DISSEMINATION APPROVED BY

January 8, 2015

Diane M. Cullen – Major Advisor, Ph.D.

John A. Yee - Committee Member, Ph.D.

Philip R. Brauer - Committee Member, Ph.D.

Mohammed P. Akhter - Committee Member, Ph.D.

David H. Nichols - Committee Member, Ph.D.

Gail M. Jensen, Ph.D., Dean
BONE RESPONSE TO CYCLOOXYGENASE INHIBITION
AND MECHANICAL LOADING

By
BRYAN HACKFORT

A DISSERTATION

Submitted to the faculty of the Graduate School of Creighton University in Partial Fulfillment of the Requirements for the degree of Doctor of Philosophy in the Department of Biomedical Sciences

Omaha, NE
January 8, 2015
Abstract

Bone is a dynamic tissue constantly adapting to daily mechanical forces. Suppressing the bone’s ability to adapt to increased forces may lead to insufficient strength and ultimately fracture. Non-steroidal anti-inflammatory drugs (NSAIDs) block the bone formation response to a single load. However, the effect of chronic NSAID use on the bone formation response to multiple loads is unknown. Since Cox-2 inhibitors are used for treating chronic pain, it is essential to investigate their effects on bone. NSAIDs inhibit the cyclooxygenase (Cox) enzyme which is the rate limiting step in prostaglandin E₂ (PGE₂) production and a key step in the bone response to loading. PGE₂ activates downstream signaling molecules of the low-density lipoprotein receptor related protein 5 (Lrp5) pathway independent of Lrp5 receptor activation. The Lrp5 pathway is a primary mediator of mechanical loading in bone. To better understand the interaction of the PGE₂ and Lrp5 pathways, Lrp5⁻/⁻ and gain of function mice (HBM) were used to test the effects of chronic Cox-2 inhibition with loading.

The results confirmed that Cox inhibitors block the bone formation response to a single load; however, chronic Cox-2 inhibition did not suppress the bone formation response to multiple loads. Furthermore there was no interaction between Cox-2 inhibition and varying levels of Lrp5 expression.

Cox-2 is the primary isoform in the bone response to a single mechanical load. To investigate the difference in response to a single versus multiple loads, Cox-1 and Cox-2 gene responses were measured. Cox-2 gene expression in osteocytes increased after loading while Cox-1 gene expression did not. However, immunohistochemistry showed
cells expressing Cox-1 increased along the periosteal and endocortical surfaces following multiple loads.

In conclusion, Cox inhibition suppressed the bone formation response to a single load but not to multiple loads. Osteocyte Cox-1 gene expression was not upregulated to compensate for Cox-2 inhibition but Cox-1 activation along the periosteal and endocortical surfaces may play a role in the adaptive response to loading. While Cox-2 is important in the initial response to loading, Cox-1 may play a role in bone adaptation to multiple loading sessions.
Acknowledgements

I would like to thank the many people that helped me get through this fun and challenging time. Without their endless hours of help and support, this would not have been possible.

First I would like to thank my major advisor and mentor, Dr. Diane Cullen. Your support has been constant and unwavering. You have taught me the critical skills necessary to conduct scientific experiments, analyze the results, and to “see the big picture”.

I would like to thank my committee members for their continuous advice and support. Drs. Jack Yee and Phil Brauer, you have taught me the importance of critical reading and reviewing articles and I appreciate your assistance in trouble-shooting my projects. Dr. Mohammed Akhter, thanks for your knowledge on bone biomechanics and for your assistance in maintaining the equipment. Dr. David Nichols, thanks for the questions and for challenging me to think about alternative approaches to obtain my goals.

I am grateful for all of the technicians and students that have made working in the laboratory fun. Thank you for all of your help with the loading studies and for all of your support, answers, and insight through the many years.

I would like to thank my family. To my parents, your support has been truly amazing. You have taught me many things but most importantly that I can accomplish anything if I’m willing to work for it. I would like to thank my mother and father in-law for all of your support and assistance with the kids. A special thanks to my brothers, brothers-in-
law, and sisters-in-law for your calls of support and your unrelenting ability to ask “is it done yet?”

Finally, I would like to thank my wife Mandy, you are my rock and without you I don’t know where I would be today. I am truly blessed you are a part of my life. Thanks to my two little miracles, Charlotte and Thomas. You have brought me so much joy and have provided the last bit of motivation needed to finish.
Contents

Abstract .................................................................................................................. iii
Acknowledgements ................................................................................................... v
List of Figures .......................................................................................................... x
List of Tables ............................................................................................................. xi
1. General Introduction ............................................................................................... 1
   1.1 Bone Cells ........................................................................................................ 2
   1.2 Bone Formation ............................................................................................... 3
   1.3 Mechanical Loading ........................................................................................ 5
       1.3.1 Loading Models ....................................................................................... 6
       1.3.2 Bone Response to Mechanical Loading .............................................. 9
       1.3.3 Bone Cell Response to Mechanical Loading .................................. 11
   1.4 Prostaglandins ............................................................................................... 12
       1.4.1 Cyclooxygenase and Prostaglandin E2 Synthesis ................................ 13
       1.4.2 The Effect of Prostaglandins on Bone .............................................. 14
       1.4.3 Prostaglandins and Mechanical Loading ........................................ 15
       1.4.4 Cyclooxygenase Inhibition ................................................................. 16
   1.5 Wnt Signaling ............................................................................................... 18
   1.6 PGE2 Interaction With β-Catenin .................................................................. 20
   1.7 Project Purpose and Goals ........................................................................... 22
2. Cyclooxygenase Inhibition on Single Versus Multiple Mechanical Loads in Mice
   2.1 Abstract: ......................................................................................................... 24
   2.2 Introduction ..................................................................................................... 25
   2.3 Materials and Methods ................................................................................... 27
2.3.1 Animals ........................................................................................................... 27
2.3.2 Tibial Compression .................................................................................. 27
2.3.3 Histomorphometry ................................................................................ 29
2.3.4 Micro-Computed Tomography (Micro-CT) Analysis .................... 30
2.3.5 Statistical Analysis ............................................................................... 30
2.4 Results ............................................................................................................. 31
2.5 Discussion ........................................................................................................ 37

3. Gene Response to Chronic Cox-2 Inhibition and Multiple Mechanical Loads ... 41
3.1 Abstract .......................................................................................................... 41
3.2 Introduction ..................................................................................................... 42
3.3 Materials and Methods ................................................................................ 43
3.3.1 Animals ....................................................................................................... 43
3.3.2 Tibial Compression .................................................................................. 43
3.3.3 Drug Treatments ..................................................................................... 43
3.3.4 Histomorphometry ................................................................................ 44
3.3.5 Tissue Collection for Gene Analysis .................................................. 44
3.3.6 qPCR ........................................................................................................ 44
3.3.7 Statistical Analysis ............................................................................... 45
3.4 Results ............................................................................................................. 48
3.4.1 Histology Study ...................................................................................... 48
3.4.2 qPCR Results .......................................................................................... 49
3.5 Discussion ........................................................................................................ 50

4. Protein Response of Cox-1 and Cox-2 to Mechanical Loading ............... 54
List of Figures

Figure 1-1 Interaction of the β-catenin and prostaglandin E₂ (PGE₂) pathways 21
Figure 2-1 Periosteal and endocortical bone formation rate in response to a
single drug treatment and a single load 33
Figure 2-2 Periosteal and endocortical bone formation rates in response to Cox-2
inhibition and multiple loads 33
Figure 2-3 Micro-CT images of proximal tibia for Lrp5⁺/⁻, WT, and HBM mice 36
Figure 3-1 Periosteal and endocortical bone formation rates in response to daily
Cox-2 inhibition and ten sessions of tibial compression 48
Figure 4-1 Relative fold expression between loaded and non-loaded tibias 59
Figure 4-2 Percent of osteocytes with positive labeling for Cox-1 and Cox-2 61
Figure 4-3 Images of immunohistochemistry staining for Cox-1 and Cox-2 62
Figure 4-4 Percent of Cox-1 and Cox-2 staining along the periosteal and
endocortical surfaces 63
Figure A-1 Images of calcein labeling 84
List of Tables

Table 1-1 Inhibitory concentrations of indomethacin and NS-398 on Cox-1 and Cox-2 in different cell populations 17

Table 2-1 Bone formation response to a single load with Cox inhibition 32

Table 2-2 Characteristics of mice in the multiple load study 35

Table 2-3 Effect of multiple injections on bone formation parameters using DMSO or NS-398 35

Table 3-1 Characteristics of mice in the histology study 46

Table 3-2 Histology results using daily Cox-2 inhibition and multiple mechanical loads 47

Table 3-3 Initial and final weights for mice in gene expression study 50

Table 3-4 Relative fold changes in gene expression after tibial compression 51

Table 4-1 Primers and probe sets used to measure gene expression 60

Table 4-2 Weights and ages for mice in the gene expression and immunohistochemistry studies 60
1. General Introduction

Osteoporosis is a disease characterized by low bone mass and an increase in fragility fractures. Approximately two million osteoporotic fractures occur each year in the United States, with 25% of hip fracture patients not living past 6 months.\(^{(1,2)}\) Bone is a dynamic tissue that constantly adapts to the daily forces exerted on it. Increasing mechanical forces through exercise is the only natural anabolic stimulus for increasing bone mass in adults. Teriparatide, a parathyroid hormone analog, is the only Food and Drug Administration approved anabolic drug treatment for increasing bone mass. Small increases in bone mineral density can lead to large increases in fracture prevention. An 8% increase in bone mass following five weeks of rat ulna loading improved fatigue resistance > 100 fold compared to non-loaded limbs.\(^{(3)}\) Inhibiting the bone formation response to loading can be harmful as well. Treatments that prevent the bone from responding to mechanical forces block an important mechanism for bone to increase mass and adapt to daily activities.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly taken for extended periods for the symptomatic treatment of osteoarthritis, rheumatoid arthritis, muscle injuries, and many other types of chronic pain. While effective at providing pain relief, NSAIDs suppress the bone hypertrophic response to a single mechanical load in rats. With the increase in long-term use of Cyclooxygenase-2 (Cox-2) inhibitors, a specific class of NSAIDs, it is important to investigate the role of long-term Cox-2 inhibition in the bone response to multiple mechanical loads.
1.1 Bone Cells

Bone modeling and remodeling are processes involving multiple cells from the mesenchymal and hematopoietic stem cell lineages. There are three main cell types involved with maintaining structural and functional stability in bone: osteoblasts, osteocytes, and osteoclasts. The interaction and communication between these cell types is essential in bone growth and maintenance.

Depending on the local environment, mesenchymal stem cells have the ability to differentiate into multiple cell types including chondroblasts, adipocytes, fibroblasts, and osteoblasts. Osteoblasts are derived from osteoprogenitor cells located adjacent to the bone surface in the periosteum or in the marrow space. Bone lining cells on the surface of quiescent bone are predetermined cells from the osteoblast lineage that are activated to osteoblasts in response to an anabolic stimulus. Osteoblasts are activated in response to osteogenic signals such as mechanical forces, hormones, cytokines, and growth factors. Activated osteoblasts on the bone surface are cuboidal in shape with an extensive rough endoplasmic reticulum. Alkaline phosphatase is a key enzyme produced by pre-osteoblasts and osteoblasts and is used as a marker for osteoblast differentiation.

Osteoblasts are responsible for synthesizing osteoid and initiating mineralization. As the osteoid accumulates around the osteoblast, the cell has three options: (1) apoptosis, (2) revert to a bone lining cell, or (3) differentiate into an osteocyte.

Osteocytes are differentiated osteoblasts located within a lacunar space and are surrounded by mineralized bone matrix. They maintain the bone quality within the surrounding area and are likely the primary mechanosensing cell within bone. Properties of osteocytes that give rise to the idea they are the primary sensor to mechanical strain in
bone include: location, signaling channels, and cell structure. Osteocytes make up 90% - 95% of the bone cells and are located within the bone matrix. Osteocytes contain several receptors and channels that have been associated with mechanical responsiveness in other tissues including Ca^{2+} channels, parathyroid receptor 1 (PTHR1), a primary cilium, and prostaglandin receptors. In vitro fluid flow models have demonstrated osteocytes are more sensitive to strain than osteoblasts and fibroblasts. Furthermore, targeted ablation of osteocytes in transgenic mice decreases the bone response to mechanical unloading. Osteocytes have unique dendrite-like processes that extend within canaliculi and communicate with other cells including osteocytes, bone lining cells, osteoblasts, and osteoclasts.

Osteoclasts are derived from the hematopoietic stem cell lineage. They resorb bone matrix in response to damage, lack of use, certain hormones, and in the modeling of bone. Osteoclasts are large, multinucleated cells that produce a ruffled border during active resorption. They tightly attach to the surface of bone that is to be resorbed using αVβ3 integrin binding to create a sealed zone. Osteoclasts release hydrogen ions into the sealed zone to lower the pH which initiates the release of calcium phosphate from the matrix. Cathepsin K is a lysosomal enzyme released by the osteoclast within the sealed zone that digests type I collagen and other matrix proteins. A proper balance between osteoblast and osteoclast activities is important in maintaining bone mass.

1.2 Bone Formation

Bone formation occurs in a three step process: (1) proliferation and differentiation of osteoblast precursor cells into activated osteoblasts; (2) osteoblast secretion of unmineralized bone matrix called osteoid; (3) mineralization of osteoid with calcium.
phosphate forming hydroxyapatite (Ca$_5$(PO$_4$)$_3$(OH)) crystals. Osteoid is predominantly comprised of type I collagen but also contains other forms of collagen, glycoproteins, proteoglycans, and γ-carboxylated proteins. As mineralization occurs, osteoblasts that become embedded within the matrix differentiate into osteocytes.

Modeling and remodeling are two separate processes by which bone formation occurs. Modeling is the process of bone growth and shaping where osteoblasts and osteoclasts work independent of each other. Modeling primarily occurs during periods of bone growth, fracture repair, and changes in mechanical usage. Loading of bone is the application of force resulting in bone deformation or strain. Natural loads are created by impact forces during locomotion, stresses at insertion sites from muscle contraction, and gravity. Increasing the strain on a bone occurs by increasing forces, as in weight lifting, or by increasing the number of cycles the bone experiences as in running. Unloading is the removal of forces which occurs during bed rest or to lack of gravity as seen with space flight. Bed rest has been shown to reduce regional bone mineral density (BMD) measurements 1.4% in the hip and up to 10% in the calcaneus$^{(15)}$ while spaceflight results in a 0.9% total BMD loss per month and 1.4% BMD loss in the femoral neck per month.$^{(16)}$

Remodeling is the life-long process where osteoblast and osteoclast activity is coupled to maintain proper bone mass, strength and mineral homeostasis. Remodeling occurs during bone damage repair and in response to an imbalance in mineral homeostasis. In response to micro cracks and minor bone damage, osteoclasts target damaged bone and resorb the area containing the micro crack. Osteoblasts then replace the removed bone. An example of remodeling involving mineral homeostasis is the bone
response to parathyroid hormone (PTH). PTH is released by the parathyroid glands in response to low calcium. PTH binds to the parathyroid hormone receptor 1 (PTHR1) on osteoblasts and osteocytes which respond by releasing receptor activator of nuclear factor kappa-B ligand (RANKL), stimulating osteoclasts to resorb bone. When serum calcium levels return to normal, PTH decreases, resorption slows, and osteoblasts refill the resorbed bone sites.

1.3 Mechanical Loading

Bone mass is maintained with normal usage and is regulated by daily forces from gravity, ground forces, and muscle contractions. In the late 19th century, Julius Wolff first proposed that bone responds to changing levels of mechanical usage and that increases or decreases in usage results in formation or bone loss. While the mathematical model proposed by Wolff was inaccurate, the idea that bone senses and adapts to applied loads remains. The bone response to strain can be investigated by in vivo loading models that control the strain magnitude, strain rate, and the number of cycles applied to the bone. Strain is a measure of how much the bone deforms during loading and is calculated by the change in length divided by the original length. Strain is a unit-less measure often reported in micro strain (µε). Deformation of 1000 µε, results in a 0.1% change from the original length. Using in vivo animal models, Harold Frost determined the set point, or amount of strain required to induce a bone formation response, to be approximately 1500 µε and that the minimum daily amount of strain being required to maintain bone was 300 µε. This set point seems to vary between species and loading models. For example, 1000 µε is sufficient to induce a formation response in the turkey ulna, 1050 µε (four-
Introduction

point bending$^{(19)}$ and $1350 \mu \varepsilon$ (ulna compression)$^{(20)}$ induces a formation response in rats, and $800-900 \mu \varepsilon^{(21,22)}$ increases bone formation using the mouse tibial compression model. Genetic variations including specific breeds within a species$^{(23-25)}$ and genetic mutations (Lrp5 point mutation) alter responsiveness to mechanical forces.$^{(21,26)}$ In general, mechanical loads of 800-1500 $\mu \varepsilon$ seem to induce a bone formation response resulting in increased bone mass across species and loading models. Ultimate fracture in response to bending forces occurs with a single load around 25,000 $\mu \varepsilon$.\(^{(27)}\)

1.3.1 Loading Models

Mechanical loading models are important tools used to investigate the bone response to strain including the cells responsible and pathways involved. Loading models apply controlled amounts of strain, strain rate, number of cycles, and waveform of loading on the bone of interest. Strains are created in bone by compression, tension, torsion, or shear forces. One type of strain often accompanies another, such as in bending where compression occurs on one side of the bone and tension occurs on the opposite side. In vivo strains are measured by attaching strain gauges to the surface of bone.$^{(28-30)}$ Several limitations with strain gauge measurements need to be considered when analyzing results. Methods of attaching the strain gauge, including scraping the bone surface and the use of bone glue, may alter or misrepresent the local environment resulting in false strain results. Placement of the strain gauge is limited by the uneven surface anatomy of the bone and the requirement to find a flat surface. This limits measurements to sites of convenience rather than sites of maximal strain. After strains are measured at convenient sites, computer generated finite element analysis estimates strains at other periosteal and endocortical surfaces.$^{(28,30)}$
Changes in strain are necessary for bone formation as static load leads to bone resorption.\cite{31} Not only are changes in strain important but the rate at which strains are applied influence the formation response.\cite{32-35} Strain rate is the measure of how fast the bone bends in response to mechanical force. Turner et al.\cite{32} found applying the same magnitude of strain but at a faster rate increases the formation response. Using the same number of cycles, applying strain at a rate of 0.013 s\(^{-1}\) increased the formation response 36\% compared to non-loaded controls while a strain rate of 0.039 s\(^{-1}\) resulted in a 133\% increase in BFR. Strain rates for loading studies presented in this dissertation can be found in Appendix II.

