMD) is a lethal severe genetic muscle disease caused by a mutation in the gene encoding dystrophin, resulting in a complete absence of this protein.1 Dystrophin is a cytoplasmic protein that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane. Many muscle proteins, such as dystroglycan, sarcoglycan, vinculin, and talin, colocalize with dystrophin at the costamere and myotendinous junction.2,3 The absence of dystrophin in DMD weakens the dystroglycan complex, increasing the susceptibility of muscle fibers to contraction-induced injuries.4

The sympathetic nervous system is a major regulator of muscle mass mediated by β-adrenoceptors (ARs).5 β2-ARs are predominantly expressed in airways and skeletal muscle. Chronic injections of β2-selective agonist induce skeletal muscle hypertrophy through cAMP and the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway.6 New generation of β2-agonists, such as formoterol, has a significant anabolic effect at micromolar concentrations7 and protects against contraction-induced muscle damage in mdx dystrophic mice.8 However, chronic treatment for months with β2-agonists is associated with some undesirable adverse effects, including increased heart rate and heart hypertrophy.9 Moreover, β2-agonists can also activate the receptor-activator of NF-κB (RANK), the receptor-activator of NF-κB ligand (RANKL), a key promoter of osteoclastogenesis and bone remodeling.10 The third protagonist, osteoprotegerin (OPG), binds to RANKL, exerting an inhibitory effect on the preosteoclastic differentiation process.11

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Thus, the potential adverse effects on heart and bone limit the use of β2-agonists for the treatment of DMD.12

Our laboratory has previously demonstrated that OPG—immunoglobulin fragment complex (OPG-Fc) treatment mitigates and restores function of fast-twitch muscles in young mdx mice.13 Because β2-agonists, as opposed to OPG-Fc, are effective for enhancing or preventing the loss of function of slow-twitch muscles, we tested whether a combined treatment of OPG-Fc with a low dose of formoterol (10 μg/kg per day i.p.) would be superior to OPG-Fc and β2-agonist alone. We show that OPG-Fc and a low dose of formoterol act synergistically on force production of slow-twitch soleus (Sol), but not on fast-twitch extensor digitorum longus (EDL) of mdx mice. We also showed that the β2-AR content of dystrophic Sol and EDL muscles was significantly lower than that of control muscles and that OPG-Fc rescued the β2-AR content of dystrophic Sol and EDL muscles. The combination of these two pharmacological agents provides a new and potentially effective treatment for rescuing the functions of slow- and fast-twitch dystrophic muscles and may counteract the negative effects of β2-agonists on bone.

Materials and Methods

Animals

Male wild-type (C57BL/10ScSnJ) and mdx dystrophic (C57BL/10ScSn-Dmdmdx/J) mice, bred in our animal facility and weighing 14 to 15 g, were used for this protocol. Food and water were provided ad libitum. Mice were injected i.p. with OPG-Fc (1 mg/kg per d; R&D Systems, Minneapolis, MN) or formoterol (10 or 100 μg/kg per d, β2-agonist; Sigma-Aldrich, Oakville, ON, Canada) alone or in combination for 10 days starting at 25 days of age. Changes in body weight and fluid consumption were adjusted to maintain the effective dose. Mice injected with equal volumes of phosphate-buffered saline were used as control. At the end of the different experimental procedures, mice were euthanized by cervical dislocation under anesthesia. All of the procedures were approved by the Universtes Laval Research Center Animal Care and Use Committee on the basis of the Canadian Council on Animal Care guidelines.

