COVER SHEET

INHIBITORS OF CYCLO-OXYGENASE-2 AND SECRETORY PHOSPHOLIPASE A_2 (GROUP IIa) PRESERVE BONE ARCHITECTURE FOLLOWING OVARIECTOMY IN ADULT RATS

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Non-standard Abbreviations:

PGHS	Prostaglandin G/H synthase
PLA_2	Phospholipase A_2
$sPLA_2$	Secretory phospholipase A_2
$cPLA_2$	Cytosolic phospholipase A_2
$iPLA_2$	Calcium independent phospholipase A_2
AA	Arachidonic acid
BMD	Bone mineral density
PTH	Parathyroid hormone
PAF	Platelet activating factor
OVX	Ovariectomy/ovariectomized
SHAM	Sham operated
DFU	[5,5-dimethyl-3-3 (3 fluorophenyl)-4-(4 methylsulphonal) phenyl-2 (5H)-furanone]
KH064	[5-(4-benzyloxyphenyl)-4S-(7-phenylheptanoylamino-pentanoic acid)]
PEG	Polyethylene glycol
NBF	Neutral buffered formalin
TRAP	tartrate resistant acid phosphatase
PLC	Phospholipase C
PLD	Phospholipase D
ET-1	Endothelin-1
T1	Timepoint 1: 72 days
T2	Timepoint 2: 98 days

ABSTRACT

Accelerated states of osteoblast and osteoclast activity are regulated by prostaglandins *in vitro*, but experimental evidence for specific roles of cyclooxygenase-2 (COX-2) and secretory phospholipase A_2 ($sPLA_2$) in bone adaptive remodeling *in vivo* is lacking. We found that treatment with a specific COX-2 or $sPLA_2$ (group IIa) inhibitor prevented ovariectomy-induced (OVX-induced) decreases in trabecular connectivity; suppressed the acceleration of bone resorption; and maintained bone turnover at SHAM levels following OVX in the rat. The $sPLA_2$ inhibitor significantly suppressed increases in osteoclast surface induced by OVX, whilst the COX-2 inhibitor only had a marginal effect. These findings demonstrate that specific inhibitors of COX-2 and $sPLA_2$-IIa effectively suppress OVX-induced bone loss in the adult rat by conserving trabecular bone mass and architecture through reduced bone remodeling and decreased resorptive activity. Moreover we report an important role of $sPLA_2$-IIa in osteoclastogenesis independent of the COX-2 metabolic pathway in the OVX rat *in vivo*.

INTRODUCTION

Prostaglandins are essential mediators of bone formation and resorption allowing the initiation of bone adaptive changes in response to mechanical (1, 2) or endocrine signals (3). Two major enzyme classes in PG synthesis are phospholipases A_2 (PLA_2), which release arachidonic acid (AA) from membrane fatty acids, and prostaglandin G/H synthases (PGHS), or cyclooxygenases (COX), which oxidize AA to PGG_2 and reduce PGG_2 to PGH_2 (4). Arachidonic acid acts as the substrate for both prostaglandins and leukotrienes, via COX and 5-lipoxygenase activities, respectively. 5-lipoxygenase metabolites increase production during inflammation and stimulate resorption *in vitro* and *in vivo* (5). The availability of AA is essential for these metabolic pathways and is dependent on the activity of PLA_2 enzymes on glycerophospholipid membranes. Three main classes of PLA_2 have been isolated: cytosolic ($cPLA_2$), calcium independent ($iPLA_2$) and a secretory ($sPLA_2$) form. Cells and tissues extracted from $cPLA_2$-deficient mice fail to produce leukotrienes, prostaglandins and platelet activating factor (PAF) *ex vivo* (6), emphasizing the importance of PLA_2 activity in the production of glycerophospholipid metabolites. A role for PLA_2 enzymes in regulating bone function has been suggested in cell and organ culture studies (7-15), but *in vivo* work is limited.