The number of cycles necessary for a bone response is associated with strain magnitude and strain rate. Rubin and Lanyon\cite{31} found that as few as 4 cycles per day is needed to maintain bone when 2000 \(\mu\varepsilon\) is applied at a strain rate of 0.010 s\(^{-1}\). A maximal bone response occurred at 36 cycles per day with no significant increase when applying 360 cycles or even 3600 cycles at the same strain. Hsieh et al.\cite{35} found increasing the strain rate decreases the threshold for an osteogenic response and increases the bone formation response to loading. When using more physiological strains of 800-1000 \(\mu\varepsilon\), the bone response becomes more dependent on cycle number.\cite{36} Loading at strains below Frost’s proposed threshold for formation can induce a bone formation response using a similar strain rate as previous studies (100 \(\mu\varepsilon\) at 30 Hz = 6,000 \(\mu\varepsilon/s\)) and a high number of cycles (108,000).\cite{37}

Early \textit{in vivo} loading models included the rabbit tibial model\cite{38} and avian ulna model.\cite{31} While these models allow for isolation and control of the bone of interest, they require invasive surgery for implanting pins and they alter normal limb use. These
models found that static load does not increase bone formation and that removing all forces leads to bone resorption.\(^{(31,39)}\)

The rat four-point bending and the ulnar compression models were developed as external, non-invasive loading models that allowed for normal cage activity when the bone was not being loaded.\(^{(40,41)}\) The rat four-point bending model creates compression forces on the lateral surface of the tibia and tension forces on the anterior-medial surface.\(^{(40,42)}\) One limitation with the four-point bending model is that soft tissue damage may occur at high forces resulting in the release of inflammatory cytokines. High forces result in a strong periosteal woven bone formation response suggesting different formation pathways are being activated.\(^{(40,43)}\) The ulnar compression model was designed to study the periosteal bone response without the possible complications related to periosteal pressure and soft tissue damage.\(^{(41)}\) Limitations of the ulna compression model include a small region of response, lack of trabecular bone in the metaphysis, and lack of an endocortical response.

The tibial compression model was designed as an alternative non-invasive loading model that applies loads in the axial plane.\(^{(44,45)}\) Loading in the axial plane is more physiological than the bending forces created by the four-point bending model.\(^{(44,45)}\) The tibial compression model increases bone formation on both the periosteal and endocortical surfaces as well as increasing the trabecular bone response in the proximal tibia.\(^{(21,26,46,47)}\) Unlike early loading models that completely isolated the bone, the rat four-point bending, rat ulnar compression, and mouse tibial compression models allow for normal cage activity (normal daily loads) after loading or in between loading sessions.
This prevents confounding effects associated with complete removal of forces between loading sessions.

1.3.2 Bone Response to Mechanical Loading

Bone contains four regions of unique microenvironments called bone envelopes. The periosteal envelope contains two distinct histological layers. The fibrous layer contains fibroblasts, collagen, elastin, sympathetic nerve fibers and micro-vessels. The cambium layer primarily contains osteogenic cells (mesenchymal stem cells, bone lining cells, and osteoblasts) with some fibrous tissue, micro-vessels and sympathetic nerves. The periosteal bone surface is the primary site for adapting to increased mechanical loads. Bone strength is a combination of bone mass and structure. The fastest way to provide added resistance to bending is to increase the overall diameter of the bone. The second moment of area \([\text{Imm}^4]\) is the measure of resistance to bending and is calculated as:

\[
I = \int_{-y_{\text{max}}}^{+y_{\text{max}}} y^2 \, dA
\]

In this equation, \(y\) is the perpendicular distance from a neutral axis and \(dA\) is the differential area. An increase in \(y\) is squared therefore the distance from the neutral axis influences the strength greater than the total area. This is demonstrated in aging bone where the total bone area decreases due to remodeling (from hormone imbalance or drug treatments) on the endocortical surface. The decrease in bone leads to increases in strains on the remaining bone cells which stimulates the modeling pathway and an increase in periosteal bone formation. The overall effect is a net decrease in bone volume, but no
loss in overall strength due to the increased periosteal expansion and increased moment of area. In the aging example, blocking the bone cells ability to respond to mechanical forces prevents an important mechanism in providing adequate bone strength.

The endocortical envelope and trabecular envelopes are similar in nature and are referred to as endosteal surfaces. Endosteal surfaces contain a single layer of osteogenic cells and lack the fibrous layer of the periosteum. The endosteum is adjacent to the marrow cavity and is exposed to a wide variety of cells that may influence the bone lining cell activity. Endosteal surfaces are more sensitive to remodeling and less responsive to mechanical loads than the periosteal surface.

Two distinct regions located in the trabecular envelope are the primary spongiosa and secondary spongiosa. The primary spongiosa begins at the growth plate and is made up of bone spicules that have begun mineralizing on the outer surface yet maintain some of their cartilage core. The primary spongiosa is driven by growth related signals and is avoided when analyzing the mechanical load response. The secondary spongiosa contains fully mineralized trabecular bone spicules. The lengths of the primary and secondary spongiosa vary depending on the age of the mouse. The cut-off between the primary and secondary spongiosa does not occur at a specific distance distal to the growth plate; however, previous studies have found the primary spongiosa to extend to approximately 0.05 mm distal to the growth plate in adult mice. \(^{(26,47,51,52)}\)

The cortical envelope contains the bone area between the periosteal and endosteal surfaces. In mice, the cortical envelope contains compact bone primarily consisting of osteocytes and mineralized matrix. Murine bone does not contain osteons as seen in human bone.
1.3.3 Bone Cell Response to Mechanical Loading

Bone cells respond immediately to mechanical loading. Early signals that respond to loading include prostaglandins,\(^{(53,54)}\) nitric oxide,\(^{(55-58)}\) insulin growth factor-1 (IGF-1),\(^{(59,60)}\) calcium (Ca\(^{2+}\)), and glucose 6-phosphate dehydrogenase.\(^{(61)}\) Using \textit{in vitro} loading models, these early signals have been shown to be increased and/or released by both osteoblasts and osteocytes. However, osteocytes have greater sensitivity and responsiveness to mechanical stimulation than osteoblasts.\(^{(12,13)}\)

The increase in cell signaling following mechanical loading leads to physical changes in bone lining cells. Osteoprogenitor proliferation and differentiation, measured by bromodeoxyuridine (BRDU), begins 24 hours after loading and peaks 48-72 hours post load.\(^{(8)}\) Alkaline phosphatase, a measure of activated osteoblasts, is increased within two to three days of mechanical loading.\(^{(62)}\) Osteoblasts secrete osteoid along activated bone surfaces and mineralization of newly synthesized matrix begins on day four to five. Mineralization is completed by day ten following a single load.\(^{(8)}\)

The bone mineralization response to mechanical loading can be measured through fluorochrome labeling or micro-computed tomography (micro-CT or \(\mu\)CT). Fluorochrome labeling involves administering a fluorescent label at two time points several days apart to allow for the measurement of new bone mineralization and the rate of mineral apposition. Alizarin red, tetracycline, and calcein are common fluorescent labels used to label mineralization \textit{in vivo}. Calcein binds to blood calcium and is incorporated into bone at sites of active mineralization. Viewed under a fluorescent microscope, calcein fluoresces allowing for the measurement of active mineralizing surface to bone surface (MS/BS) and the mineral apposition rate (MAR). The total bone
formation rate (BFR) is calculated from the MS/BS and the MAR (Appendix I). One benefit of calcein labeling is that small changes in osteoblast activation and activity can be detected as early as four to five days after mechanical loading.

Micro-CT measures the total amount of mineral in a sample and can be useful in determining the overall structure of bone. Micro-CT scanners use x-ray technology to scan the bones at high resolution (5 – 16 µm) and store the images on a computer. The images are assembled to form a three dimensional (3D) model of the bone’s architecture. One advantage of µCT is that it can be used in longitudinal studies and measurements can be made on the same bone before and after loading in living animals. Disadvantages of µCT are that large changes in bone mass are needed to resolve differences and mineralization needs to be completed before changes can be detected. Unlike dynamic histomorphometry, µCT doesn’t differentiate between increased formation or decreased resorption therefore it gives an end result but not the mechanism by which the result was obtained. This is problematic when measuring the primary spongiosa, because growth related changes may interfere with the formation response due to loading. Cross sectional studies of different legs are limited by measurement site and variability of bone area with respect to location on the tibia.

1.4 Prostaglandins

Prostanoids are local hormones derived from arachidonic acid and synthesized as needed by almost all mammalian cells and therefore are not stored within the cell. They are synthesized through a three step process: (1) mobilization of arachidonic acid; (2) conversion arachidonic acid to prostaglandin endoperoxide (PGH₂) through the activity of cyclooxygenase and; (3) rearrangement or reduction by specific prostanoid
Resulting prostanoids are prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), prostacyclin (PGI\textsubscript{2}), prostaglandin D\textsubscript{2} (PGD\textsubscript{2}), prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}), and thromboxane (TxA\textsubscript{2}). While several prostanoids are involved in the bone formation pathway, PGE\textsubscript{2} has been shown to have the greatest influence on the bone response to loading.\textsuperscript{(53,64-66)}

1.4.1 Cyclooxygenase and Prostaglandin E\textsubscript{2} Synthesis

Stimulators of prostaglandin release by bone cells include hormones, cytokines, and mechanical loading. The process starts with the enzyme phospholipase A\textsubscript{2} (PLA\textsubscript{2}), which converts phospholipids into arachidonic acid. Arachidonic acid is oxygenated by Cox-1 or Cox-2 to form the intermediate prostaglandin G (PGG), followed immediately by reduction to prostaglandin endoperoxidase H (PGH) by the peroxidase activity of Cox-1 or Cox-2.\textsuperscript{(63,67)} Prostaglandin endoperoxidase is further converted to PGE\textsubscript{2} by prostaglandin endoperoxide synthase (PGES).

As briefly noted above, cyclooxygenase is the rate limiting enzyme responsible for the conversion of all prostanoids from arachidonic acid.\textsuperscript{(63)} Three isoforms of Cox have been isolated: Cox-1 is constitutively expressed in most mammalian cells and plays an important role in maintaining the gastric mucosa. Cox-2 is induced by inflammatory stimuli in most cells and is increased in response to mechanical forces in bone.\textsuperscript{(68-73)} Cox-3 (Cox-1b) is a splice variant of Cox-1 with no known cyclooxygenase activity.\textsuperscript{(74-76)} Cox-1 and Cox-2 are localized to the endoplasmic reticulum and nuclear envelope; however, Cox-2 has twice the concentration on the nuclear envelope than in the endoplasmic reticulum.\textsuperscript{(77)} Cox-1 and Cox-2 share 60-65\% homology and both isoforms are homodimers consisting of two 70 kDa subunits.\textsuperscript{(69,78)} Each subunit contains a cyclooxygenase and a peroxidase active site;\textsuperscript{(79,80)} however, only one subunit can be
activated at a time. Both isoforms are inactivated through a suicide inactivation sequence. While many similarities exist between Cox-1 and Cox-2, the amino acid sequence in the binding site differs, providing the possibility for selective inhibition of cyclooxygenase activity. Another difference is that Cox-1 protein is more stable in vivo ($t_{1/2} = 24$ hours) than Cox-2 protein which is rapidly turned over within the cell ($t_{1/2} = 2$ hours).

1.4.2 The Effect of Prostaglandins on Bone

Prostaglandins were originally described as activators of bone resorption suggesting that they had a catabolic effect on bone. These early studies used continuous treatments of PGE$_1$ ($10^{-8} – 10^{-5}$ M) or PGE$_2$ ($10^{-8}$ M). It was later discovered that daily administration of PGE$_2$ (0.1-3 mg/kg, 4.48 x $10^{-6}$ M - 1.34 x $10^{-4}$ M) in rats had a positive effect on bone formation. PGE$_2$ activates the remodeling pathway, thus it stimulates bone resorption followed by bone formation. Further investigation of PGE$_2$ effects on bone found both intermittent and continuous PGE$_2$ (1-3 mg/kg, 4.56 x $10^{-5}$ M – 1.34 x $10^{-4}$ M) treatments stimulated bone formation on the periosteal surface but that continuous PGE$_2$ treatment led to an increase of resorption parameters on the endosteal surfaces that exceeded the positive effects on the periosteal surface resulting in a net decrease in bone mass. This shows the periosteal and endocortical surfaces respond differently to PGE$_2$ treatment.

PGE$_2$ binds to one of the four E-type prostanoid receptors (EP1-4) which are G protein coupled receptors. Primary cultures of osteoblast cells isolated from mouse calvaria express mRNA for all four EP receptors. EP1 has been shown to inhibit osteoblast differentiation. EP3 activates inhibition of the G-protein signaling ($G_{ia}$) on
osteoblasts resulting in decreased adenylate cyclase and a decrease in proliferation. EP3 also is present on osteoclasts and activation leads to an increase in osteoclast motility.\(^{94,95}\) EP2 and EP4 have both been shown to be responsible for the bone’s anabolic response to PGE\(_2\).\(^{92,96-98}\) EP2 stimulates the protein kinase A (PKA) pathway activating cAMP response element binding protein (CREB).\(^{97-99}\) EP4 signals through the phosphotidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway in osteoblasts.\(^{97}\) EP4 is expressed on osteoclasts and directly inhibits bone resorption.\(^{94,95,100}\) The osteocyte-like MLO-Y4 cells release PGE\(_2\) in response to mechanical loading which acts in an autocrine manner through the EP2 and EP4 receptors.\(^{98,99}\) The availability of the prostaglandin receptors on bone cells suggests PGE\(_2\) has the ability to directly influence osteoblasts, osteocytes and osteoclasts. The anabolic response to mechanical loading is primarily mediated through the EP2 and EP4 receptors.

1.4.3 Prostaglandins and Mechanical Loading

Prostaglandins and mechanical loading are osteogenic stimuli when applied to bone individually. PGE\(_2\) has the ability to activate bone formation through the remodeling and modeling pathways while mechanical loading activates bone formation through the modeling pathway. To test the effects of combining PGE\(_2\) with mechanical loading, Tang et al.\(^{101}\) conducted a dose response study of PGE\(_2\) with different strain magnitudes using the rat four-point bending model. The minimum concentration of PGE\(_2\) needed to increase the bone formation in non-loaded legs was 1 mg/kg/day (4.56 \times 10^{-5} M). The minimum mechanical strain required to stimulate bone formation in the loaded leg was 1360 \(\mu\varepsilon\) at the periosteal and 580 \(\mu\varepsilon\) at the endocortical surfaces when loaded for 36 cycles at 2 Hz.\(^{101}\) The combination of loading and PGE\(_2\) had a synergistic periosteal
response and an additive response on the endocortical surface demonstrating an interaction of the PGE$_2$ and mechanical loading pathways on the periosteal surface. The mechanism behind the synergistic effect is unknown but may involve the Wnt signaling pathway (Figure 1-1 and Section 1.6).

1.4.4 Cyclooxygenase Inhibition

Confirming the importance of PGE$_2$ in the bone formation response to loading, NSAID treatment suppresses the bone response to a single mechanical load in vivo$^{65,66,102}$ and in vitro$^{72,73}$ NSAIDs target the Cox enzymes resulting in a decrease in prostaglandins and a decrease in inflammation, fever, and pain. Traditional NSAIDs inhibit both Cox-1 and Cox-2 to varying degrees. Coxibs, a selective class of NSAIDs, inhibit Cox-2 to a greater extent than Cox-1. Coxibs are used in the treatment of chronic inflammatory conditions such as arthritis and pain because they are not associated with gastrointestinal side effects that Cox-1 inhibitors can exhibit.$^{103-105}$

Traditional NSAIDs inhibit Cox-1 greater than Cox-2 but the degree of inhibition for either enzyme varies between drugs. Indomethacin is classified as a non-selective NSAID, meaning it inhibits both Cox-1 and Cox-2. However, a wide range of inhibitory concentrations for 50% effectiveness (IC$_{50}$) have been reported for indomethacin ranging from no difference between Cox-1 and Cox-2 to inhibiting Cox-1 40 times more effectively than Cox-2 (Table 1-1). Indomethacin has a half-life of 4.5 hours meaning all traces of inhibitor should be cleared within 24 hours following administration.$^{106}$

N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398) was one of the first Cox-2 inhibitors developed.$^{104,107}$ NS-398 has an IC$_{50}$ for Cox-1 of 0.08 - 125 µM and for Cox-2 of 0.0095 -5.6 µM in various cell types and with various methods of
measuring IC\textsubscript{50} values (Table 1-1).\textsuperscript{(107-112)} Hence, NS-398 inhibits Cox-2 activity 22-1263 fold greater than Cox-1 activity (Table 1-1). NS-398 binds Cox-2 in a time dependent, irreversible manner.\textsuperscript{(111,113)} Since Cox-2 protein has a short half-life within the cell, NS-398 bound to Cox-2 is cleared with the Cox-2 protein.

