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Reduced gravitational loading does not account for the skeletal effect of botulinum toxin-induced muscle inhibition suggesting a direct effect of muscle on bone

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ABSTRACT

Intramuscular injection of botulinum toxin (botox) into rodent hindlimbs has developed as a useful model for exploring muscle–bone interactions. Botox-induced muscle inhibition rapidly induces muscle atrophy and subsequent bone loss, with the latter hypothesized to result from reduced muscular loading of the skeleton. However, botox-induced muscle inhibition also reduces gravitational loading (as evident by reduced ground reaction forces during gait) which may account for its negative skeletal effects. The aim of this study was to investigate the skeletal effect of botox-induced muscle inhibition in cage control and tail suspended mice, with tail suspension being used to control for the reduced gravitational loading associated with botox. Female C57BL/6J mice were injected unilaterally with botox and contralaterally with vehicle, and subsequently exposed to tail suspension or normal cage activities for 6 weeks. Botox-induced muscle inhibition combined with tail suspension had the largest detrimental effect on the skeleton, causing the least gains in midshaft tibial bone mass, cortical area and cortical thickness, greatest gains in midshaft tibial medullary area, and lowest proximal tibial trabecular bone volume fraction. These data indicate botox-induced muscle inhibition has skeletal effects over and above any effect it has in altering gravitational loading, suggesting that muscle has a direct effect on bone. This effect may be relevant in the development of strategies targeting musculoskeletal health.

Introduction

Skeletal tissue adapts to its mechanical environment by transducing mechanical stimuli into a cellular response via a process referred to as mechanotransduction [1]. The mechanical stimulus driving mechanotransduction is believed to derive from two primary sources—exogenous gravitational forces and endogenous muscle forces. Debate persists as to the relative roles of these two forces in mechanotransduction and their subsequent contributions to skeletal health [2–4]. In particular, the contribution of muscle forces to skeletal health has proven difficult to elicit as muscle and bone are inextricably linked genetically, mechanically, and molecularly.

Focusing on the mechanical link between muscle and bone, muscle-derived forces have been suggested to be both causative and protective of bone loading. Muscles attach close to axes of motion and thereby have small lever arms. They consequently need to generate and transmit high forces to the skeleton in order to produce a desired torque at the end of a lever (i.e. bone). It has subsequently been proposed that muscle-derived forces are the primary source of mechanical loading for bone [5,6], providing not only peak loads that generate the highest bone strains, but also low-magnitude high-frequency stimuli to which bone tissue may also respond [7].

It has also been hypothesized and modeled that muscle is protective of bone loading [8,9]. During impact loading, muscle is believed to act as an active shock absorber helping to attenuate impact loads as they are transmitted proximally along the kinetic chain. When muscles are dysfunctional (weakened, fatigued, or altered in their activation patterns) their ability to absorb loads becomes compromised, potentially leading to increased loading on the skeleton. For instance, laboratory-based studies have shown that muscle fatigue increases bone loading, as indicated by elevated bone strain magnitudes and rates [10–13]. Similarly, cross-sectional and prospective clinical studies report that susceptibility to mechanical overload-induced skeletal injury (i.e. stress fracture) is heightened in individuals with reduced indices of muscle performance [14–18].

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A novel means of exploring the muscle–bone interaction has been to study the skeletal effects of intramuscular botulinum toxin (botox) injection. Botox blocks neuromuscular transmission by inhibiting the release of acetylcholine leading to locally reduced muscle activity, and resultant muscle atrophy and reduced strength. Previous studies have consistently demonstrated negative skeletal effects of botox-induced muscle inhibition in rodent models [19–32]. However, botox injected into the hindlimb muscles of rodents also reduces exogenous gravitational loading, as indicated by reduced vertical ground reaction forces (GRFs) during gait [27]. It remains unclear whether the reduction in GRFs associated with botox accounts for its skeletal effects. Manske et al. [25] contributed to this question by exploring the skeletal effects of combined Achilles tenotomy and botox-induced muscle inhibition; however, tenotomy incompletely controls for the effects of exogenous gravitational forces as partial weight bearing remains possible.

The aim of the current study was to investigate whether reduced gravitational loading accounts for the skeletal effects of botox-induced muscle inhibition by exploring the effects of botox in cage control and tail suspended mice. Tail suspension effectively removes exogenous loading of the hindlimbs (i.e. GRFs) such that all skeletal loading in the hindlimbs of tail suspended animals is generated endogenously by muscle. If the skeletal changes occurring following botox injection are due to the associated decrease in GRFs there would be no difference between muscle intact and botox inhibited hindlimbs in tail suspended animals. Conversely, if botox-induced muscle inhibition directly influences bone, skeletal status in muscle intact and inhibited hindlimbs of tail suspended animals would differ.

Methods

Animals

Forty virgin female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were acclimated until 14 weeks of age. Animals were maintained under standardized environmental conditions at all times with ad libitum access to standard mouse chow and water. Procedures were performed with approval from the Institutional Animal Care and Use Committee of Indiana University.