Isometric Contractile Properties

Mice received buprenorphine as an analgesic (0.1 mg/kg) at least 15 minutes before being anesthetized with pentobarbital sodium (50 mg/kg). The right Sol and EDL were dissected and incubated in a buffered physiological salt solution (Krebs-Ringer), as previously described by Dufresne et al.14 The maximum tetanic tension (P0, g) values were obtained using a 305B-LR dual-mode lever arm system controlled by dynamic muscle control and data acquisition software (Dynamic Muscle Data Analysis software version 6.1; Aurora Scientific, Aurora, ON, Canada), and the maximum specific tetanic tension (sP0, N/cm²) was obtained by dividing the wet weight by the optimal muscle length, multiplied by the muscle density (1.06 g/cm³), multiplied by the fiber/muscle length ratio. At the end of the contractile property measurements, the tendons were removed, and the muscles were weighed, embedded in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC), frozen in liquid nitrogen, and stored at −80°C until used for the immunohistochemical and immunofluorescence assays.

Immunohistochemical and Immunofluorescence Analyses

Transversal sections (10 μm thick) of Sol and EDL were immunolabeled for 2 hours at room temperature with anti–pan-macrophage F4/80 (1:100; Serotec, Oxford, UK) or anti-neutrophil Ly-6G and Ly-6C (Gr-1) antibodies (1:300; BD Pharmingen, Mississauga, ON, Canada) to identify macrophages and neutrophils, respectively. Positive cells were counted at ×400 magnification, and the total areas of the sections were determined and multiplied by the thicknesses to express the number of each cell type per cubic millimeter. The concentrations of inflammatory cells were measured in duplicate in two different sections of muscles. Fiber typing and areas occupied by type I, IIA, IIX, and IIB fibers were determined, as described by Schiaffino et al.15 Briefly, muscle sections were incubated with the following antibodies: anti-MyHC I (Novus Biological, Oakville, ON, Canada), anti-MyHC IIA (SC-71; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-MyHC IIX (6H1; Developmental Studies Hybridoma Bank), anti-MyHC IIB (BF-F3; Developmental Studies Hybridoma Bank), and Alexa Fluor secondary antibodies (Invitrogen, Waltham, MA). The myofiber cross-sectional area of each fiber type was analyzed with ImageJ software version 1.41 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

Hematoxylin and Eosin Staining and Serum Creatine Kinase Assays

Hematoxylin and eosin staining (Sigma-Aldrich) was used to visualize the number of centrally nucleated fibers and muscle integrity and quantify the extent of muscle damage on approximately 100 myofibers per muscle. The damaged area was defined as an area not occupied by normal or regenerating muscle fibers. Sections were examined with an inverted microscope (Nikon, Mississauga, ON, Canada) and analyzed with ImageJ software version 1.41.

Blood collection was performed by cardiac puncture, and samples were centrifuged at 10,000 × g at 4°C for 10 minutes. The serum creatine kinase activity was determined according to the manufacturer’s instructions (BioVision, Milpitas, CA). The activity was expressed as U/L and used to detect indirectly muscle damage.

Western Blots

For Western blotting, Sol and EDL muscles from mice treated for 10 days with OPG-Fc (1 mg/kg per day) or

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formoterol (10 or 100 µg/kg per day) alone or in combination with OPG-Fc were injected into mdx mice for 10 days. The data were analyzed by two-way analysis of variance (ANOVA) to determine whether the variations among the experimental groups were significant. InStat software version 3.1 from GraphPad (La Jolla, CA) was used to perform the analysis. A posteriori test was performed with a Turkey protected least-significant differences test. The level of significance was set at P < 0.05.

**Results**

**OPG-Fc Rescues the Expression of β2-Adrenergic Receptor**

The β2-AR content was significantly reduced by 17% and 28%, respectively, in dystrophic SOL and EDL muscles compared with wild-type muscles. OPG-Fc treatment completely rescued β2-AR expression in both dystrophic muscles, whereas the low and high doses of formoterol treatment had no significant impact on β2-adrenergic receptor content in dystrophic muscles (Figure 1).