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell type</th>
<th>Indomethacin (µM) IC\textsubscript{50}</th>
<th>Cox-2/Cox-1</th>
<th>NS-398 (µM) IC\textsubscript{50}</th>
<th>Cox-1/Cox-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitchell 1994</td>
<td>Murine macrophages</td>
<td>0.028</td>
<td>1.12</td>
<td>40</td>
<td>NA</td>
</tr>
<tr>
<td>Kawai 1998</td>
<td>Human platelets/ human synovial cells</td>
<td>0.013</td>
<td>0.044</td>
<td>3.38</td>
<td>12</td>
</tr>
<tr>
<td>Kato 2001</td>
<td>Human monocytes</td>
<td>0.009</td>
<td>0.31</td>
<td>34.4</td>
<td>125</td>
</tr>
<tr>
<td>Futaki 1994</td>
<td>Hematin/arachidonate</td>
<td>0.74</td>
<td>0.97</td>
<td>1.31</td>
<td>No effect</td>
</tr>
<tr>
<td>Gierse 1995</td>
<td>Sf21</td>
<td>0.1</td>
<td>0.9</td>
<td>9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Pilbeam 1997</td>
<td>Pyla (Cox-1) and ROS (Cox-2)</td>
<td>0.03</td>
<td>0.03</td>
<td>1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Cox-2 inhibitors suppress the bone formation response to loading more than non-selective NSAIDs.\textsuperscript{(65,66)} NS-398 suppresses the bone formation response to a single mechanical load \textit{in vivo} using the rat four-point bending and rat ulnar loading models,\textsuperscript{(65,66)} and reduces fluid flow induced PGE\textsubscript{2} production \textit{in vitro}.\textsuperscript{(72,114)}

Retrospective human studies comparing NSAID users with non-users have been inconclusive in determining the overall effect on NSAIDs on bone density and fracture risk.\textsuperscript{(115-120)} Bauer, Carbone, and Morton\textsuperscript{(115-117)} have found regular NSAID use leads to an increase in bone mineral density while van Staa\textsuperscript{(118)} reported an increased fracture risk with regular NSAID use. However, NSAIDs are reported to increase the incidence of complications with fracture healing, including non-union and infection rates.\textsuperscript{(120-122)} NSAIDs used in pain management after fracture repair have been shown to delay fracture
healing\(^{(123,124)}\) and prevent fracture healing.\(^{(125)}\) Cox-2 is the isoform responsible for fracture repair as Cox-2 inhibitors prevent fracture healing more than non-selective Cox inhibitors.\(^{(126)}\) Moreover, Cox-2\(^{-/-}\) mice do not heal in response to femur fracture but Cox-1\(^{-/-}\) heal similar to wild-type controls.\(^{(126)}\) In summary, Cox inhibition suppresses the bone formation response to a single load as well as delays or prevents fracture repair. Conflicting results on bone quality associated with long-term use of NSAIDs have been reported demonstrating the need for further research on the long-term safety of Cox inhibition.

1.5 Wnt Signaling

Wnt signaling plays a major role in bone formation and the bone response to mechanical forces. Briefly, the canonical Wnt signaling pathway involves Wnt binding to the co-receptors low-density lipoprotein related receptor protein 5 (Lrp5) and Frizzled. After Wnt binds, glycogen synthase kinase-3 beta (GSK-3\(\beta\)) is inactivated through phosphorylation resulting in an upregulation of \(\beta\)-catenin in the cytoplasm and translocation into the nucleus. \(\beta\)-catenin binds to the T-cell factor (TCF) and Lymphoid enhancer families (LEF) of transcription factors activating many genes involved in cell proliferation, differentiation, and growth including the upregulation of Runx2, Wnt, \(\beta\)-Catenin, PTGS2 (Cox-2) and the downregulation of inhibitors of Wnt/Lrp5 including Sclerostin (SOST) and Dickoff-1 (Dkk-1).\(^{(127)}\)

In the middle 1990’s, researchers at Creighton University discovered a family that had bone mass four to five standard deviations above normal.\(^{(128)}\) It was determined that the high bone mass phenotype was due to a point mutation in the Lrp5 gene.\(^{(129)}\)
Independent studies from a separate family found a similar mutation resulting in the same phenotype.\textsuperscript{(130)} The gain in function mutation caused a glycine to valine substitution at amino acid 171 of the Lrp5 receptor.\textsuperscript{(129)} A similar high bone mass (HBM) phenotype is expressed in mice when the G171V mutation is generated in a mouse model.\textsuperscript{(131)} Bones from HBM mice are the same length and shape as WT littermates, but have greater total area, cortical area, trabecular number, BMD, and strength than their WT littermates.\textsuperscript{(131,132)} Moreover, HBM mice respond to a lower strain threshold than their WT littermates and have a greater bone formation response at strain levels required to induce a response in WT mice.\textsuperscript{(21,26)}

Mutations resulting in the loss of function in the Lrp5 receptor have the opposite phenotype compared to HBM mice. Lrp5\textsuperscript{\textminus/\textminus} mice have bones with smaller area, less trabecular volume and number, and lower BMD compared to WT littermates.\textsuperscript{(133-135)} Male Lrp5\textsuperscript{\textminus/\textminus} mice lack a load induced cortical bone formation response\textsuperscript{(26)} while studies on the response to loading with female Lrp5\textsuperscript{\textminus/\textminus} mice have resulted in inconsistent responses. Sawakami \textit{et al.}\textsuperscript{(47)} have shown that Lrp5 is essential for the bone response in female mice however, 1900 με was the maximum strain Lrp5\textsuperscript{\textminus/\textminus} mice were loaded at. Previous studies at Creighton have shown Lrp5\textsuperscript{\textminus/\textminus} mice require a higher stimulus than WT mice with formation occurring at 1500 με.\textsuperscript{(21)} Furthermore, Saxon \textit{et al.}\textsuperscript{(26)} have reported female Lrp5\textsuperscript{\textminus/\textminus} mice have a significant load response for cortical area, marrow area, and trabecular thickness at moderate to high strains, but no response at lower strains. Results from this laboratory and from Saxon’s suggest Lrp5\textsuperscript{\textminus/\textminus} mice have a robust formation response to loading at high physiological forces (greater than 2000 με).
1.6 PGE₂ Interaction With β-Catenin

PGE₂ interacts with the Wnt signaling pathway by inhibiting GSK-3β (Figure 1-1). PGE₂ binding to the EP2 or EP4 receptor activates the downstream signaling molecules PKA or AKT, which inhibit GSK-3β resulting in an increase in β-catenin and TCF/LEF signaling. Further perpetuating the response, β-catenin signaling results in upregulation of PTGS2 (Cox-2) and Wnt and downregulation of several inhibitors of the Wnt signaling pathway including SOST and Dickoff-1. Because PGE₂ release is an immediate response to loading, it may be initiating β-catenin signaling independent of the Lrp5 receptor. This overlap of pathways could be responsible for the synergistic bone formation response on the periosteal surface with mechanical loading and PGE₂ injections reported by Tang et al. In WT mice, PGE₂ activation of β-catenin following a single load may jump start the Wnt signaling pathway. In Lrp5⁻/⁻ mice, PGE₂ activation of β-catenin may be responsible for the load-induced formation response when exposed to high mechanical strains.
Figure 1-1 The interaction of the β-catenin (β-Cat) and prostaglandin E₂ (PGE₂) pathways. PGE₂ pathway includes Cyclooxygenase-2 (Cox-2) converting arachidonic acid to PGE₂. This can be blocked by using the Cox-2 inhibitor NS-398. PGE₂ binds to the E-type prostanoid receptors 2 or 4 (EP2/4) initiating multiple signaling cascades including Protein kinase C (PKC) activation which increases intra-cellular Calcium (Ca²⁺) release and/or activates protein kinase B (AKT), both of which can increase osteoprogenitor proliferation, osteoblast differentiation, and increase other genes for matrix production. Gαs activates adenylate cyclase (AC) increasing cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA). PKA can phosphorylate cAMP response element-binding protein (CREB) which activates gene transcription by binding to CRE. PKA and AKT have been shown to inhibit the glycolgen synthase kinase-3β (GSK-3β) complex resulting in increased β-catenin signaling. The Wnt pathway includes Wnt binding to the co-receptors LRPS and Frizzled which inhibits the GSK-3β destruction complex. Active GSK-3β phosphorylates β-cat leading to ubiquitination and destruction of β-cat. Inhibited GSK-3β allows for β-cat accumulation and translocation into the nucleus where it binds the T-Cell Factor/Lymphoid Enhancing Factor (TCF/LEF) family of transcription factors and increases osteoblast proliferation, osteoblast differentiation, decreases osteocyte apoptosis, and decreases the inhibitors of Lrp5/Wnt signaling, SOST and Dkk-1.
1.7 Project Purpose and Goals

The bone formation response to mechanical loading is the bone’s way of adapting to provide adequate support and strength for weight bearing function. Treatments that block this response may result in insufficient support for daily activities and eventually fracture. Several studies have demonstrated that NSAIDs suppress the bone response to a single mechanical load; however, Cox-2 inhibitors were developed for long-term use. At the onset of this project, there were no reports on the long-term effects of Cox-2 inhibition on the bone response to loading even though they are used for long-term treatments. The purpose of this project is to determine the effect of Cox inhibition on the murine bone response to tibial compression. The primary hypothesis is that Cox-2 inhibition suppresses the bone formation response to multiple mechanical loads in mice and that the load response will be suppressed the greatest in Lrp5\textsuperscript{+/-} mice.

Alternatively, if Cox-2 inhibition does not suppress the bone response to multiple mechanical loads, it was hypothesized that Cox-1 is upregulated in response to chronic Cox-2 inhibition and mechanical loading.

The following specific aims were used to test these hypotheses:

Chapter 2:

- Determine if Cox inhibition blocks the load response to a single mechanical load in mice using the tibial compression model
- Determine if Cox-2 inhibits the bone response to multiple mechanical loads
- Determine if the Lrp5 and PGE\textsubscript{2} pathways interact in response to multiple mechanical loads
Chapter 3:

- Determine the effects of daily Cox-2 inhibition on multiple mechanical loads
- Determine if the Lrp5 and PGE2 pathways interact in response to multiple mechanical loads with daily Cox-2 inhibition
- Determine the Cox-1 and Cox-2 gene response to mechanical loading with Cox-2 inhibition

Chapter 4:

- Determine the gene response to tibial compression in mice over time
- Determine the Cox-1 and Cox-2 protein response to tibial compression over time
2. Cyclooxygenase Inhibition on Single Versus Multiple Mechanical Loads in Mice

2.1 Abstract:

Mechanical loads induced by exercise maintain and increase bone strength. An initial response to loading is PGE$_2$ release, which can be blocked by non-steroidal anti-inflammatory drugs (NSAIDs). Cox-2 inhibitors are a class of NSAIDs that are commonly taken for extended periods to treat chronic pain and their long-term use may lead to an increase in fractures. This study examined the effects of acute and chronic Cox inhibition on the bone formation response to mechanical loading. In the acute study, adult C57Bl6 mice were injected with NS-398, indomethacin, or vehicle (dimethyl sulfoxide (DMSO) or saline) 3 hours prior to the right leg being mechanically loaded (1450 µε for 360 cycles at 2 Hz) by tibial compression. After a single session, loading increased the periosteal bone formation rate (BFR) 72% (saline) and 78% (DMSO) in the vehicle control groups while Cox inhibition by both indomethacin and NS-398 suppressed the load response.

The effect of chronic Cox-2 inhibition on the bone formation rate with multiple mechanical loads was then determined. The interaction of the PGE$_2$ pathway and the Lrp5 pathway was also investigated using Lrp5$^{-/-}$, WT, and high bone mass (HBM) mice. Mice were given NS-398 or DMSO and loaded three days per week for 3.5 weeks for a total of ten loads. Loads were selected to induce a moderate bone formation response in each genotype. Mechanical loading increased bone formation in the DMSO groups after 28 days for all genotypes and in the NS-398 treated WT and HBM groups. Surprisingly,
the WT mice had a greater BFR response to loading (40 to 60 μm³/µm²/yr) in NS-398 treated mice compared to DMSO. The Lrp5−/− mice did not have a load response in periosteal BFR when treated with NS-398. Therefore, Cox inhibition in mice suppressed the periosteal bone formation response to a single load but bone adapted to multiple mechanical loads regardless of Cox-2 inhibition when Lrp5 was functional.

2.2 Introduction

Non-steroidal anti-inflammatory drugs are commonly taken for extended periods for the symptomatic treatment of osteoarthritis, rheumatoid arthritis, muscle injuries, and many other types of chronic pain. While providing pain relief, NSAIDs have been shown to suppress the bone hypertrophy response to mechanical forces.\(^{65,66}\) Prostaglandin E\(_2\) (PGE\(_2\)) is an early and important responder to mechanical loading\(^{53,87,101,139}\) and increases the bone formation response when added to loading regimens.\(^{87-89,101}\) Bone is a dynamic tissue that adapts to daily forces or loads. Suppressing the bone’s ability to adapt to increased forces, through chronic Cox-2 inhibition, may lead to insufficient strength and ultimately fracture.

Both \textit{in vivo} and \textit{in vitro} studies consistently show that Cox-2 inhibition prevents the bone response to a single mechanical load stimulus. However, in the only mouse study using a selective Cox-2 inhibitor, the bone formation response was not suppressed after multiple loads.\(^{51}\) Furthermore, Alam \textit{et al.}\(^{46}\) reported that genetically knocking out Cox-2 in mice had no influence on the osteogenic response to two mechanical loading sessions. From these studies, it is not clear if Cox-2 is essential to the \textit{in vivo} bone formation response to loading in mice or if bone cells have the ability to compensate for the lack of Cox-2 with repeated loads.
To test if Cox inhibition suppresses the bone formation response to a single load in mice similar to other species (rats and roosters), the first study examined Cox inhibition on the *in vivo* bone formation response to a single load using the mouse tibial compression model. The bone formation response to a single load was suppressed in the presence of Cox inhibition which is similar to the response in other species. The long-term effect of Cox-2 inhibition on multiple mechanical loads over a 25 day period was then determined.

To determine if PGE2 release interacts with the Lrp5 pathway, Lrp5\(^{-/-}\) and HBM mice were treated with NS-398 and loaded for ten loading sessions. Lrp5\(^{-/-}\) mice lack a bone formation response to mechanical loading at physiological loads however, high strains induce a formation response. AKT and PKA, downstream signals of PGE2 binding, activate β-catenin independent of the Lrp5 receptor. This study will determine the bone formation response to loading when inhibiting both the Lrp5 and PGE2 pathways.

HBM mice have increased sensitivity to mechanical strain. Treating HBM mice with a Cox-2 inhibitor prior to multiple loads may determine if PGE2 is necessary or if the increased activity of the Wnt/β-catenin pathway overcomes the lack of PGE2.

The multiple load study investigated the *in vivo* interaction of the Wnt/β-catenin pathway with the PGE2 pathway using Lrp5\(^{-/-}\), WT, and HBM mice and Cox-2 inhibition in response to mechanical loading. It was hypothesized that Cox-2 inhibition would suppress the bone formation response and that suppression would be the greatest in the Lrp5\(^{-/-}\) mice.
2.3 Materials and Methods

2.3.1 Animals

Wild-type (C57Bl6), homozygous Lrp5<sup>−/−</sup> (134) and heterozygous Lrp5<sup>+/−</sup>/G171V high bone mass mice<sup>(131)</sup> were housed until adulthood in the animal facility at Creighton University (Omaha, NE, USA). Mice were allowed food and water ad libitum and exposed to a 12 hour light/dark cycle. All procedures were approved by Creighton University Institutional Animal Care and Use Committee.

2.3.2 Tibial Compression

For each study, the right tibia was loaded in compression (ELF 3200, Bose Corporation, Eden Prairie, MN, USA). The load apparatus was designed to apply force through the knee and ankle with the leg positioned vertically.<sup>(44,45)</sup> Proper leg position was maintained by applying a 0.5 newton (N) preload. Left tibias were collected as contralateral non-loaded controls. For all loading sessions, mice were anesthetized via inhalation of 2-3% isoflurane (Patterson Veterinary, Devens, MA, USA). The loads used for each genotype were selected to create a similar bone formation response across genotypes.

Single load study: The first study tested the effects of Cox inhibition on the bone response to a single mechanical load. This study compared the effects of a non-selective Cox-1 inhibitor, indomethacin (Bedford Labs, Bedford, OH, USA) and a Cox-2 selective inhibitor, NS-398 (Cayman Chemical, Ann Arbor, MI, USA). Indomethacin has an IC<sub>50</sub> of 0.009 µM for Cox-1 and 0.31 µM for Cox-2 in cultured human monocytes<sup>(110)</sup> indicating a preference for Cox-1 inhibition. NS-398 has an IC<sub>50</sub> of 12-125 µM for Cox-1 and 0.009-5.6 µM for Cox-2<sup>(107,109,110)</sup> and was used as a Cox-2 selective inhibitor.<sup>(65,66)</sup>
Female WT, C57Bl6 mice (4.9 ± 0.6 months, 24.4 ±1.8 g) were loaded in one session for 360 cycles at 2 Hz and 1450 µε. Mice were injected sub-cutaneously three hours prior to tibial compressions with indomethacin (10 mg/kg, 2.79 x10⁻⁵ Mol/kg), NS-398 (10 mg/kg, 3.18 x 10⁻⁵ Mol/kg), or their vehicle controls, 0.9% saline (Hospira, Inc, Lake Forest, IL, USA) or dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA), respectively. The total volume injected was 0.03 ml. Saline, the vehicle-control for indomethacin, was used to control for possible side-effects related to DMSO. Calcein (8 mg/kg) (Sigma, St. Louis, MO) was injected on days 5 and 9 to label bones for histomorphometry with collection on day 12.