Muscle intervention

The right hindlimb of each animal was injected at baseline with *Clostridium botulinum* type A neurotoxin (BOTOX®; Allergan, Inc. Irvine, CA) (botox group). On the day of use, 100 U of botox was reconstituted in 4 ml of 0.9% sterile saline to create a solution with 0.025 U/μl. Animals were anesthetized using inhalation anesthesia, and the right quadriceps, hamstring, gastrocnemius and tibialis anterior muscles were injected with 20 μl (5 μl/muscle group) using a 25 μl Hamilton syringe equipped with a 30 G needle (Hamilton Co., Reno, NV). The left quadriceps, hamstring, gastrocnemius and tibialis anterior muscles were injected with an equivalent volume of 0.9% sterile saline (vehicle group) and served as internal controls.

Gravitational intervention

Immediately following muscle intervention, animals were randomly divided into two activity groups: 1) cage control and 2) tail suspended. The cage control group was allowed normal cage activities throughout the study, whereas the tail suspended group was tail suspended continuously for 6 weeks as previously described [33]. Half of a metal paper clip was made into a U-shape and its open end attached to the sides of the mouse tail with superglue. The rounded end of the paperclip was attached to a swivel and hung from an overhead wire. The wire height was adjusted to maintain the mice at 30° of head down tilt so that the hindlimbs but not forelimbs were elevated above the cage floor. The swivel allowed animal pivoting and slid freely on the wire to permit side-to-side movements. The cage floor was sparsely lined with bedding to absorb excretions. Suspended animals were floor fed.

In vivo peripheral quantitative computed tomography

In vivo skeletal and muscle assessments were performed under inhalation anesthesia at baseline and following 6 week intervention using a peripheral quantitative computed tomography (pQCT) machine equipped with software version 6.20C (Stratec XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany). Following a scout scan, a transverse midshaft scan was taken of each tibia using a 70 μm voxel size. This voxel size is relatively large compared to the cortical thickness of the mouse tibial midshaft increasing the potential for partial volume effects. However, good agreement has previously been shown between pQCT, micro-computerized tomography (μCT) and histological measures of cortical bone properties in mice [34].

Bone properties were obtained by placing a region of interest around the tibia and assessing using cortical mode 1 with a threshold of 400 mg/cm^3. Total bone mineral content (BMC, mg/cm), total bone area (Tt.Ar, mm^2), cortical area (Ct.Ar, mm^2), cortical thickness (Ct.Th, mm) and polar moment of inertia (I_P, mm^4) were recorded, and medullary area (Me.Ar, mm^2) derived as Tt.Ar minus Ct.Ar. Muscle cross-sectional area (mCSA, cm^2) was assessed by placing a region of interest around the entire leg (including the posterior, lateral and anterior muscle compartments), and using contour mode 3 (threshold, −100 mg/cm^3) to locate the skin surface and peel mode 2 (threshold, 40 mg/cm^3) to locate the subcutaneous fat–muscle boundary. A 3×3 kernel filter to filter all voxels between −500 and 500 mg/cm^3 followed by a 5×5 kernel filter to filter all voxels between −500 and 300 mg/cm^3 (F03F05 filter) was used to remove noise. All in vivo pQCT measures were expressed as percent change from baseline ([final − baseline]/baseline×100).

Ex vivo micro-computed tomography

Animals were euthanized following 6 week intervention (animal age = 20 weeks), and the right and left tibias dissected free and placed in 10% neutral buffered formalin for 48 h before being stored in 70% ethanol. A desktop μCT machine (SkyScan 1172 high-resolution μCT; SkyScan, Kontich, Belgium) scanning with a source voltage of 59 kV and 11.8 μm isotropic voxel size was used to assess cortical bone properties at the midshaft tibia and trabecular bone properties within the proximal tibial metaphysis. For cortical bone properties, a single

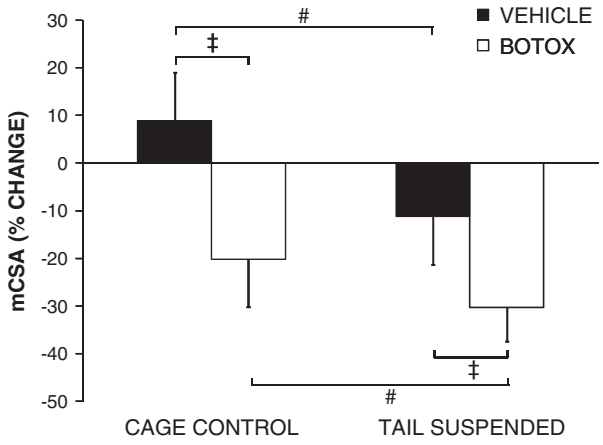

Fig. 1. Effect of gravitational and muscle interventions on in vivo percent change in muscle cross-sectional area (mCSA) at the level of the midshaft tibia. There was a significant gravitational×muscle group interaction ($p<0.01$). ‡Botox reduced gains in mCSA in both cage control and tail suspended animals and #tail suspension reduced gains in mCSA in both vehicle and botox injected hindlimbs (all $p<0.0125$). Bars represent mean±SD.