**OPG-Fc Acts Synergistically with Formoterol to Enhance Dystrophic Slow-Twitch Muscle Function**

We next investigated the effect of OPG-Fc and/or low or high doses of formoterol on the contractile properties of dystrophic muscles.
Osteoprotegerin (OPG)–immunoglobulin fragment complex (OPG-Fc) and formoterol significantly improve muscle integrity and reduce muscle inflammation in slow-twitch Sol muscles of mdx mice. mdx mice were injected with phosphate-buffered saline (PBS) or OPG-Fc and/or formoterol for 10 days and included five groups: i) mdx PBS-injected mice (mdx PBS), ii–iii) mdx formoterol-injected mice at two different doses, 10 or 100 μg/kg (mdx FORMO-10 and mdx FORMO-100, respectively), iv) mdx OPG-injected mice at a dose of 1 mg/kg, or v–vi) mdx injected with a combination of OPG and two different doses of formoterol (mdx FORMO-10 + OPG and mdx FORMO-100 + OPG). Representative hematoxylin and eosin staining (A), quantification of muscle damage (B), serum creatine kinase (CK) activity (C), and percentage of centrally nucleated fibers of Sol (D) and EDL (E) showed that OPG-Fc (1 mg/kg per day) or the high dose of formoterol (100 μg/kg per day) or OPG-Fc plus a low dose of formoterol (10 μg/kg per day) reduce cell infiltration and damage in dystrophic Sol muscles. Immunohistochemical analyses demonstrated that the numbers of neutrophils (anti-Ly6C/Ly6G; F) and pan-macrophages (anti-F4/80; G) are reduced significantly after OPG-Fc and/or formoterol treatments. Healthy muscles from wild-type mice are shown as controls. The dotted line is the visual representation of wild-type (WT) data. All data are expressed as means ± SEM (B–G). n = 6 to 8 independent experiments per group (B–G). *P < 0.05, **P < 0.01. Scale bar = 100 μm (A).
dystrophic Sol and EDL muscles (Figure 2). A high dose of formoterol (100 µg/kg) increased the maximum specific force of dystrophic Sol and EDL muscles by 100% and 65%, respectively, whereas a low dose (10 µg/kg) had no significant effect. OPG-Fc (1 mg/kg) was significantly superior to any dose of formoterol, increasing the maximum specific force of dystrophic EDL muscles by 120%. However, the effect of OPG-Fc alone on the function of dystrophic Sol muscles was similar to that of the high dose of formoterol (100 µg/kg; 81% gain in force). More important, the combination of a low dose of formoterol (10 µg/kg) plus OPG-Fc (1 mg/kg) had a synergistic effect that was significantly superior to OPG-Fc alone in restoring the function of dystrophic Sol muscles (Figure 2). Interestingly, the effect of a high dose of formoterol plus OPG-Fc was similar to that of a low dose of formoterol plus OPG-Fc on force production by dystrophic Sol muscles. On the other hand, the effect of OPG-Fc alone on dystrophic EDL muscles was significantly superior to any dose of formoterol, whereas the combination of the two pharmacological agents did not provide any additional benefit for dystrophic EDL muscles. Like specific force, the maximum absolute isometric tetanic force (P0) showed similar differences between groups for Sol and EDL muscles (Supplemental Table S1). However, OPG-Fc and/or low or high doses of formoterol had no significant impact on time-to-peak twitch tension, half-relaxation time, muscle mass, and body weight (Supplemental Table S1).

**OPG-Fc Is as Effective as Formoterol in Reducing Muscle Damage and Inflammation**

The dystrophic features (centrally nucleated myofibers, necrotic myofibers, and serum creatine kinase) were considerably reduced in dystrophic mice treated with OPG-Fc (1 mg/kg) or a high dose of formoterol or OPG-Fc and a low dose of formoterol (Figure 3, A–E). However, treatments had no impact on myofiber size and phenotype in Sol and EDL muscles (Supplemental Tables S2 and S3). Because chronic inflammation is a major problem in muscular dystrophy and contributes significantly to disease progression in DMD, we next compared the efficacy of OPG-Fc, formoterol, and both combined in reducing neutrophil and macrophage infiltrations. Consistent with the extent of muscle damage and creatine kinase activity, the number of neutrophils and macrophages was significantly reduced in dystrophic muscles from mice treated with OPG-Fc or a high dose of formoterol or OPG-Fc combined with a low dose of formoterol compared with phosphate-buffered saline–treated mdx mice (Figure 3B). In terms of muscle damage and inflammation, the combined treatment of OPG-Fc with a low dose of formoterol was not superior to OPG-Fc or a high dose of formoterol alone.