Multiple load study: This study examined the effects of Cox-2 inhibition with multiple mechanical loading sessions on 3 genotypes: homozygous Lrp5⁻/⁻, WT, and heterozygous HBM mice. While indomethacin suppressed the bone formation response with a single load, it was not used due to the gastro-intestinal side effects associated with long-term, high dose Cox-1 inhibition. Female adult mice (5.0 ± 0.5 months, 23.2 ± 2.1 g) were loaded in tibial compression for 100 cycles at 2 Hz, Mon-Wed-Fri for 3.5 weeks for a total of 10 loads. Applied forces and their predicted strains (Lrp5⁻/⁻ = 6 N, 1500 µε; WT = 8 N, 1060 µε; HBM = 9 N, 650 µε) were based on a previous study where these loads produced a similar lamellar bone response between genotypes. Sub-cutaneous injections with NS-398 (3.18 x 10⁻⁵ Mol/kg) or vehicle (DMSO) were given three hours prior to mechanical loading. Calcein (8 mg/kg, Sigma) was injected on days 15 and 22 with tissue collected on day 25.
2.3.3 Histomorphometry

For both studies, the soft tissue was carefully removed to avoid damaging the periosteum. Tibias were fixed in 70% ethyl alcohol, pre-stained with Villanueva Osteochrome Bone Stain (Polysciences, Inc, Warrington, PA) for 72 hours, dehydrated and embedded in methyl methacrylate. Tibias were cross-sectioned (80 µm thick) 1-3 mm proximal to the tibia fibula junction (TFJ) on a saw microtome (Model 1600, Leica, Germany). Two sections from each tibia were blind coded for histomorphometry and the measurements were averaged.

Measurements were made with a light/epifluorescent microscope and a video camera interfaced with BioQuant Osteo V7.20.10 (BioQuant, Nashville, TN, USA). The cortical bone cross-sections were measured at: 40X for total and marrow area (Tt.Ar., Ma.Ar.) and periosteal and endocortical bone perimeter (Ps.Pm and Ec.Pm); 200X for double (dL.Pm.) and single (sL.Pm.) labeled calcein perimeter and woven bone perimeter (Wo.Pm); 400X for inter label width (Ir.Wi.) at double labeled sites. The following calculations were made for each section: cortical bone area (Tt.Ar - Ma.Ar), single calcein-labeled (sLS/BS), double calcein-labeled (dLS/BS), and woven bone (WoS/BS) bearing surfaces. Percent mineralizing surface (MS/BS x 100) is the sum of the three calcein labeled surfaces ((sLS/2+dLS+WoS)/BS). Mineral apposition rate (MAR in µm/d) was calculated by dividing Ir.L.Th / Ir.L.t (inter label time). Bone formation rate (BFR) was calculated as MAR*(MS/BS). For non-loaded tibias that did not have double label, a MAR of 0.3 was used for calculating the lamellar response.\(^{142}\) This rate was below the level detected at any visible double label site. MAR for woven bone was estimated at 3.5 µm/day. This value is lower than maximal lamellar apposition rates.
measured (3.8 µm/day). BFR calculated by this method for regions of tightly compacted labeled woven bone correlated with BFR calculated from woven area ($R^2 = .92$).

Examples of calcein labeled surfaces and woven bone surface can be found in Appendix I (Figure A-1).

2.3.4 Micro-Computed Tomography (Micro-CT) Analysis

Micro-CT (µCT) analysis was conducted to evaluate bone structural properties on the proximal tibia metaphysis of six randomly selected left-right pairs from each group in the multiple load study. Tibias were scanned in a desktop µCT (micro-CT-40, Scanco Medical AG, Bassersdorf, Switzerland) at 16 µm resolution. Three-dimensional (3-D) isotropic images of the proximal tibia were collected with an integration time of 300 ms. The region of interest (ROI) started 3 slices (0.048 mm) distal to the growth plate and included 60 slices (0.96 mm) to 1.008 mm distal to the growth plate, similar to the ROI from previous studies (Figure 2-3).$^{(26,51)}$ A customized thresholding technique (Scanco) was used which provided the best segmentation of the bone tissue. Three-dimensional µCT data included bone volume to total volume fraction (BV/TV, %), trabecular number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp). In addition, structural model index (SMI), connectivity (Conn.D), apparent (App.D) and tissue densities (Tiss.D) of the trabecular bone were measured.

2.3.5 Statistical Analysis

Data analyses for body weight and comparisons between histomorphometry were conducted using SPSS version 21 (SPSS IBM Corp, Armonk, NY, USA). Statistics for Study 1 were conducted using the general linear model (GLM) repeated measures with load/non-load as within subject variable and drug as fixed factor. Statistics for Study 2
and µCT were done using a three way GLM repeated measures (genotype x drug x load/non-load) to examine all variables. The data were then split by genotype and GLM repeated measures with load/non-load x drug were tested to measure differences within a genotype.

For each study, significant effects due to loading were followed up using paired t-tests and effects due to drug interaction were followed up using a one way analysis of variance (ANOVA) using difference (load – non-load) scores. Differences identified by one way ANOVA were further examined by least significant difference post hoc tests. For all data, P values < 0.05 were considered significant.

2.4 Results

Study 1: Bone response to a single load with Cox inhibition

There were no differences in initial weights or ages between groups. Periosteal bone formation indices increased due to loading in both vehicle groups but not with Cox suppression (Table 2-1, Figure 2-1). In the non-loaded control, BFR was ~19% MS/BS and MAR of 0.6 µm/day. In the loaded leg, MS/BS was increased 57% (saline, P < 0.001) and 55% (DMSO, P = 0.002) in the vehicle groups. Differences in MS/BS with Cox inhibition were 20% (indomethacin, n.s.) and 31% (NS-398, P = 0.038). MAR differences with loading were not significant but tended to increase for all groups (P > 0.072, Table 2-1). Periosteal BFR was increased due to loading in both the saline (P < 0.001) and DMSO (P = 0.019) groups (Figure 2-1). Cox inhibition using indomethacin and NS-398 prevented the increase in the BFR to loading that occurred in response to loading (Figure 2-1).
Endocortical BFRs were variable ranging from 100 to 225 µm³/µm²/yr and were greater than periosteal rates. Cox inhibition had no consistent effect on the endocortical response (Table 2-1, Figure 2-1). The saline and indomethacin groups had an endocortical load response for MS/BS, MAR, and BFR (P < 0.05). Loading increased the MAR in the DMSO and NS-398 groups (P < 0.012) but the MS/BS and BFR were not increased (Table 2-1, Figure 2-1). There were no differences in the load responses between saline and DMSO on the periosteal or endocortical formation in non-loaded tibia.

Study 2: Bone response to Cox-2 inhibition and multiple loads

Lrp5⁻/⁻ mice had lower initial weights (-2.5 g) and were younger (-0.8 months) than WT and HBM groups (Table 2-2) so mice were randomized within each genotype so there were no initial differences in age or weight. There were no changes in final weight due to drug treatment. Total bone area varied between genotypes with Lrp5⁻/⁻ mice having
Cox Inhibition: Single vs Multiple Loads

Figure 2-1 Periosteal (A) and endocortical (B) bone formation rate (BFR) in response to a single injection of drug and a single load session in wild-type mice. White bars are non-loaded (left) legs and black bars are loaded (right) legs. Right legs were loaded at 1450 µε, 360 cycles, 2 Hz. Data represents mean ± standard error of the mean. N = 16-17. a = Load effect within a group (P < 0.05).

Figure 2-2 Periosteal (A) and endocortical (B) BFR in response to multiple mechanical loads and Cox-2 inhibition. Data represents three genotypes with varying levels of Lrp 5 function loaded at different micro strains for 100 cycles and 2 Hz to induce a similar load response between genotypes. White bars represent non-loaded leg and black bars represent loaded leg. Data represents means ± standard error of the mean. N = 14-15. a = Load effect within a group (P < 0.05); b = Interaction of drug and loading (P < 0.05).

25% less area than WT and HBM mice having 22% more area than WT (P < 0.001; Table 2-2). Neither mechanical loading nor NS-398 increased the total area as compared to contralateral non-loaded tibia (Table 2-2). Tibia lengths averaged 17.4 ± 0.4 mm and did not differ between genotype or with loading.

For the non-loaded legs, the periosteal BFR in the WT-DMSO group was greater and the endocortical BFR was lower than in the single load experiment (Figures 2-1 and
2-2). The periosteal BFR for the Lrp5\(^{--}\)-DMSO was similar to WT vehicle but HBM mice had less formation demonstrating variability in genotypes and cohorts. The WT-DMSO periosteal response to ten load sessions was similar to the response to a single load where BFR averaged 100 to 120 µm\(^3/\mu m^2/yr\). Periosteal MS/BS was increased due to loading in both WT (40 – 50%) and HBM (160 – 400%) genotypes (P < 0.003, Table 2-3) regardless of Cox inhibition. The MAR was increased in the WT-NS-398 group and both HBM groups (P < 0.037; Table 2-3). Periosteal BFR was increased by loading in all DMSO treated groups (P < 0.007). Unexpectedly, periosteal BFR was increased in response to mechanical loading in the NS-398 treated groups for the WT (P < 0.001) and HBM (P = 0.004) genotypes (Figure 2-2). WT mice had an interaction where NS-398 and loading increased the periosteal MAR (P = 0.012) and BFR (P = 0.035) more than mechanical loading alone (Table 2-3; Figure 2-2). The MS/BS tended to increase in the Lrp5\(^{--}\)-NS-398 group (P = 0.062), but paired comparisons between loaded and non-loaded tibia showed no response in MAR and BFR. Woven bone formation was measured only in Lrp5\(^{--}\) mice, representing half of the load response in the Lrp5\(^{--}\)-DMSO group (Table 2-3).

The endocortical response to loading was highly variable. The endocortical MS/BS was increased 25-33% in response to loading in the NS-398 treated groups for all genotypes (P < 0.025), but there was not a consistent load response in the DMSO treated groups (Table 2-3). NS-398 treatment and loading had an interaction in MS/BS for the Lrp5\(^{--}\) and WT genotypes, with a lower MS/BS in the vehicle and greater MS/BS in NS-398 treated groups (Table 2-3). The endocortical MAR was less in the loaded legs in the HBM-DMSO group (P = 0.040) but was not different from the non-loaded leg in all other
Table 2-2 Characteristics of mice in the multiple load study

<table>
<thead>
<tr>
<th></th>
<th>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>WT</th>
<th>HBM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO NS-398</td>
<td>DMSO NS-398</td>
<td>DMSO NS-398</td>
</tr>
<tr>
<td>Weight Initial (g)</td>
<td>21.9 (2.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.4 (1.7)</td>
<td>23.9 (1.0)</td>
</tr>
<tr>
<td></td>
<td>22.5 (2.6)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.2 (3.2)</td>
<td>23.8 (2.4)</td>
</tr>
<tr>
<td>Weight Final (g)</td>
<td>21.5 (1.4)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.2 (1.6)</td>
<td>23.6 (1.1)</td>
</tr>
<tr>
<td></td>
<td>22.5 (2.6)</td>
<td>24.5 (2.2)</td>
<td>23.5 (2.0)</td>
</tr>
<tr>
<td>Age (months)</td>
<td>4.6 (0.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3 (0.3)</td>
<td>5.1 (0.5)</td>
</tr>
<tr>
<td></td>
<td>4.5 (0.3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2 (0.4)</td>
<td>5.0 (0.5)</td>
</tr>
<tr>
<td>Total Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.65 (0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86 (0.05)</td>
<td>1.05 (0.11)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.62 (0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85 (0.04)</td>
<td>1.03 (0.15)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cortical Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.44 (0.05)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57 (0.03)</td>
<td>0.74 (0.09)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.43 (0.05)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55 (0.02)</td>
<td>0.73 (0.11)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, N = 14-15.

<sup>a</sup> = P < 0.05 versus WT.

<sup>b</sup> = Weight final different than weight initial by paired t-test P < 0.05.

Table 2-3 Effects of multiple injections on bone formation parameters using DMSO or NS-398 and 10 mechanical loading sessions over 22 days

<table>
<thead>
<tr>
<th></th>
<th>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>WT</th>
<th>HBM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO NS-398</td>
<td>DMSO NS-398</td>
<td>DMSO NS-398</td>
</tr>
<tr>
<td>Periosteal Surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>Non-loaded 18.9 ± 23.7</td>
<td>32.61 ± 21.1</td>
<td>10.7 ± 11.2</td>
</tr>
<tr>
<td></td>
<td>Loaded 25.9 ± 26.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.57 ± 17.6</td>
<td>26.0 ± 12.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>Non-loaded 0.56 ± 0.44</td>
<td>0.63 ± 0.18</td>
<td>0.40 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Loaded 0.57 ± 0.36</td>
<td>0.68 ± 0.11</td>
<td>0.52 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wo.BS (%)</td>
<td>Non-loaded 0.91 ± 3.28</td>
<td>0.77 ± 0.35</td>
<td>0.83 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Loaded 3.27 ± 7.85</td>
<td>0.79 ± 0.17</td>
<td>0.74 ± 0.15</td>
</tr>
<tr>
<td>Endocortical Surface</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>Non-loaded 33.7 ± 12.7</td>
<td>31.1 ± 13.3</td>
<td>29.7 ± 14.8</td>
</tr>
<tr>
<td></td>
<td>Loaded 30.0 ± 14.7</td>
<td>28.1 ± 12.3</td>
<td>37.6 ± 11.4</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>Non-loaded 0.91 ± 0.30</td>
<td>0.83 ± 0.17</td>
<td>0.74 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Loaded 0.76 ± 0.44</td>
<td>0.85 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.65 ± 0.13</td>
</tr>
</tbody>
</table>

Data represents mean ± SD, N = 14-15 per group.

<sup>a</sup> = P < 0.05 versus non-loaded control

<sup>b</sup> = P < 0.05 for an interaction with NS-398 and loading within a genotype.
groups. There was an interaction in MAR between loading and NS-398 in the Lrp5\(^{-/-}\) (P = 0.045) and HBM (P = 0.023) groups, tending to be less in vehicle and stable or greater in NS-398 groups with loading (Table 2-3). The Lrp5\(^{-/-}\) mice had an increase in total BFR due to loading in the NS-398 group (P = 0.022) and a drug interaction with loading and NS-398 (P = 0.018). The WT and HBM genotypes did not have a BFR response to load or drug.

Micro CT Results

In the Lrp5\(^{-/-}\) and WT genotypes, µCT results were not analyzed due to the overwhelming effect of primary spongiosa on the region of interest and the lack of trabecular bone in the secondary spongiosa. The bone volume over total volume (BV/TV) of the region analyzed averaged 5% in the Lrp5\(^{-/-}\) mice and 7% in the WT proximal tibias (Figure 2-3). Trabecular bone in the secondary spongiosa for Lrp5\(^{-/-}\) and WT mice was limited with almost no trabecular bone remaining at 0.51 mm distal to the growth plate (Figure 2-3). HBM mice averaged 24.9% BV/TV but a positive load response was not detected for any of the parameters analyzed.

Figure 2-3 Micro CT images of the non-loaded proximal tibia for Lrp5\(^{-/-}\), WT, and HBM mice. Top row: Lateral view of the region of interest 0.048 mm-1.008 mm distal to the growth plate. Bottom row: Cross section at 0.51 mm distal to the growth plate. For all sections, the anterior bone surface is on the left side.
2.5 Discussion

The purpose of these studies was to investigate the role of Cox inhibition on the bone response to a single mechanical load in mice and then to determine the effects of long-term Cox-2 inhibition on the response to multiple loads in Lrp5−/− and HBM mice. These experiments show that the bone formation response to mechanical loading differs between acute Cox-2 inhibition and chronic Cox-2 inhibition in mice. This single load experiment is the first *in vivo* experiment to demonstrate pharmacological Cox-2 inhibition suppresses the bone response to a single load in mice, consistent with the response to a single load in other species and cell cultures. Similar amounts of suppression occurred on the periosteal surface when using either a non-selective Cox inhibitor (indomethacin) or a Cox-2 selective inhibitor (NS-398), consistent with previous findings in the rat ulna compression model and rat four-point bending model. There was no difference in the periosteal or endocortical response between the DMSO and saline vehicle groups, suggesting DMSO did not suppress or enhance the periosteal bone formation response in the single load experiment.

Unlike previous studies in rats, the endocortical response to a single load and Cox inhibition was not different from the vehicle controls. This may be related to differences in the loading model where four-point bending used stronger loads that initiated woven bone formation, indicating that different pathways were activated than in a normal lamellar response. McKenzie *et al.* have published that Cox-2 mRNA is upregulated 1.9 fold after a load stimulus that creates a lamellar response in rats, but is upregulated 16.9 fold after a load stimulus that creates a woven bone response, suggesting PGE2 may play a bigger role in the woven bone response to loading. In the
multiple load study, woven bone formation was limited to the periosteal surface of five Lrp5^/- mice (4 DMSO, 1 NS-398) due to loading and one non-loaded tibia. The increased incidence of woven bone in the DMSO group compared to the NS-398 group supports the results reported by McKenzie et al. that Cox-2 may be more involved in the woven bone response than in lamellar loads.