Prostaglandins are rapidly removed and degraded *in vivo,* necessitating auto-amplification to produce a sustained PG response (16). Cytosolic PLA_2 is an arachidonyl-selective phospholipase and is the main regulatory phospholipase that modulates cellular AA release and PGE_2 production in osteoblasts (17). Secretory PLA_2 enzymes show little preference for AA, hydrolyzing other more abundant fatty acids such as oleic acid and linoleic acid (13). By binding to specific receptors on osteoblasts, however, $sPLA_2$ enzymes indirectly increase the release of AA via $cPLA_2$ activity (12). In this way, $sPLA_2$ acts as an important auto- and trans-cellular amplifier of eicosanoid production (18). Inhibition of osteoblastic and osteocytic $sPLA_2$ activity abolishes loading-related increases in PGE_2 and PGI_2 secretion *ex vivo* (9), whereas inhibition of $cPLA_2$ prevents loading-induced release of PGE_2 but not PGI_2 (9). This suggests that $sPLA_2$ can act independently, whilst also augmenting

the activity of $cPLA_2$. Its role may therefore be cell- and agonist-specific, acting through a capacity to attach to cell surface binding proteins in addition to its hydrolyzing activity (12). With multiple phospholipases A_2 available to liberate AA for eicosanoid production, the importance of $sPLA_2$-IIa, the principal $sPLA_2$ found in synovial joints of arthritis sufferers, in the bone microenvironment has not been extensively investigated.

Two COX isoforms have been identified in human tissues, the regulatory COX-1 enzyme and the inducible COX-2, both of which act on AA to synthesize prostaglandins. Studies utilizing specific COX-2 inhibitors, or COX-2 deficient mice, demonstrate the importance of COX-2 in inflammatory events (19, 20) and in accelerated states of bone formation and resorption (1, 2, 21-23). For example, blockade of COX-2 activity can retard bone adaptation, preventing loading-induced bone formation (1, 2); or delay healing in bone fractures (21-23). Bone resorption induced by IL-1 and IL-6 also occurs via stimulation of COX-2 dependent PGE_2 production in osteoblasts, acting to increase osteoclastogenesis (24, 25). This COX-2 specific regulation of resorption is not necessary for wild-type bone development, but plays a critical role in activated states of bone resorption, such as those stimulated by vitamin D and PTH (3). In the latter case, treatment with propionate-based NSAIDs is associated with higher bone mineral density (BMD) in women aged between 44 and 85. These positive changes are independent of lifestyle, medication factors, and degree of osteoarthritis (26). Although the reasons for the greater BMD have not been established, they are consistent with maintenance of trabecular bone architecture observed in ovariectomized rats treated with the non-selective COX-2 inhibitor, Naproxen (27). The specific role of COX-2 in mediating activated states of bone remodeling *in vivo* has not been investigated and requires further scrutiny.

Here we show that specific inhibitors of $sPLA_2$-IIa and COX-2 effectively inhibited OVX-induced bone loss in rats by conserving trabecular bone mass and architecture through reduced bone remodeling and decreased resorptive activity. These *in vivo* data support epidemiological evidence for a bone sparing effect of COX-2 inhibitors in postmenopausal women. Moreover, the inhibitor of

$sPLA_2$-IIa significantly prevented OVX-induced rises in osteoclast number, suggesting an important role for $sPLA_2$-IIa in osteoclastogenesis, independent of the COX-2 metabolic pathway. This $sPLA_2$ may therefore play a central role in the amplification of PG synthesis associated with osteoclast differentiation.

MATERIALS & METHODS

Ovariectomy

One hundred and twenty-four female Wistar rats (385 ± 52 g) were anaesthetized using ketamine (Parnell, 60mg/kg i.p.) and xylazil-20 (Ilium, 30mg/kg i.p.), and bilaterally ovariectomized (OVX) or SHAM-operated (SHAM) at an age ranging between 8 and 9 months. The SHAM-operation involved the exposure of the ovaries without full extraction, leaving the ovaries intact, whilst OVX rats underwent full removal of both the right and left ovaries. The University of Queensland Animal Ethics Committee approved all experiments.

