COX2-independent and COX2-dependent effects of naproxen on bone quality, osteocytes, and fatigue fracture healing in male and female mice

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INTRODUCTION: Non-steroidal anti-inflammation drugs (NSAIDs) are the most commonly consumed medication in the world. These medications effectively relieve pain, inflammation, and fever through the inhibition of cyclooxygenase enzymes, COX1 and/or COX2. COX1 is constitutively active and critical for several physiological functions, such as gastric cytoprotection, whereas COX2 expression is induced for prostaglandin synthesis in scenarios of inflammation, injury, and disease. Nonetheless, growing evidence in both clinical and preclinical studies has linked regular NSAID use to higher stress fracture risk, through mechanisms including impaired strain-adaptive bone remodeling as well as loss of bone toughness. However, it is not clear if these effects are due to on-target COX2 inhibition or off-target COX2-independent signaling. To investigate this knowledge gap, we used Ptgs2-Y385F mice, which lack cyclooxygenase 2 (COX2) enzyme activity, to determine which effects of naproxen that increase stress fracture risk are COX2-independent. Specifically, we hypothesized that naproxen increases fatigue fracture risk by diminishing strain adaptive bone remodeling through COX2-dependent mechanisms as well as decreasing bone toughness through COX2-independent mechanisms that influence osteocytes and their dendritic networks.

METHODS: All animal procedures were approved by the Thomas Jefferson University IACUC (Protocol #01919). Ptgs2-Y385F (Strain #008101) and C57BL/6J (Strain #000664) mice were obtained from Jackson Laboratory. NSAIDs were administered through drinking water, with treatment groups including vehicle (ddH_2O), low dose naproxen sodium (10.9 mg/kg/day), or high dose naproxen sodium (41.6 mg/kg/day), assuming consumption of 4 mL of water/day/mouse. Axial forelimb compression was used to induce lamellar bone formation using 100 cycles of a 2 Hz rest-inserted sinusoidal waveform with a peak force of 3.0 N. Separately, axial forelimb compression of 3.3N until 75% of the total displacement needed for fracture (relative to the 10^{th} cycle) was used to generate fatigue fractures. Bone mass and density were quantified by microCT (Bruker Skyscan 1275, 1 mm Al filter, 55 kV, 181 uA, 74ms exposure, 13 µm voxel), with trabecular and cortical regions analyzed separately. Three-point bending was used to assess mechanical properties, with stress-strain conversion using microCT-based geometry. Calcein (10 mg/kg) and alizarin red (30 mg/kg) injections (IP) were performed on days 5 and 12, with PMMA sections imaged by confocal microscopy for dynamic histomorphometry according to ASBMR standards. Collagen was evaluated in paraffin-embedded tibias stained with picrosirius red and imaged with a circularly polarized light microscope. Osteocyte dendrites were visualized by staining frozen sections with phalloidin conjugated to AlexaFluor 488, then quantified using manual and custom semi-automated methods. Sample size varied between experiments (range: 8 to 38). Male and female mice were analyzed separately and are both presented (full circles for females, empty circles for males).