Gene expression arrays and qPCR have shown Cox-2 but not Cox-1 is upregulated in response to mechanical loading in vivo and in vitro, suggesting chronic Cox-2 inhibition may block bone adaptation to loading. This multiple load experiment was the first to examine dynamic histomorphometric effects of chronic Cox-2 inhibition with loading on bone formation over a 25 day period. This study was also the first to examine the interaction of chronic Cox-2 inhibition using mice expressing varying levels of Lrp5 activity. Cox-2 inhibition did not suppress the periosteal bone formation response to multiple loads in the WT and HBM genotypes, similar to previous work analyzing bone mass differences with µCT in C57Bl6 mice. Contrary to expectations, WT mice in the multiple load study had an interaction where NS-398 treatment and loading resulted in a greater formation response than loading alone. The fact that NS-398 did not inhibit the formation response to multiple loads suggests bone cells have the ability to compensate for Cox-2 inhibition. Cox-1 may compensate for long-term Cox-2 inhibition as Cox-1 increases in response to two loading sessions in Cox-2^/- mice.

Sugiyama et al. reported a decrease in trabecular bone volume due to NS-398 in non-loaded legs after two weeks. In the current study, NS-398 tended to lower the cortical bone formation rate on the periosteal and endocortical surfaces in the non-loaded leg after 25 days in WT mice suggesting PGE2 has a greater role in bone remodeling than
the bone response to multiple lamellar loads. NS-398 did not decrease the baseline bone formation rates in non-loaded legs in the Lrp5<sup>−/-</sup> mice or HBM mice.

I predicted that NS-398 would suppress the bone formation response to a greater degree in the Lrp5<sup>−/-</sup> group compared to the other genotypes. While NS-398 did not suppress the periosteal bone formation rate with multiple loads in the WT and HBM genotypes, there was not a response to multiple loads in the Lrp5<sup>−/-</sup>-NS-398 mice (P = 0.201). Lrp5<sup>−/-</sup> mice respond to loading but required higher strain (1500 µε) than WT (960 µε) mice to generate a bone response. In this study, Lrp5<sup>−/-</sup>-DMSO mice loaded at 1500 µε had a similar periosteal response to WT mice loaded at 1060 µε. This was contrary to previous results by others that have reported Lrp5 as essential to the bone response to strain. Saxon et al. have also reported diminished load responses in female Lrp5<sup>−/-</sup> mice but reported load effects at strains greater than 2000 µε.

Micro-CT analysis of the proximal tibia showed very little trabecular bone in Lrp5<sup>−/-</sup> and WT mice 0.51 mm distal to the growth plate (Figure 2-3). The WT mice in this study had a small amount of trabecular bone (7%) compared to that in the Sugiyama study of the same region (17%). The lack of a trabecular bone response in HBM mice to loading is in contrast to a positive increase in periosteal BFR measured by histomorphometry. BFR increases as measured by histomorphometry were 30 to 60 µm³/µm²/yr which equates to 2–4 µm of new bone that would be added after 25 days. The small increase in total bone is within measurement and sampling errors associated with μCT measurements.

The endocortical response was highly variable. A possible explanation for the lack of an endocortical response is that the loads were not high enough to create a
Cox Inhibition: Single vs Multiple Loads

constant response on the endocortical surface. Strain gauge measurements can only be made on the periosteal surface and computer modelling estimated endocortical strains.

PGE$_2$ can activate the Wnt/β-catenin intracellular signaling pathway through AKT inhibition of glycogen synthase kinase 3β (GSK-3β) therefore bypassing the Lrp5 receptor.\textsuperscript{(137)} It was expected that eliminating both the PGE$_2$ response through Cox-2 inhibition and the Lrp5 pathway by genetic knockout of the Lrp5 receptor would be most effective at eliminating the bone response to loading. Consistent with this hypothesis, the only group that did not have a significant load response was the Lrp5$^{-/-}$ mice on the periosteal surface with NS-398 treatment. Yet, Lrp5$^{-/-}$-NS-398 treated mice still showed an endocortical response to loading, again demonstrating that the endocortical surface responds differently from the periosteum.

In conclusion, Cox inhibition suppressed the periosteal bone formation response to mechanical stimulation after a single load but did not suppress the periosteal bone formation response to multiple mechanical loading sessions in the WT and HBM mice. This suggests that long-term therapeutic use of Cox-2 inhibitors may not have a negative impact on bone adaptation to loading. To better understand the bone response to loading, future studies need to focus on pathways that may compensate for long-term Cox-2 inhibition with Cox-1 being a likely candidate. The results from the long-term study support the hypothesis that suppressing both the PGE$_2$ and Lrp5 pathways has the greatest effect on suppressing the bone response to mechanical loading. This suggests PGE$_2$ may activate bone formation in the absence of the Lrp5 receptor.
3. Gene Response to Chronic Cox-2 Inhibition and Multiple Mechanical Loads

3.1 Abstract

Chapter 2 showed Cox inhibition suppressed the bone formation response to a single mechanical load while Cox-2 inhibition did not suppress the response to ten loading sessions. One of the caveats of the long-term study is that Cox-2 inhibitor was only given on days of loading. The current study determined the effects of mechanical loading on bone formation using daily Cox-2 inhibition in Lrp5−/−, WT, and high bone mass (HBM) mice. Mice were given daily injections of a Cox-2 inhibitor (NS-398) or control (DMSO) and loaded three days per week for 3 weeks and a total of 10 loads. Mechanical loading increased the periosteal bone formation rate for WT (54 μm³/μm²/yr) and HBM (45 μm³/μm²/yr) mice regardless of Cox-2 inhibition. Lrp5−/− mice did not have a significant formation response to loading.

To determine if Cox-1 compensated for chronic Cox-2 inhibition, the gene expression for Cox-1 and Cox-2 was measured four hours following one, five, or ten mechanical loads. Cox-2 expression increased 1.7 fold after a single load in both DMSO and NS-398 treated mice while Cox-1 increased 1.3 fold in DMSO treated mice regardless of genotype. Cox-1 gene expression was not increased at any time point with chronic Cox-2 inhibition. This study confirmed chronic Cox-2 inhibition does not suppress the bone formation response to multiple mechanical loads and demonstrates Cox-1 mRNA was not upregulated to compensate for Cox-2 inhibition.
3.2 Introduction

Coxibs, a special class of NSAIDs used in the treatment of chronic inflammatory conditions such as rheumatoid arthritis, inhibit the Cox-2 isoform greater than the Cox-1 isoform. My previous study (Chapter 2) used NS-398, a Cox-2 selective inhibitor, to block PGE2 prior to mechanical loading. However, NS-398 was only given on days of loading and not daily. The current study uses daily injections of NS-398 to examine the bone formation response to multiple mechanical loads over 25 days.

Cox-2 mRNA but not Cox-1 mRNA is upregulated in response to a single mechanical load in vitro\(^{72,73}\) and in vivo.\(^{46,143}\) However, Alam et al.\(^{46}\) measured an increase in Cox-1 mRNA expression in response to two days of mechanical loading in Cox-2 knockout mice suggesting Cox-1 may compensate when Cox-2 is deleted. To measure the gene response to loading and test whether Cox-1 is upregulated in response to chronic Cox-2 inhibition, PTGS1 (Cox-1) and PTGS2 (Cox-2) mRNA levels were measured using quantitative polymerase chain reaction (qPCR) after one, five, and ten loads. It is hypothesized that Cox-1 mRNA will be upregulated in response to chronic Cox-2 inhibition and multiple mechanical loads.

PGE\(_2\) binds to the EP2 and/or EP4 receptors increasing both AKT and PKA signaling.\(^{97,137}\) These downstream signals of PGE\(_2\) inhibit GSK3-\(\beta\) and increase \(\beta\)-catenin independent of the Lrp5 receptor.\(^{97,137}\) Increased PGE\(_2\) signaling via activation of \(\beta\)-catenin may be responsible for the bone formation response observed using high strains in Lrp5\(^{-/-}\) mice. The current study used Lrp5\(^{-/-}\), WT, and HBM mice to compare the effects of Cox-2 inhibition on the bone response to loading in mice with varying Lrp5
function. The Cox-1 and Cox-2 gene responses were measured to determine if they are differentially regulated with changes in Lrp5 expression and mechanical loading.

3.3 Materials and Methods

3.3.1 Animals

Adult WT, Lrp5−/−, and HBM mice (5.3 ± 0.4 months) were housed as described in Chapter 2. All procedures were approved by Creighton University Institutional Animal Care and Use Committee.

3.3.2 Tibial Compression

The right tibias of female, virgin mice were loaded in compression using a previously described protocol (Chapter 2). For this study, each load session comprised of 100 cycles at 2 Hz at a force that would induce the following predicted micro strains for each genotype: Lrp5−/− = 2000 µε, WT = 1450 µε, HBM = 1080 µε. The forces were chosen as the maximal force to induce a lamellar bone response for each genotype while preventing soft tissue damage.

3.3.3 Drug Treatments

Daily, sub-cutaneous injections of NS-398 (3.18 x 10^-5 Mol/kg) or vehicle (DMSO) were given at the same time each morning. Load was applied three hours after injections based on previously published data demonstrating the greatest suppression response at this time point.(65) Mice used for histomorphometry were injected with calcein (8 mg/kg) on days 15 and 22 with tissue collected on day 25.
3.3.4 Histomorphometry

Tibias were collected and processed as described in Chapter 2. Histomorphometry measurements and analyses were made using the same magnifications and calculations as described in section 2.3.3 (Chapter 2).

3.3.5 Tissue Collection for Gene Analysis

Loaded (right) and non-loaded (left) tibias were collected and processed separately four hours after the final load and immediately placed in cold RNA Later (Life Technologies, Grand Island, NY, USA). Soft tissue including the periosteum was removed and cuts were made distal to the growth plate and at the tibia fibula junction to expose the marrow space. Marrow was flushed out using cold RNA Later and the bones were stored in fresh RNA Later and flash frozen in liquid nitrogen. Bones were stored in -80 degrees Celsius until further processing.

3.3.6 qPCR

Bones were pulverized in liquid nitrogen using a metal mortar and pestle. Crushed bone fragments were added to 1 mL denaturation solution from Totally RNA Kit (Life Technologies, Grand Island, NY, USA) and homogenized using hard tissue tips and a tissue homogenizer (Omni, Inc, Kennesaw, GA, USA). RNA was extracted (QIAshredder and RNeasy Plus Mini-Kit, Qiagen, Valencia, CA, USA) according to manufacturer protocol and full length cDNA was obtained using 200 ng of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA).

Quantification of mRNA levels was performed by real time PCR (7500 Fast Real-Time PCR System, Life Technologies) using the following primers and probes: GAPDH forward: CAAGAAGGTGGTGGAAGCAGGC; reverse: AGGTGGAAAGTGAGGAGTGC; probe:
AGGGCATCTTGGGCTACACTGAGGAC. PTGS1 forward: GGTGCCCTCACAGTCAATC; reverse: CGGACACAGACACCCCTGGTT; probe: CTGTTGTTACTATCGTGCCA. PTGS2 forward: GTAACAGTCCATCTCTCAATGCAA; reverse: AATGGGCTGGAAGACATATCAA; probe: ATCAGTGGCCTCGTGAGCTTCTTCACA. Primer and probe sets were designed using the National Center for Biotechnology Information Gene and Blast sites. Primers were deigned to generate short amplicons (70-120 base pairs) with the probe (20-25 base pairs) binding to the amplicon. Primers and probes were ordered from Integrated DNA Technologies (Iowa City, Iowa, USA).

3.3.7 Statistical Analysis

Data analyses for body weight and comparisons between histomorphometry were conducted using SPSS version 21. Statistics were conducted using a three way general linear model (GLM) repeated measures (genotype x drug x load/non-load) to examine all variables. The data were then split by genotype and GLM repeated measures with load/non-load x drug were tested to measure differences within a genotype. Significant effects due to load were followed up using paired t-tests and effects due to interaction were followed up using a one way analysis of variance (ANOVA) using difference (load – non-load) scores. Differences identified by one way ANOVA were further examined by least significant difference post hoc tests. For all data, P values < 0.05 were considered significant.

Gene expression results were analyzed using single sample paired t-tests with the test variable set to 1. Paired t-tests were conducted separately for each gene with all time points pooled to see an overall effect and then with each individual time point.
Table 3-1 Characteristics of mice in histology study

<table>
<thead>
<tr>
<th></th>
<th>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th></th>
<th>WT</th>
<th></th>
<th>HBM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>NS-398</td>
<td>DMSO</td>
<td>NS-398</td>
<td>DMSO</td>
<td>NS-398</td>
</tr>
<tr>
<td>Weight Initial (g)</td>
<td>23.1 (2.8)</td>
<td>22.9 (3.0)</td>
<td>23.3 (1.3)</td>
<td>24.7 (2.8)</td>
<td>23.8 (2.4)</td>
<td>23.7 (2.0)</td>
</tr>
<tr>
<td>Weight Final (g)</td>
<td>22.0 (2.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.5 (2.9)</td>
<td>23.1 (1.2)</td>
<td>23.7 (2.3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.3 (1.4)</td>
<td>22.7 (1.3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>Non-Loaded</td>
<td>0.69 (0.07)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66 (0.06)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91 (0.05)</td>
<td>0.91 (0.06)</td>
<td>1.15 (0.09)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Loaded</td>
<td>0.69 (0.07)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.67 (0.10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90 (0.05)</td>
<td>0.93 (0.08)</td>
<td>1.15 (0.08)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cortical Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>Non-Loaded</td>
<td>0.44 (0.04)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43 (0.03)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 (0.04)</td>
<td>0.57 (0.04)</td>
<td>0.80 (0.04)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Loaded</td>
<td>0.46 (0.03)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 (0.05)&lt;sup&gt;b, c&lt;/sup&gt;</td>
<td>0.58 (0.03)</td>
<td>0.59 (0.05)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.81 (0.05)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is represented as means (standard deviation). N = 16/group

<sup>a</sup> = P < 0.05 Paired t-test different from initial weight
<sup>b</sup> = P < 0.05 Different from WT
<sup>c</sup> = P < 0.05 Paired t-test for load effect
### Table 3-2: Histology results for daily Cox-2 inhibition and multiple mechanical loads

<table>
<thead>
<tr>
<th></th>
<th>Lrp5&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>WT</th>
<th>HBM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>NS-398</td>
<td>DMSO</td>
</tr>
<tr>
<td><strong>MS/BS (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Loaded</td>
<td>10.3 (12.2)</td>
<td>16.3 (15.7)</td>
<td>18.7 (24.5)</td>
</tr>
<tr>
<td>Loaded</td>
<td>20.5 (18.1)</td>
<td>26.9 (15.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.2 (17.3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>MAR (μm/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Loaded</td>
<td>0.40 (0.11)</td>
<td>0.41 (0.12)</td>
<td>0.41 (0.15)</td>
</tr>
<tr>
<td>Loaded</td>
<td>0.44 (0.14)</td>
<td>0.49 (0.16)</td>
<td>0.59 (0.09)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Wo.BS (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Loaded</td>
<td>0.0 (0.0)</td>
<td>2.3 (7.5)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>Loaded</td>
<td>0.9 (3.7)</td>
<td>1.0 (4.1)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td><strong>Endocortical Surface</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MS/BS (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Loaded</td>
<td>24.9 (11.2)</td>
<td>21.9 (12.7)</td>
<td>14.9 (8.5)</td>
</tr>
<tr>
<td>Loaded</td>
<td>30.1 (9.1)</td>
<td>27.2 (10.9)</td>
<td>17.8 (7.3)</td>
</tr>
<tr>
<td><strong>MAR (μm/day)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Loaded</td>
<td>0.56 (0.10)</td>
<td>0.53 (0.18)</td>
<td>0.43 (0.13)</td>
</tr>
<tr>
<td>Loaded</td>
<td>0.59 (0.09)</td>
<td>0.57 (0.14)</td>
<td>0.46 (0.15)</td>
</tr>
</tbody>
</table>

Mice were injected daily with DMSO or NS-398 and the right tibia was loaded on M, W, F for a total of 10 loads over 22 days.

Data represented as means (standard deviation) N = 16/group

<sup>a</sup> = P < 0.05 Paired t-test for load effect
3.4 Results

3.4.1 Histology Study

There were no differences in initial weights between genotypes or groups (Table 3-1). Overall, mice lost weight (< 1g) but the effect was not due to drug treatment or genotype. There were no differences in tibia lengths between genotypes or with loading (17.4 ± 0.4). Lrp5 function was associated with 26% greater total area and cortical areas (Lrp5<sup>−/−</sup> < WT < HBM; P < 0.001; Table 3-1). Total area did not change with load but cortical area was greater in the loaded leg after 3 weeks of loading compared to the non-loaded leg (Table 3-1; P = 0.001). Paired t-tests analyzing load differences in cortical area resulted in significant differences in the Lrp5<sup>−/−</sup>-NS-398 and WT-NS-398 groups.

Mechanical loading resulted in an overall difference within subjects for periosteal MS/BS, MAR, and BFR (P < 0.001). Genotype differences were significant for between subject effects for MS/BS, MAR, and BFR (P < 0.005). After splitting the data by genotype, MS/BS was greater in loaded legs compared to non-loaded legs for all groups (P < 0.03) except Lrp5<sup>−/−</sup>-DMSO treated mice (P = 0.064, Table 3-2). Mineral apposition rates were greater after loading in DMSO and NS-398 treated groups in the WT and HBM mice (P < 0.001) but...
not in Lrp5^{−/−} mice (Table 3-2). The total BFR was greater in the loaded leg in the WT and HBM genotypes regardless of NS-398 treatment (Figure 3-1). No periosteal load response was detected in the Lrp5^{−/−} mice. Periosteal woven bone formation occurred after loading in three mice, two Lrp5^{−/−} and one WT mouse. Woven bone was also detected in the non-loaded leg of two Lrp5^{−/−} mice.