**Discussion**

The RANK/RANKL/OPG pathway and the β-adrenergic system communicate and regulate bone homeostasis.10 RANKL/RANK interactions and adrenergic stimulation also control Ca2+ content and mobilization as well as SERCA [sarco(endo)plasmic reticulum Ca2+ ATPase, an ATP-dependent Ca2+ pump] activity in skeletal muscles.16,17 The loss of Ca2+ homeostasis leads to muscle degeneration, inflammation, and fibrosis, a signature of muscular dystrophy.18 The present study provides compelling evidence that
the combination of a low dose of formoterol plus OPG-Fc is sufficient to rescue the function of slow- and fast-twitch dystrophic skeletal muscles during the first peak of muscle degeneration at 3 to 5 weeks of age in mdx mice.

Previous studies have shown that 1000 mg/kg per day of clenbuterol\textsuperscript{19} or 25 to 100 mg/kg per day of formoterol,\textsuperscript{20} given for 4 weeks, is required to induce muscle hypertrophy and protect against muscular dystrophy. Mechanistically, $\beta_2$-agonists activate, among others, protein kinase A, which phosphorylates phospholamban on serine 16 and activates SERCA ($\beta_2$-agonists–protein kinase A–SERCA pathway).

Interestingly, our findings showed that low doses of OPG-Fc potentiate the positive effects of formoterol on slow-twitch Sol muscles in young mdx mice. Indeed, the combination of formoterol and OPG-Fc increased the force production of dystrophic Sol muscles to a greater extent than the sum of the effects of OPG-Fc and formoterol alone. However, the possible cross talk between OPG-Fc and formoterol in skeletal muscles is poorly understood. Given that OPG-Fc increased the density of $\beta_2$-AR and thus the number of potential binding sites for formoterol, it is possible that OPG may strengthen the downstream effect of $\beta_2$-agonists on phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin signaling and/or the cAMP-dependent pathway by promoting protein synthesis and SERCA activity (Figure 4). However, the fact that OPG-Fc alone was largely superior to any dose of formoterol in rescuing fast-twitch dystrophic EDL muscles suggests that the inhibition of RANKL/RANK interactions by OPG-Fc is important in preventing subsequent NF-kB activation and the progression of muscular dystrophy\textsuperscript{21} (Figure 4). OPG-Fc reduced the number of neutrophils and macrophages in dystrophic muscles but had no effect on muscle force production by healthy mice, suggesting that a pathological state is required for OPG-Fc to be effective. Although the mechanism by which OPG-Fc and formoterol work requires further investigation, our findings provide the first evidence that much less formoterol concentration is required to obtain the same beneficial effect in skeletal muscles, with potentially no or fewer adverse effects on heart and bone functions.

The present study convincingly shows that a low dose of formoterol combined with OPG-Fc acts synergistically on force production of Sol muscles and mitigates muscular dystrophy. The effects of prolonged OPG-Fc and $\beta_2$-agonist treatments should be investigated in a more severe mouse model of muscular dystrophy. Because osteoporosis and muscle atrophy/degeneration worsen in tandem during neuromuscular diseases and because OPG-Fc has a beneficial effect on skeletal muscle and bone functions, we have high hopes that this combined treatment may open the way for new treatments for DMD patients.

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S.S.D. and J.F. conceived the project and its design; S.S.D. performed experiments and data analysis; S.S.D., A.B.-P., and J.F. wrote the manuscript; and all authors checked for scientific content and approved the final manuscript.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2016.11.006.

**References**