RESULTS: First, we characterized the skeleton of the Ptgs2-Y385F mouse model. Using microCT and standard three-point bending analysis of unfixed, non-loaded femurs, we found that Ptgs2-Y385F mice are comparable to WT littermates at skeletal maturity (16-18 weeks of age) in bone length, size, and mechanical performance. We note WT and Ptgs2-Y385F mice also had similar body mass and appear generally healthy, unlike COX2 null mice. We also assessed aged male mice (52 weeks) – here, the effect of age on cortical bone geometry (polar moment of inertia) and mechanical performance (ultimate moment) was stronger in Ptgs2-Y385F mice as compared to WT controls. Next, we quantified bone formation in response to six bouts of uniaxial forelimb compression over a period of 15 days. Here, relative measures of periosteal and endosteal bone formation parameters – including mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS) – were not significantly different between genotypes. Next, we used Ptgs2-Y385F mice to determine the COX2-dependent and COX2-independent effects of naproxen. To do so, Ptgs2-Y385F and WT mice received naproxen sodium (10.9 mg/kg) or vehicle drinking water for 15 days in addition to the 6 bouts of non-damaging axial forelimb compression. Here, WT mice displayed the expected decrease in relative periosteal bone formation rate per bone surface (rPs.BFR/BS) with treatment, but Ptgs2-Y385F mice did not. In contrast, three-point bending demonstrated that naproxen significantly decreased ultimate bending energy and toughness in both genotypes (**Fig. 1**). Surprisingly, WT female mice treated with ~3.8x higher dose of naproxen (41.6 mg/kg/day) lost the same amount of toughness as those treated with the lower dose. To determine the mechanism of this COX2-independent effect of naproxen, we assessed non-loaded tibias from Ptgs2-Y385F and WT mice of both sexes histologically. Here, we observed that osteoblast number per bone perimeter in trabecular bone was significantly increased with naproxen treatment in WT mice but not Ptgs2-Y385F mice. However, naproxen increased empty osteocyte lacunae in both genotypes. In addition, the number of fluorophore-labeled osteocytes near the endosteal, but not the periosteal, surface of ulnar bone was significantly increased by naproxen treatment in WT mice. Using picrosirius red stained slides, we found that naproxen treatment significantly altered collagen fibril thickness in only Ptgs2-Y385F mice. In both genotypes, we found altered osteocyte dendritic networks in response to naproxen treatment using Phalloidin staining. Finally, naproxen pre-treatment of fatigue fractures had modest and sexually dimorphic effects – female WT mice given naproxen before injury trended towards a decreased cycle number to fracture compared to vehicle, consistent with diminished bone toughness. Furthermore, continued treatment during fracture healing only decreased bone callus volume in female WT and Ptgs2-Y385F mice.

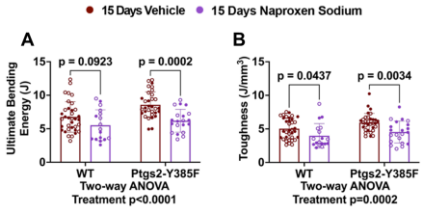

Figure 1. Naproxen negatively affects ultimate bending energy and toughness in both WT and Ptgs2-Y385F mice.

DISCUSSION: Regular non-steroidal anti-inflammatory drug (NSAID) use increases stress fracture risk, but the mechanisms remain unclear. Using Ptgs2-Y385F mice, which lack COX2 activity, we tested whether naproxen acts through COX2-dependent or COX2-independent pathways. Naproxen reduced strain-adaptive bone formation only in WT mice, consistent with a COX2-dependent effect, but decreased bone toughness in both genotypes, indicating a COX2-independent mechanism. Naproxen also altered osteocyte dendritic networks in a sexually dimorphic manner and produced mild, sex-specific effects on fatigue fracture initiation and healing, with females showing greater detriment. Collectively, these findings demonstrate that naproxen compromises skeletal health through both COX2-dependent and COX2-independent mechanisms. Therefore, regular use of both selective and non-selective NSAIDs may increase risk for skeletal injury, underscoring the need for new pain management strategies. Our future work will focus on determining whether NSAID cessation can reverse or mitigate skeletal alterations as well as assess how naproxen impacts fracture callus composition and long-term healing outcomes.

SIGNIFICANCE/CLINICAL RELEVANCE: The NSAID naproxen can affect bone through both COX2-dependent and COX2-independent mechanisms. Collectively, the data suggest that chronic administration of NSAIDs, whether non-selective or COX2-selective, may elevate the risk of skeletal fatigue injuries. As a result, NSAID use should be minimized in individuals at high risk for fatigue-related skeletal injuries and alternative drug classes or administration methods without these effects in bone must be uncovered to address musculoskeletal pain for these individuals.