Endocortical MS/BS was greater overall in response to mechanical loading (P = 0.046), however MS/BS was not significantly greater in any individual group (Table 3-2). The endocortical MARs and total BFRs were not different with loading when compared to the non-loaded legs in any of the groups.

3.4.2 qPCR Results

There were no differences in initial weights between groups. Mice lost weight (< 1 g) in the five load and ten load groups independent of genotype or drug treatment (Table 3-3). There were no overall load differences or genotype differences for PTGS1 at any time point. Across all genotypes, NS-398 treated mice had a lower PTGS1 expression in response to loading compared to DMSO treated mice (P = 0.012). PTGS1 expression was greater in response to loading in the Lrp5^{−/−}-DMSO group following 10 loads (P = 0.046). The overall fold increase in response to loading was greater in PTGS2 (1.7 fold) than PTGS1 (1.3 fold); however, there was not a significant PTGS2 response with loading, drug treatment, or between genotypes (Table 3-4).
Table 3-3 Initial and final weights for mice in gene expression study

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>1 Load</th>
<th>5 Load</th>
<th>10 Load</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DMSO</td>
<td>NS-398</td>
<td>DMSO</td>
</tr>
<tr>
<td>Lrp5-/</td>
<td>Initial</td>
<td>23.5 (1.6)</td>
<td>22.0 (1.0)</td>
<td>21.9 (2.2)</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>21.3 (1.7)</td>
<td>20.9 (1.8)</td>
<td>21.7 (1.1)</td>
</tr>
<tr>
<td>WT</td>
<td>Initial</td>
<td>24.6 (1.4)</td>
<td>24.3 (1.3)</td>
<td>24.3 (2.6)</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>23.8 (1.7)</td>
<td>23.8 (1.5)</td>
<td>23.0 (1.5)</td>
</tr>
<tr>
<td>HBM</td>
<td>Initial</td>
<td>22.4 (1.6)</td>
<td>22.7 (1.7)</td>
<td>22.8 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>22.4 (1.8)</td>
<td>23.2 (1.2)</td>
<td>22.4 (1.3)</td>
</tr>
</tbody>
</table>

\( a = P < 0.05 \) Paired t-test for difference between initial and final weights. Data represent means (SD).

3.5 Discussion

Results from Chapter 2 showed Cox-2 inhibition blocks the periosteal bone formation response to a single mechanical load in mice, which is similar to the Cox-2 inhibition response in rats.\(^{(65,66)}\) Mice were then treated with a Cox-2 inhibitor before loading three days per week for ten loads, and bone formation was not suppressed (Chapter 2). One of the caveats with the previous long-term study was that NS-398 was only used on days the mice were loaded. Cox-2 inhibitors are used daily in the treatment of chronic conditions. This study used daily NS-398 treatment to rule out the possibility of a rebound effect and to test the bone response to daily Cox-2 inhibition. Similar to my previous study, Cox-2 inhibition did not suppress the periosteal formation response to multiple loads over a 25 day period in the WT and HBM groups. This is consistent with Sugiyama’s findings where two weeks of Cox-2 inhibition did not suppress the bone response to mechanical loading in WT C57Bl/6 mice.\(^{(51)}\) In the current study, the Lrp5⁻/⁻ mice did not have an increased response to loading. The Lrp5⁻/⁻ response has been variable in multiple studies. I previously measured an increase in bone formation in
### Table 3-4 Relative changes in gene expression after tibial compression in mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>1 Load</th>
<th>5 Loads</th>
<th>10 Loads</th>
<th>Avg. for all Loads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>NS-398</td>
<td>DMSO</td>
<td>NS-398</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lrp5−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| PTGS1  | 1.10 (0.42) | 0.95 (0.29) | 1.33 (0.45) | 1.03 (0.39) | 1.55 (0.43)

^a^ = P < 0.05 Relative load effect different than 1

Data represented as the relative mean fold difference (SD) between the right (loaded) and left (non-loaded) tibia, N = 4-5/group
Lrp5<sup>−/−</sup> mice after ten loads using this level of strain<sup>(21)</sup> and measured a periosteal bone formation response in Lrp5<sup>−/−</sup> mice with lower strains (Chapter 2). The lack of a bone formation response in Lrp5<sup>−/−</sup> mice in this study is similar to what Sawakami and Saxon found with lower strains.<sup>(26,47)</sup> Overall, the Lrp5<sup>−/−</sup> response to loading seems to be variable and unpredictable between gender, studies, and strains.

The strain for each genotype was based on results from a previous strain-response study and selected to induce a similar load response among the different genotypes. The WT and HBM mice had a similar, significant periosteal load response to mechanical loading but neither genotype had an endocortical response. The lack of an endocortical response was unexpected as I have previously measured a response at the strains used.<sup>(21)</sup> A maximal amount of strain that did not cause joint or bone injury was used for each genotype. The trabecular bone response was not measured in the proximal tibia because a previous study showed minimal trabecular bone in Lrp5<sup>−/−</sup> (5%) and WT (7%) genotypes and trabecular bone volume did not increase in HBM mice (Chapter 2).

Cox-1 upregulation is one possible mechanism that compensates for Cox-2 inhibition. Alam<sup>et al.</sup><sup>(46)</sup> reported that Cox-1 expression was upregulated in response to multiple mechanical loads in Cox-2<sup>−/−</sup> null mice but not in WT mice. In this study, Cox-1 mRNA increased 1.4 fold in DMSO treated mice in response to loading; however, Cox-1 mRNA expression did not increase in response to loading in NS-398 treated mice following one, five, or ten loads. Therefore I reject my hypothesis that Cox-1 is upregulated in response to mechanical loading and chronic Cox-2 inhibition.
Nitric oxide\textsuperscript{(55,57,144)} and parathyroid related protein (PTHrP)\textsuperscript{(145)} are two additional early responders to mechanical loading. Nitric oxide has been associated with regulation of PGE\textsubscript{2} in response to loading.\textsuperscript{(55)} PTHrP binds to the PTH1 receptor, and is increased in response to mechanical forces in osteoblast cells\textsuperscript{(145)} resulting in an anabolic effect on bone. To test if NO and/or PTHrP compensated for Cox-2 inhibition and loading, mRNA for endothelial nitric oxide synthase (eNOS) and PTHrP were measured after one, five, and ten loads. Neither eNOS nor PTHrP were upregulated four hours after a single load or were upregulated to compensate for Cox-2 inhibition after multiple loads in Lrp5\textsuperscript{−/−}, WT, or HBM mice (data not shown).

One limitation to my study is that the mRNA response was measured only at four hours after loading and the peak change in mRNA could have occurred at a different time point. PGE\textsubscript{2}, NO, and PTHrP are all released shortly after mechanical loading suggesting that precursors for their synthesis are already made and stored.

In conclusion, the histomorphometry results support previous studies that chronic Cox-2 inhibition does not suppress the bone response to multiple mechanical loads when using strains that induce lamellar bone formation. PTGS1, eNOS, and PTHrP mRNA expression were not upregulated in response to Cox-2 inhibition four hours after loading. This suggests bone adaptation occurs in response to lamellar forces in patients using Cox-2 inhibitors for long-term treatment.
4. Protein Response of Cox-1 and Cox-2 to Mechanical Loading

4.1 Abstract

Cox inhibition suppressed the bone response to a single load but did not suppress the response to multiple loads (Chapters 2 and 3). Furthermore it was hypothesized that Cox-1 would compensate for Cox-2 inhibition in response to loading. Cox-1 mRNA expression measured in osteocytes did not increase in response to loading regardless of Cox-2 inhibition. Gene expression was measured at only one time point following loading (four hours). The studies in this chapter measured the gene response and the Cox-1 and Cox-2 protein responses to tibial compression at 1, 6, 12, and 24 hours following one load or 24 hours following 2 or 3 loading sessions on consecutive days. The Cox-1 and Cox-2 protein responses were detected by immunohistochemistry.

Cox-2 mRNA in cortical osteocytes was upregulated 2.7 fold 1 hour after loading and remained increased 24 hours post load. Cox-1 mRNA expression did not increase with loading. In the non-loaded leg, Cox-2 antibody binding was detected in 0.8% of the osteocytes while Cox-1 antibody binding was detected in 15-20% of the osteocytes. Mechanical loading did not increase labeled osteocyte number for either isoform. In the non-loaded leg, Cox-2 antibody binding was detected in cells along 3% of the periosteal surface and 7% of the endocortical surface while Cox-1 antibody stain was present in 2-3% of the periosteal and endocortical surfaces. Cox-2 antibody binding did not increase with loading on the periosteal or endocortical surfaces however, Cox-1 antibody binding increased 2-3 fold following three loads along the periosteal and endocortical surfaces. In
conclusion, osteocyte Cox-2 mRNA expression increased 2 fold (n.s) in response to tibial compression while no increase was detected for Cox-1 mRNA. The increase in Cox-2 mRNA following loading did not translate to an increase in osteocytes or bone surface cells binding Cox-2 antibody. The increase in Cox-1 antibody binding along the bone surface following multiple loads may be a mechanism for bone adaptation in response to chronic Cox-2 inhibition.

4.2 Introduction

The bone response to mechanical loading is complex with many contributing pathways. Prostaglandin E$_2$ (PGE$_2$) is released within minutes of mechanical loading and is one contributing factor in the bone response to loading.$^{(53,54,64)}$ Inhibiting PGE$_2$ production with Cox inhibitors prevents the bone formation response to a single load (Chapter 2).$^{(65,66,72,102)}$ While Cox-2 is necessary for a single load, Cox-2 inhibition does not suppress the response to multiple loads suggesting Cox-2 is not required for multiple loads or another factor is compensating.

Cox-1 gene expression increased in response to two mechanical loads in Cox-2$^{-/-}$ mice suggesting Cox-1 may compensate for Cox-2 inhibition.$^{(46)}$ My previous study (Chapter 2) did not measure an increase in Cox-1 mRNA in response to loading at four hours post load. However, immunohistochemistry showed both Cox-1 and Cox-2 isoforms are present in osteoblasts and osteocytes (Ot) in non-loaded bones and that both isoforms are immediately increased in response to mechanical loading using the rat four-point bending model.$^{(146)}$ Furthermore, Cox-1 was detected in more osteocytes (102 Ot/mm$^2$) than Cox-2 (13 Ot/mm$^2$) immediately following loading.$^{(146)}$ Forwood suggested
Cox-1 is important for the immediate response to loading and that Cox-2 may be important for the adaptive response to loading.

The purpose of this study was to test the Cox-1 and Cox-2 gene response and protein response to tibial compression in wild-type mice. This study measured Cox gene regulation in osteocytes and the protein regulation of Cox-1 and Cox-2 in osteocytes and bone lining cells at multiple time-points following one, two, or three mechanical loading sessions.

4.3 Materials and Methods

4.3.1 Animals

Wild-type C57Bl6 mice were bred and housed in the animal research facility at Creighton University as previously reported (Chapter 2). All procedures were approved by Creighton University Institutional Animal Care and Use Committee.

4.3.2 Tibial Compression

The right legs of female, virgin mice (23.7 ± 1.7 g, 4.8 ± 0.5 months) were loaded in tibial compression as described in Section 2.3.3 (Chapter 2). Mechanical loads were applied at 11.5 N (1450 µε) for 360 cycles at 2 Hz. Mice in the 48 and 72 hour groups were loaded 24 hours apart for two or three consecutive days.

4.3.3 Tissue Collection for Gene Analysis

Loaded (right) and non-loaded (left) tibias (N = 10/group) were collected following the same procedure as described is Section 3.3.5 (Chapter 3). Loaded or non-loaded tibias from two mice were pooled for a final sample size of five per time point.
4.3.4 qPCR

Tibial mid-shafts were processed as described in Section 3.3.6 (Chapter 3). Quantification of Cox-1 and Cox-2 mRNA levels was performed by real time PCR using the primers and probes as designed in Section 3.3.6 (Table 4-1). Gene expression for two alternative early responders to mechanical loading, parathyroid hormone related protein (PTHrP) and nitric oxide (NO), were measured to test their response to tibial compression.

4.3.5 Immunohistochemistry

Prior to euthanasia, mice (N = 7/group) were anesthetized by inhalation of isoflurane and perfused via cardiac puncture with cold 4% paraformaldehyde (PFA) for 5 - 10 minutes. After perfusion, the right and left tibias were collected and stored in cold 4% PFA under vacuum for 36 hours. Bones were decalcified in 7% ethylenediaminetetraacetic acid (EDTA) for two weeks. Tibias were dehydrated using graded alcohol concentrations, cleared with Citrisolv, and paraffin embedded.

Tibias were sectioned 7 µm thick in the region 2-3 millimeters proximal to the tibia fibula junction. Following antigen retrieval (Trypsin at 37° C), sections were rinsed and incubated in 1% goat serum in phosphate buffered saline. Primary antibody was applied as a 1:200 dilution of rabbit polyclonal anti-Cox-1 (Cayman Chemical, Ann Arbor, MI, USA) or 1:200 dilution of rabbit polyclonal anti-Cox-2 (Cayman Chemical) in phosphate buffered saline containing 1% normal goat serum and 0.1% Tween 20 at 37° C for one hour. Sections were rinsed and endogenous endoperoxidases were quenched using 0.3% hydrogen peroxide. Secondary antibody consisting of a biotinylated goat anti-rabbit (1:500) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) in
phosphate buffered saline containing 1% normal goat serum and 0.1% Tween 20 was applied at room temperature for two hours. Antibody binding was visualized using the Vectastain-Horse Radish Peroxidase system (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. During each staining day, primary and/or secondary antibody was not applied to a section as negative control. Positive controls for Cox-1 included pyloric cells of the mouse gastric mucosa while positive controls for Cox-2 included the apical cytoplasm of epithelial cells in the proximal gastro-intestinal tract. Load/non-load pairs were processed at the same time. Two sections from each bone were stained on different days to reduce variation. Sections were blind coded and measurements were made on a light microscope for total area, marrow area, percent periosteal labeled surface, percent endocortical labeled surface, total number of lacunar spaces, total number of osteocytes, and number of positive stained osteocytes.

4.3.6 Statistical Analysis

All statistical analyses were conducted using SPSS version 21. Gene expression results were analyzed using single sample paired t-tests with the test variable set to 1. A test variable of 1 would indicate equal amounts of gene expression normalized to GAPDH between loaded and non-loaded legs. Paired t-tests were conducted between right and left legs with the four time points pooled for each gene to test the overall effect followed by paired t-tests for each time point. The immunohistochemistry (IHC) data was analyzed by two way analysis of variance (load/non-load x time) to test differences between time points followed by Bonferoni post hoc analysis. Paired t-tests were conducted to test for load differences within each time point.
4.4 Results

There were no differences in weights or ages between groups or studies for mRNA expression or IHC (Table 4-1). PTGS2 expression was increased 2.7 fold (n.s.) one hour after loading and relative expression remained at least 1.7 fold (n.s.) higher through 24 hours (Figure 4-1). The relative gene expression for PTGS1, eNOS, and PTHrP were not different in response to loading at any time point (Figure 4-1).

The average lacunae per bone area from all sections analyzed was 1051 ± 189/mm². Empty lacunae represented about 10% of total lacunae (data not shown). There were no differences in lacunar density or empty lacunae between groups. In the non-loaded leg, Cox-1 antibody was detected in 16.4% of osteocytes one hour after loading and did not differ at any of the other time points (Figure 4-2). In the loaded leg, Cox-1 antibody binding was detected in 13.4% of osteocytes one hour after loading. Cox-1 positive osteocytes tended to increase over time in the loaded leg but were never statistically different than the non-loaded control. Overall, Cox-1 stain was present in 17.9% of osteocytes in the non-loaded tibia and 19.1% of osteocytes in loaded bones.
Table 4-1 Primers and probe sets used to measure gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CAAGAAGGTGGTGAAGCAGGC</td>
<td>AGGTGGAAGAGTGGGAGTTGC</td>
<td>AGGGCATCTTGGGCTACACTGAGGAC</td>
</tr>
<tr>
<td>PTGS1</td>
<td>GGTGCCCTCACCATGCAATC</td>
<td>CCGACACAGACACCCCTGGTT</td>
<td>CTGTGTTATCTATCCGTGCCA</td>
</tr>
<tr>
<td>PTGS2</td>
<td>GTAACAGTCCATCTCTCAATGCAA</td>
<td>AATGGGCTGGAAGACATATCAA</td>
<td>ATCAGTGCGCTCGTAGCTTCTTCACA</td>
</tr>
<tr>
<td>PTHrP</td>
<td>AAGGGCAAGTCCATCCAGAG</td>
<td>GATCTCCCGCGATGGT</td>
<td>TTGC GCCGCCCACCCTTCTTCCTC</td>
</tr>
<tr>
<td>NOS3</td>
<td>CTGGCCCAGAAAATACCTGGTT</td>
<td>ACCGAACGAAGTGACACAATCC</td>
<td>ACAGTCTTCTCCCCCTCCAGTTCCCG</td>
</tr>
</tbody>
</table>

Primers and probe sets used for quantifying mRNA expression. GAPDH was used to control for variation in cell number. Cox-1 (PTGS1), Cox-2 (PTGS2), Parathyroid hormone related protein (PTHrP), and endothelial nitric oxide synthase (NOS3).

Table 4-2 Weights and ages for mice in the gene expression and immunohistochemistry (IHC) studies

<table>
<thead>
<tr>
<th></th>
<th>1 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
<th>48 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>4.9 (0.5)</td>
<td>4.8 (0.5)</td>
<td>4.8 (0.5)</td>
<td>4.8 (0.5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gene</td>
<td>23.9 (1.8)</td>
<td>22.5 (1.3)</td>
<td>24.1 (2.2)</td>
<td>23.4 (1.3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IHC</td>
<td>25.1 (1.9)</td>
<td>24.2 (2.2)</td>
<td>23.5 (1.4)</td>
<td>24.3 (1.4)</td>
<td>24.4 (0.9)</td>
<td>23.2 (2.5)</td>
</tr>
</tbody>
</table>

ND = No data, samples were lost during RNA extraction
Data represented as means (SD)
N = 3 or 4 for groups in gene expression analysis and N = 7 for IHC groups
Cox-2 antibody binding in the non-loaded leg was detected in 1.0% of osteocytes and did not change in any of the groups (Figure 4-2). Cox-2 antibody binding was detected in 0.8% of osteocytes after 1 hour of loading and was not greater at any of the time points. Antibody binding for Cox-2 was detected in 2.6% of osteocytes 6 hours after loading however, this increase was not significant (Figure 4-2).

Cox-1 immunopositive staining along the periosteal surface was limited in the non-loaded leg with an average of 1.7% of positive stain along the surface for all groups. Detection of Cox-1 antibody stain did not increase with loading until the 72 hour/3-load group where Cox-1 antibody binding was detected along 4.6% of surface. Cox-1 antibody staining was more prevalent along the endocortical surface but followed the same pattern. Percent immunopositive staining of the periosteum and endosteum for Cox-1 was greater at 72 hours after loading than all other time points (P < 0.03, Figures 4-3 and 4-4).
Cox-2 stain was detected along 2.6% of the periosteal surface in the non-loaded leg and along 4.4% of the surface in the loaded leg one hour following loading (Figures 4-3 and 4-4). Periosteal Cox-2 stain did not differ in any of the time points in the non-loaded or loaded legs. Cox-2 was detected along 2.8% of the periosteum in non-loaded tibias and 3.9% of loaded tibias. In the non-loaded leg, Cox-2 antibody stain was detected in 10.5% of osteocytes, while only 8.5% of osteocytes had Cox-2 staining in the loaded leg 1 hour post load. Staining did not differ significantly at any of the time points in the non-loaded leg.
or loaded legs. Overall, Cox-2 was detected along 8.6% of the non-loaded legs and 9.2% of the loaded leg’s endocortical surface (Figure 4-4).

### Figure 4-4

Top row: Percent positive staining along the periosteal surface for Cox-1 (A) and Cox-2 (B) stain. Bottom row: Percent positive staining along the endocortical surface for Cox-1 (C) and Cox-2 (D). White bars represent non-loaded tibias while black bars represent loaded tibias. a = P < 0.03. N = 7.

#### 4.5 Discussion

The PGE$_2$ response to a single mechanical load has been demonstrated to be Cox-2 dependent based on Cox inhibition studies and gene expression responses to loading.$^{(46,64-66,72,143)}$ Here Cox-2 mRNA was reported to be expressed 2.7 fold (n.s.) more in the loaded leg compared to the non-loaded leg one hour post load and remained approximately 2-fold higher at 6, 12, and 24 hours post load. Alam et al.$^{(46)}$ reported PTGS1 increases in response to two mechanical loads in Cox-2 knockout mice. PTGS1 expression in the current study did not increase with a single load at any of the time...
points supporting previous studies that measure no load response in PTGS1.

Unfortunately, the RNA extraction failed in the samples from 48 and 72 hours post
loading therefore I was unable to measure a gene response from tibias loaded multiple
times. No differences in PTHrP or eNOS gene expression were measured in response to
loading. This differs from what has been reported by others.\(^{55-58,143,145,147}\) \textit{In vivo}
measurements of the mRNA expression for eNOS and PTHrP were previously measured
after loading at extremely high forces suggesting they are more involved in the woven
bone response than in the lamellar load response.

PGE\(_2\) is released within minutes following mechanical loading. While Cox-2 gene
expression is increased in response to loading, it would take hours before the new
message is converted to protein for the enzymatic conversion of arachidonic acid to
PGE\(_2\). Cox-1 and Cox-2 protein was therefore visualized using immunohistochemistry to
measure the protein response to loading. Cox-1 immunostaining was previously reported
to be present at levels 10-fold higher than Cox-2 immunostaining in non-loaded rat
osteocytes (43.6 Ot/mm\(^2\) vs 4.1 Ot/mm\(^2\)) and along the endocortical surface (82.7% and
9.7%). Cox-1 positive osteocytes increased to 102 Ot/mm\(^2\) and Cox-2 positive osteocytes
increased to 14 Ot/mm\(^2\) immediately following four-point bending with no change in
length of endocortical surface stain.\(^{146}\) The presence of Cox-1 in 10 times the number of
cells as Cox-2 suggests that Cox-1 is immediately available in more osteocytes than Cox-
2 to synthesize PGE\(_2\) at the time of loading. The current study found that Cox-1 was
detected in 25 fold more cells in non-loaded osteocytes than Cox-2 (177.0 ± 80.7 Ot/mm\(^2\)
vs 6.9 ± 6.5 Ot/mm\(^2\)). Unlike Forwood’s results, no increase in osteocyte Cox-1 or Cox-
2 antibody binding was measured in response to loading. One possible explanation for
this difference is the strain used in the current study (1450 µε) was less than what was used in Forwood’s study (3100 µε) suggesting PGE₂ produced by osteocytes is more involved in the response to high strains than physiological forces.

While the number of osteocytes expressing Cox-1 or Cox-2 did not increase in response to loading, an increase in Cox-1 positive cells occurred along the periosteal and endocortical surfaces 72 hours after the initial load. This delayed increase in Cox-1 expression may be a mechanism that prepares the bone surface for future loads. Cox-2 inhibitors suppress the bone formation response to a single load but fail to suppress the response to multiple loads. While Cox-2 may be essential for the response to a single load, the increase in Cox-1 surface staining suggests Cox-1 may play an important role in the adaptive response to loading at physiologic strains.

Osteocytes are regarded as the mechano-responsive cells that reside within lacunae in bone. The lacunar density is a measure of the maximum number of osteocytes that could reside in bone. The lacunar density reported here (1051 Ot/mm²) is similar to what has previously been reported in C57Bl6 mice (1260 Ot/mm²), yet greater than the lacunar density of 160 -250/mm² reported in humans. Osteoblasts, cuboidal cells on the periosteal and endocortical surface, were present at a density of 129 cells/mm. Based on the average perimeter lengths and areas measured in this study, this would equate to a total of 479 periosteal and 309 endocortical surface cells for a total of 787 cells lining the bone surface and 529 osteocytes on a 2D section. Osteocytes have been reported to be the most abundant cells in bone representing 90-95% of bone cells. Here we report 33% more osteoblasts and bone lining cells on bone surfaces than osteocytes in a bone cross section. Not only are lining cells more prevalent but cells on
the periosteum would be exposed to greater strains than osteocytes, which are embedded within mineralized matrix, suggesting that lining cells may play a larger role in the bone response to mechanical forces than previously expected.

A limitation to the gene expression results in this study and previous studies is that the periosteal and endocortical surfaces were removed. This means the mRNA results represent the osteocyte response only. The increase in cells that stain positive for Cox-1 along the bone surface 72 hours after the initial load, suggest this population of cells provides a delayed response to loading which may missed by the current methods of gene analysis. Furthermore, the greater number of cells lining the bone surface compared to the number of osteocytes and the exposure to higher strains suggests that the role of bone lining cells and surface osteoblasts should not be ignored in future gene analysis. Not only should the periosteal response be measured but the gene response to loading should be followed for a minimum of 72 hours.

In conclusion, Cox-2 gene expression in osteocytes was 2.7-fold (n.s) higher in loaded legs one hour following loading and remained elevated at 24 hours while Cox-1 gene expression did not change in response to load. The increase in gene expression did not translate to an increase in osteocytes or bone surface lining cells expressing Cox-2 detected by immunohistochemistry. This suggests the increase in osteocyte gene expression was due to the activated cells producing more Cox-2 mRNA and not due to an increased number of cells producing Cox-2. In non-loaded osteocytes, Cox-1 antibody staining was more prevalent than Cox-2 demonstrating that Cox-1 is more available for an immediate response than Cox-2. The increase in Cox-1 immunostaining on the periosteal and endocortical surfaces after three loads suggests Cox-1 may be a
mechanism to prepare the cell for future loads which may be missed by current gene analysis techniques.
5. Discussion

The purpose of this project was to investigate the effects of cyclooxygenase inhibition on the bone response to mechanical loading in mice and to determine if the effects were altered by varying levels of Lrp5 expression. Administration of either a non-selective or a Cox-2 selective inhibitor suppressed the periosteal bone formation response to a single mechanical load in wild-type mice (Chapter 2) which is similar to what has been reported in rats and roosters. However, long-term administration of the Cox-2 selective inhibitor, NS-398, did not suppress the bone formation response to multiple loads over 22 days (Chapters 2 and 3). HBM mice have an increased load response to strain but did not differ from WT mice in response to Cox-2 inhibition. Lrp5−/− knockout mice have decreased mechanosensitivity which resulted in a variable load response between the two long-term studies independent of NS-398 treatment (Chapters 2 and 3).

5.1 Periosteal Envelope

The periosteal envelope is the primary surface for adapting to mechanical loads. In the single load study, the non-selective Cox inhibitor, indomethacin, and the Cox-2 selective inhibitor, NS-398, suppressed the periosteal formation response to a single load. This is similar to what others found in the loaded avian ulna, rat four-point bending, and rat ulnar compression models. While both inhibitors suppressed the periosteal loading response, the BFR in indomethacin treated mice was less compared to the saline control whereas loaded tibias in NS-398 treated mice were not different than their vehicle control (Chapter 2). In rats, NS-398 suppressed the bone formation response more than indomethacin. One possibility to explain why indomethacin had such a strong
effect in the current study is that the dose used was five times greater than what was used in rats. To ensure DMSO does not inhibit bone formation, DMSO treated mice were compared to saline treated mice. Mice in both groups had a similar periosteal load response demonstrating DMSO did not influence the periosteal response to loading. Therefore DMSO was used as the vehicle for both long-term studies.

In both multiple load studies, Cox-2 inhibition did not suppress the periosteal bone formation response to loading in WT or HBM mice (Chapters 2 and 3). In the first study, mice were loaded to create moderate strains on the bone while in the second multiple load study mice were loaded at a higher force to create greater strains on the tibias resulting in a bigger formation response. Loaded tibias in the second study had a greater change in BFR (56-65 µm³/µm²/yr) compared to mice in the first long-term study (30-61 µm³/µm²/yr).

No interaction between the PGE2 pathway and increased Lrp5 signaling related to the high bone mass G171V mutation was detected in response to mechanical loading. In the first multiple load study, HBM mice had low baseline bone formation in the non-loaded leg (56 µm³/ µm²/yr) compared to the Lrp5⁻/⁻ and WT genotypes (~115 µm³/µm²/yr). However, the response due to loading was similar for all groups (30-45 µm³/µm²/yr) except in the WT-NS-398 treated mice. This indicated the range of forces selected were appropriate to induce a similar load response between genotypes. In the second multiple load study, HBM mice had a similar response as the WT mice. In the HBM groups, the relative BFRs were similar between the first load study (33-45 µm³/µm²/yr) and the second (42-46 µm³/µm²/yr) suggesting the increase in force had no additional load effect. In HBM mice, the similar response in the DMSO treated groups
and NS-398 treated groups for both studies suggests PGE$_2$ may not play an essential part in the bone formation response to multiple loads when using forces that create a lamellar bone formation response.

In Lrp5$^{-/-}$ mice, the first multiple load study (Chapter 2) indicates PGE$_2$ plays an important role in the adaptive response to multiple loads however, the second multiple load study (Chapter 3) lacked a load induced bone formation response in DMSO and NS-398 mice. In the first multiple load study, Lrp5$^{-/-}$ mice had a periosteal formation response in the DMSO treated group but not in the NS-398 treated group (P = 0.201) suggesting Cox-2 inhibition suppressed the bone formation response to multiple loads (Chapter 2). While the response in the NS-398 treated group looks similar to the DMSO group, the lack of a significant load response in the Lrp5$^{-/-}$-NS-398 mice may be due to a high variation when comparing loaded tibias to their contra-lateral counterparts using paired t-tests. Differences of the means and standard deviations between non-loaded and loaded tibias were 40 ± 42 $\mu$m$^3$/µm$^2$/yr for DMSO treated mice and 33 ± 90 $\mu$m$^3$/µm$^2$/yr for NS-398 treated mice. The large variation was related to several individual mice having no load response while others had a prolific bone response to loading due to woven bone formation. In the second multiple load study, Lrp5$^{-/-}$ mice did not have a periosteal load response for either DMSO or NS-398 treated groups, even with higher loading forces. The lack of a periosteal formation response in the second multiple load study may be related to the difference in size and age of Lrp5$^{-/-}$ animals compared to the first long-term study. Mice were older (0.8 mo) and heavier (0.8 g) in the second study. While their tibial lengths were the same, the total area and cortical areas were larger in the second long-term study suggesting the strains may not have been as high as predicted.
One way that the bone may adapt to multiple loads is through upregulation of Cox-1 protein as visualized by antibody staining (Chapter 4). The amount of Cox-1 immunostaining detected along the periosteal surface was low (~1.7%) in all non-loaded tibias and in loaded tibias at 1, 6, 12, 24, and 48 hours post load. However, Cox-1 antibody staining doubled along the periosteal surface in the three load group collected 72 hours after the initial load. Whether the doubling of Cox-1 immunostaining along the periosteum was related to multiple loads, the time after the load or the combination of both is not clear from this study. To investigate this further, another group could be loaded once and collected 72 hours post load. Kidd et al.\(^{151}\) reported that Cox-1 gene expression is a delayed response to fracture that peaks four days following a large loading stimulus. The increase in Cox-1 protein may be a way for the bone lining cells to prepare for more loads. If the cell has more available Cox protein at the time of load, it would be able to convert more arachidonic acid to PGE\(_2\). While gene expression studies analyzing osteocytes have found Cox-2 to be increased in response to loading, no increase in Cox-2 immunostaining was detected along the periosteal surface with loading (Chapter 4).

A limitation of the gene response studies conducted in chapters 3 and 4 is that the periosteum was physically removed from the tibias prior to RNA extraction. My hypothesis that Cox-1 would be upregulated to compensate for Cox-2 inhibition with multiple loads was initially rejected based on the results from the gene expression studies. However, the increase in Cox-1 staining in response to multiple loads on the periosteal surface suggests Cox-1 may have a role in the adaptive bone formation response to loading. To fully test if Cox-1 is upregulated in response to Cox-2 inhibition or if it is a normal, physiological response to multiple loads, the time course study would need to be
repeated using NS-398 or another Cox-2 selective inhibitor while maintaining the periosteum. The current study detected higher levels of Cox-1 staining than Cox-2 staining in response to multiple mechanical loads without comparing the effects of Cox-2 inhibition. The increase in Cox-1 staining on the periosteum suggests it may play a role in the bone formation response to loading in the osteoblasts and bone lining cells, independent of or different from the osteocyte response.

For each of these studies, mice were loaded at a force that would induce lamellar bone formation. Woven bone formation was measured on the periosteal surface of five Lrp5\(^{-/-}\) mice in the first multiple load study and in three mice (2 Lrp5\(^{-/-}\), 1 WT) in the second multiple load study. Woven bone is an emergency formation response where osteoblasts secrete matrix rapidly and in an unorganized manner. McKenzie et al.\(^{143}\) published that PTGS2 gene expression in osteocytes is increased 2-fold in response to a lamellar formation stimulus and that PTGS2 increased 16-fold in response to a formation stimulus that induces woven bone formation. In McKenzie’s study, the large increase in PTGS2 expression in the bones loaded to induce a woven bone response suggests Cox-2 has a bigger role in the formation response at higher strains. However, NS-398 did not have an effect in regards to preventing woven bone formation in my studies. Of the tibias that had woven bone formation, five received DMSO treatment and three received NS-398. Seven of the eight mice that had woven bone formation were Lrp5\(^{-/-}\) mice suggesting that the woven bone response occurs independent of the Lrp5 receptor and that woven bone formation may be a secondary pathway activated at high mechanical loads. There was no difference in bone formation between WT and HBM mice in response to Cox-2
inhibition on the periosteal surface. HBM mice were loaded at higher forces than WT mice yet had less predicted strains on the tibia due to the larger bone size in HBM mice.

5.2 Endosteal Envelope

The baseline endocortical formation rates and the overall responses to loading were extremely variable between the studies. In the non-loaded legs of mice in the single load study, baseline endocortical bone formation rates were high (~ 200 $\mu$m$^3$/ $\mu$m$^2$/yr, Chapter 2) which may account for the lack of load response. The baseline endocortical rates in the two multiple load studies (20 – 125 $\mu$m$^3$/ $\mu$m$^2$/yr, Chapters 2 and 3) were less than the single load study but still had high variation between the groups. The ages of the WT mice were the same in all studies, therefore age related growth should not have been a factor. The average ages for mice in all studies ranged from 4.5 to 5.3 months. At 4 months of age, mice are considered to be skeletally mature meaning formation rates on non-loaded bone surfaces should be at a steady, adult level related to normal bone turnover and not related to growth.

Cox inhibition in the single load study did not affect the endocortical bone formation response (Chapter 2). Indomethacin treated mice had a bone formation response to a single mechanical load on the endocortical surface as did their saline controls. NS-398 treated mice did not have a load response but the vehicle control group also did not have a load response. The lack of an inhibitory response to a single load on the endocortical surface in indomethacin treated mice is contrary to previous published studies in roosters and rats that used different loading models and higher strains (2700 - 3600 $\mu$ε) that caused a woven bone response on the periosteal surface. (65,66,102) This
suggests PGE₂ may be more involved in the bone response to supra-physiologic mechanical loads on the endocortical surface.

No endocortical bone formation response for any of the groups in the two multiple load studies was measured except in the first study’s Lrp5/–/-NS-398 group which had high variation between mice (Chapters 2 and 3). An endocortical bone formation response was expected for all genotypes because the forces used for each genotype resulted in an endocortical response (29 – 109 μm³/μm²/yr) in a previous study. While the increased baseline formation rate may be a factor in the single load study, baseline BFRs were not exceptionally high in either multiple load study. A possible explanation is that the loading regimen (force, strain rate, and cycle number) chosen for the multiple load studies was not strong enough to elicit a load response.

One of the reported benefits of using the tibial compression model compared to the ulnar compression or four-point bending models is the ability to investigate the effects of pharmacological treatments combined with loading on trabecular bone. However, in our C57Bl6 mice, µCT analysis in the proximal tibia showed little trabecular bone compared to C57Bl6 mice in other labs. The region of interest analyzed (0.048 mm to 1.008 mm distal the growth plate) was based on previous µCT studies in C57Bl6 mice. The primary spongiosa is reported to extend to approximately 0.05 mm in C57Bl6 mice therefore the region measured should have avoided the primary spongiosa. Selection of where to start analysis is subjective to the operator’s judgment on where the growth plate ends. Complicating matters is variation in measurement that occurs due to each slice being 0.016 mm thick. Depending on where the growth plate ends in the last section before analysis, measurements can be shifted in the region of interest. Sugiyama
Discussion

et al.\textsuperscript{(51)} demonstrated an increase in trabecular and cortical bone formation associated with tibial compression following two weeks of loading (M, W, F). Sugiyama et al. reported 17\% BV/TV in non-loaded legs whereas the WT mice analyzed in this study had 7\% BV/TV. Moreover, there was little to no trabecular bone measured by μCT past 0.51 mm distal to the growth plate suggesting the region measured should have ended prior to 0.51 mm distal to the growth plate (Chapter 2).

Immunostaining on the endocortical surface detected more positive surface staining for Cox-1 and Cox-2 than on the periosteal surface. Cox-2 antibody staining was detected along ~9\% of the endocortical surface and did not change with loading which is similar to previous reports in rats where 3-16\% of surface expressed Cox-2 and no load response was detected.\textsuperscript{(146)} Cox-1 followed the same pattern as the periosteum where there was no difference detected in Cox-1 antibody staining with loading until the 3 load group, where Cox-1 immunostaining was detected along three times more surface than in the non-loaded leg (Chapter 4). Cox-1 staining in the non-loaded bones was detected along the endosteum (5\%) at lower levels than previous findings in rats (80-98\%).\textsuperscript{(146)} Differences in Cox-1 expression between species could be related to the antibody specificity used for binding Cox-1. Forwood et al.\textsuperscript{(146)} used a monoclonal mouse antibody against ovine PGHS-1 to stain for Cox-1 in rat while a polyclonal rabbit antibody against murine PGHS-1 was used to detect Cox-1 in the present study.

5.3 Cortical Envelope

In mice, the cortical envelope consists of mineralized matrix and osteocytes which have been reported to be the mechanosensing cells in bone. Histomorphometry measures active bone formation that can be a secondary or an indirect measure of osteocyte activity.
but fails to directly measure the response in osteocytes. After the second multiple load study confirmed that Cox-2 inhibition does not suppress the bone formation response to multiple loads, gene expression in osteocytes was measured to directly detect their response to loading and to investigate possible alternative pathways that may compensate for Cox-2 inhibition.

While Cox-2 has been demonstrated as the isoform responsible for the load response to a single load, Cox-1 has been reported to be upregulated in Cox-2\(^{-/-}\) mice in response to loading.\(^{(46)}\) In a fracture loading model, Cox-1 gene expression has a delayed increase with peak levels measured four days following fracture.\(^{(151)}\) Combining the gene expression response in WT mice from all experiments, Cox-2 gene expression increased 2.7 fold (n.s.) one hour after loading and the relative expression remained elevated approximately two-fold through 24 hours. The two fold increase in PTGS2 expression is consistent with McKenzie’s results of a 1.9 fold increase when a lamellar load regimen was applied to the rat ulna.\(^{(143)}\) PTGS2 expression was not different from a single load after five or ten load sessions and chronic Cox-2 inhibition did not alter the PTGS2 gene response compared to DMSO. While gene expression is not a direct indication that there will be an increase in PGE\(_2\), it suggests osteocytes were being stimulated and that Cox-2 gene expression was being initiated as a response to loading. NS-398 inhibits the Cox-2 enzyme activity so the similar response between DMSO and NS-398 treatment was expected. PTGS2 gene expression was not expected to change in response to Cox-2 inhibition. If PTGS2 expression changed I would have expected an increase in expression as a product related feedback pathway to increase PGE\(_2\) to compensate for the lack of enzyme activity. In the time course study, PTGS2 expression was significantly increased.
when data from all time points was pooled; however, there was no significant increase at any individual time point (Chapter 4). Several samples were lost during RNA extraction so the final sample size in each group was only 3 or 4. Increasing the sample size may have led to load related differences at individual time points.

The increase in Cox-2 gene expression did not translate to an increase in the number of osteocytes expressing Cox-2 protein as detected by immunohistochemistry (Chapter 4). In the non-loaded leg, Cox-2 staining was detected in approximately 1% of osteocytes or 9.5 Ot/mm² and did not change in response to loading at any of the time points tested. Forwood et al.\textsuperscript{(146)} reported an increase in Cox-2 immunostaining in rats immediately following loading (10 minutes post-load) from 4.1 to 13.8 Ot/mm² but reported < 2 Ot/mm² at all other time points.

Cox-1 gene expression (PTGS1) did not increase in response to a single load at any time point. This result is consistent with previous studies.\textsuperscript{(72,143)} PTGS1 mRNA was not increased in response to multiple loads in the DMSO controls or with chronic Cox-2 inhibition. This finding did not support the hypothesis that Cox-1 compensates for Cox-2 inhibition, yet the IHC results on the periosteal and endocortical surfaces detected an increase in Cox-1 immunostaining in response to multiple loads in the absence of NS-398. Kidd et al.\textsuperscript{(151)} reported Cox-1 gene expression is a delayed response to fracture loading. The current study did not measure Cox-1 mRNA expression beyond 24 hours. Further studies would need to measure the PTGS1 expression levels at 3-4 days post loading to see if Cox-1 increases in response to lamellar loads or if the Cox-1 increase reported by Kidd et al. was dependent on fracture. No Cox-1 gene response was measured in response to lamellar loads after five or ten sessions. Overall, PTGS1
expression was 1.33 – 1.52 fold greater (n.s.) in the loaded leg compared to the non-loaded leg in DMSO treated mice. The relative PTGS1 expression in osteocytes was less in NS-398 treated mice (0.95-1.19) in response to loading, suggesting Cox-1 is not compensating for Cox-2 inhibition using lamellar loads.

A limitation to the multiple load study is that RNA expression was analyzed only four hours post loading. PTGS2 gene expression has been demonstrated to increase as soon as 1 hour after mechanical loading\(^{72,73,143}\) and remain elevated at one and three days post loading.\(^{143}\) In the short term gene expression study, no differences were detected in PTGS1 expression or PTGS2 expression at 1, 6, 12, or 24 hours after loading therefore the four hour time point should have been sufficient to detect changes in gene expression.

Other investigators have suggested that increased release of PGE\(_2\) is an immediate early response that initiates bone formation by activating \(\beta\)-catenin signaling.\(^{10,53,64}\) My studies show that PGE\(_2\) was important in initiating the response to a single load but the formation response still occurs with multiple loads when PGE\(_2\) was presumably blocked. Two other early responders to mechanical loading, parathyroid hormone related protein (PTHrP) and endothelial nitric oxide synthase, were examined as possible candidates that mediate loading effects when PGE\(_2\) production is prevented by Cox-2 inhibition. PTHrP and eNOS mRNA expression were unchanged after one, five, or ten loads with or without Cox-2 inhibition. In the short term gene expression study, PTHrP mRNA expression and eNOS mRNA expression were not different between loaded and non-loaded legs at any time point.
Gene levels for PTGS1 or PTGS2 were similar in osteocytes for each genotype suggesting Cox is not upregulated to compensate in Lrp5\(^{-/-}\) mice and that Cox plays a similar role in HBM mice even with increased β-catenin signaling.

5.4 Conclusion

Cox-2 is an immediate early pathway that has a major role in the bone response to a single load. However, when Cox-2 is inhibited, the cell has other means of adapting to multiple mechanical loading sessions. I believe that following a single load other pathways such as Cox-1 may be increasing the osteoblasts/bone lining cells sensitivity to loading by preparing it for further loads. One example is that the number of cells expressing Cox-1 protein is increased along the bone surface following loading. By having more Cox-1 available at the time of loading, the cell is more prepared to adapt to loads.

While osteocytes may be the primary cells for sensing mechanical strain, osteoblasts along the bone surfaces are responsible for the synthesis of osteoid and new bone formation. Cox-1 may play a larger role in osteoblasts and in the production of new matrix as evidenced by the increase in Cox-1 antibody stain on the periosteal and endocortical surfaces 72 hours after the first load. Future in vivo studies should focus on the delayed response along the bone surface to determine the role of bone lining cells and osteoblasts in compensating for Cox-2 inhibition.

Current in vitro studies allow only for analysis after a single load and no in vitro model is available that allows the investigator to study the formation or cell response to multiple loads. While it is important to understand the signaling response to a single stimulus, bone constantly adapts to force. Therefore, future studies need to investigate the
bone response to multiple loads. It is also essential to investigate the interaction of the “mechanosensing” osteocyte and the matrix producing osteoblast (which also senses loads).

It was predicted that Lrp5−/− mice would have the greatest suppression in response to Cox-2 inhibition and loading. While Cox inhibition tended to suppress the periosteal load response in the first long-term loading study, the lack of a load response in the second long-term study prevented confirmation. No differences were measured in gene expression after 1, 5, or 10 loads in Lrp5−/− mice compared to WT mice suggesting the lack of Lrp5 function does not influence the Cox-1 or Cox-2 gene responses to loading. HBM mice had similar bone formation rates and Cox-1 and Cox-2 gene expression levels as WT mice. Therefore, having increased Lrp5/β-catenin capacity did not seem to alter the bone response compared to WT mice other than decreasing the initial amount of strain needed to induce a response.

In summary, Cox inhibition suppressed the bone formation response to a single load but did not suppress the formation response to multiple loads in mice. I was unable to identify a gene that compensates for chronic Cox-2 inhibition in the response to multiple mechanical loads. Contrary to my hypothesis, Cox-1 mRNA expression did not increase in response to loading, however, Cox-1 antibody staining increased in response to multiple loads on the periosteal and endocortical surfaces 72 hours after the initial load suggesting Cox-1 may play a role in the adaptive response.
Appendix I: Abbreviations and Calculations

β  Beta
ε  Strain
μ  Micro
AA  Arachidonic acid
AKT  Protein kinase B
ANOVA  Analysis of variance
Ar  Area
B  Bone
BFR  Bone formation rate
BMD  Bone mineral density
Ca^{2+}  Calcium
cAMP  Cyclic adenosine monophosphate
Cox  Cyclooxygenase
CREB  cAMP response-element binding protein
CT  Computed tomography
D  Density
Dkk-1  Dickoff-1
dL.  Double label
DMSO  Dimethyl sulfoxide
Ec  Endocortical
EDTA  Ethylenediaminetetraacetic acid
En  Endosteal
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EP</td>
<td>E-type prostanoid receptor</td>
</tr>
<tr>
<td>F</td>
<td>Formation</td>
</tr>
<tr>
<td>Frz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>g</td>
<td>Gram(s)</td>
</tr>
<tr>
<td>GLM</td>
<td>Generalized linear model</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>HBM</td>
<td>High bone mass</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Inhibitory concentration of 50%</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin growth factor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Ir.</td>
<td>Inter label</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LEP</td>
<td>Lymphoid Enhancer-binding Factor</td>
</tr>
<tr>
<td>Lrp5</td>
<td>Low-density lipoprotein receptor-related protein 5</td>
</tr>
<tr>
<td>M</td>
<td>Molar; Mineral(izing)</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter(s)</td>
</tr>
<tr>
<td>MAR</td>
<td>Mineral apposition rate</td>
</tr>
<tr>
<td>MC3T3</td>
<td>Mouse calvarial cells 3T3</td>
</tr>
<tr>
<td>MLOY4</td>
<td>Murine long-bone osteocyte Y4</td>
</tr>
<tr>
<td>MU</td>
<td>Mechanical usage</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>N.S.</td>
<td>Not significant</td>
</tr>
<tr>
<td>NS-398</td>
<td>N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>Ot.</td>
<td>Osteocyte</td>
</tr>
<tr>
<td>P</td>
<td>Periosteal (eum)</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>Pm</td>
<td>Perimeter</td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin-endoperoxide synthase or cyclooxygenase</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHR1</td>
<td>Parathyroid hormone receptor 1</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Rate</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>S</td>
<td>Surface</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sL.</td>
<td>Single label</td>
</tr>
<tr>
<td>SOST</td>
<td>Gene for sclerostin</td>
</tr>
</tbody>
</table>
TCF  Transcription factor (T-Cell factor)
TRAP  Tartrate resistant acid phosphatase
Tt.  Total
V  Volume
Wi.  Width
Wo.  Woven
WT  Wild-type

Calculations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>(½ sL + dL + Wo.B)/2</td>
</tr>
<tr>
<td>FS</td>
<td>MS/BS</td>
</tr>
<tr>
<td>MAR</td>
<td>Interlabel thickness/days between labels</td>
</tr>
<tr>
<td>BFR</td>
<td>FS*MAR</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone volume/total volume</td>
</tr>
</tbody>
</table>

Figure A-1. Ultraviolet images for calcein labeling show (A) double labeling (dL) and (B) woven bone (Wo.B) formation. EcS, endocortical surface. PsS, Periosteal surface. Represents original image at 200x with scale bar = 50 µm.
Appendix II: Strains and Strain Rates for Loading

Strain is the measure of deformation that occurs when a force is applied. Strain is calculated as:

\[ Strain \ (\varepsilon) = \frac{L - L_O}{L_O} \]

where \( L \) is the final length and \( L_O \) is the original length.

Strain rate is the speed of deformation over time. For all studies, mice were loaded at 2 cycles per second (2 Hz). This means the deformation was applied or removed in 0.25 second increments.

\[ strain \ rate = \frac{deformation}{time} \]

For the WT mice in the single load study:

\[ strain \ rate = \frac{1450 \ \mu\varepsilon}{0.25 \ s} = 5800 \ \mu\varepsilon/s \]
<table>
<thead>
<tr>
<th></th>
<th>Single Load Study</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>με</td>
<td>rate (με/s)</td>
<td></td>
</tr>
<tr>
<td>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1500</td>
<td>6000</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1450</td>
<td>5800</td>
<td></td>
</tr>
<tr>
<td>HBM</td>
<td>650</td>
<td>2600</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Long-term Study 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>με</td>
<td>rate (με/s)</td>
<td></td>
</tr>
<tr>
<td>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1060</td>
<td>4240</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1450</td>
<td>5800</td>
<td></td>
</tr>
<tr>
<td>HBM</td>
<td>1080</td>
<td>4320</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Long-term Study 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>με</td>
<td>rate (με/s)</td>
<td></td>
</tr>
<tr>
<td>Lrp5&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>2000</td>
<td>8000</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1450</td>
<td>5800</td>
<td></td>
</tr>
<tr>
<td>HBM</td>
<td>1080</td>
<td>4320</td>
<td></td>
</tr>
</tbody>
</table>
Appendix III: Molar Concentrations

Maximum molar concentration calculations based on the total amount of drug injected and a total blood volume of 2.0 mL^152^)

**Indomethacin maximum calculations**

5 mg/ml * 0.06 ml = 0.3 mg (0.0003 g) indomethacin injected

0.0003 g / 357.79 g per mole of indomethacin = 8.39 * 10^-7 Moles per injection

8.39 * 10^-7 / .002 L (estimated blood volume) = 0.000419 Mol/L

**NS-398 maximum calculations**

10 mg/ml * 0.03 ml = 0.3 mg (0.0003 g) NS-398 injected

0.0003 g / 314.4 g per mole of NS-398 = 9.54 * 10^-7 Moles per injection

9.54 * 10^-7 / 0.002 L = 0.000477 Mol/L
References


osteocytes—a cytoskeleton-dependent process. Biochem Biophys Res Commun


(26) Saxon LK, Jackson BF, Sugiyama T, Lanyon LE, Price JS. Analysis of multiple bone responses to graded strains above functional levels, and to disuse, in mice in vivo show that the human Lrp5 G171V High Bone Mass mutation increases the osteogenic response to loading but that lack of Lrp5 activity reduces it. Bone 2011;49(2):184-193.


Bryan T. Hackfort


(65) Li J, Burr DB, Turner CH. Suppression of prostaglandin synthesis with NS-398 has different effects on endocortical and periosteal bone formation induced by mechanical loading. Calcif Tissue Int 2002;70(4):320-329.


(74) Chandrasekharan NV, Dai H, Roos KLT, Evanson NK, Tomsik J, Elton TS, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other