Housing

Removal of the ovaries is accompanied by an increase in body mass in the rat. So that the extra load did not suppress bone loss, food consumption was restricted by giving all rats 18g of standard laboratory pellets per day, commencing a few days before surgery [Howard Morris, Personal Communication; (27)]. Any food remaining the next day was discarded and replaced with a new amount of weighed food. Rats were caged in pairs and had constant access to water. Temperature was maintained between 18˚C and 23˚C, and a diurnal light control allowed daily light and dark periods of 12 hours each.

Drug Administration

Eight rats were euthanized prior to surgery as a baseline control group, and 8 rats were euthanized two weeks post-OVX as an OVX-control group. The remainder commenced treatment 14 days after surgery with either Vehicle (VEH), a selective COX-2 inhibitor (DFU [5,5-dimethyl-3-3 (3 fluorophenyl)-4-(4 methylsulphonal) phenyl-2 (5H)-furanone], Merck, Sharpe and Dohme, USA) at 0.02 mg.kg.d^{-2} and 2.0 mg.kg.d^{-2}; or a sPLA$_2$-IIa inhibitor (KH064, [5-(4-benzyloxyphenyl)-4S-(7-phenylheptanoylamino-pentanoic acid)], University of Queensland) at 0.4 mg.kg.d^{-2} and 4.0

mg.kg.d^{-2}. DFU inhibits the arachidonic acid-dependent production of PGE_2 in CHO cells with at least 1,000 fold selectivity for COX-2 (IC_{50} = 41+14 nM) over COX-1 (IC_{50} > 50 μM) (28). KH064 is two-fold more potent against the group $sPLA_2$-IIa human enzyme [IC_{50} 29nM; (29)] than a previously published inhibitor (LY311299), but 170-fold more selective for the group IIa isoform over the group V human $sPLA_2$ enzyme (30), and does not bind to human $cPLA_2$. It also does not bind to some 30 receptors and enzymes screened by MDS Pharma Services (Fairlie, unpublished). We consider that KH064 is a selective inhibitor of human $sPLA_2$-IIa (31). Polyethylene glycol (PEG) was used as the vehicle treatment and both DFU and KH064 were prepared in PEG. At the start of the treatment period, DFU was administered i.p. and KH064 by oral gavage. One month after commencement of treatment, all rats were treated by oral gavage in the interests of animal welfare. This was to avoid potential adverse effects of prolonged i.p injections. Treatment continued daily until rats were euthanized by CO_2 inhalation at 70 days (T1) or 98 days (T2) post-OVX.

For dynamic histomorphometry, fluorescent labels (Calcein 7.0 mg.kg^{-1} i.p.; Sigma Chemical Co, St Louis MO) were administered 7 and 2 days before euthanasia. At euthanasia the uterus was removed to verify the success of OVX.

Tissue Processing

The right and left tibiae were extracted and stored in 10% neutral buffered formalin (NBF) for structural and cellular histomorphometry, respectively. Right tibiae were sectioned transversely, 13 mm distal to the proximal epiphysis and were fixed in cold NBF (10%) for 24 hours. Tibiae were then washed in 70% ethanol and embedded in methylmethacrylate for structural bone analysis. Left tibiae were similarly cut and fixed, but then decalcified using a series of EDTA washes. Once decalcification was confirmed by x-ray, tibiae were embedded in paraffin and sectioned for cellular histomorphometry.

Structural Histomorphometry

Thick sections (70 – 100 μm) were cut from right tibiae with a diamond-blade microtome (Leitz 1600 Saw Microtome, Leica, Wetzlar) and mounted unstained on glass slides for light and fluorescence microscopy using an Olympus BX-60 Fluorescence microscope, at magnification of X 400. The microscope was connected to a Macintosh computer via a digitizing tablet (Calcomp Drawing Slate, Calcomp Scottsdale, AZ) that allowed the following variables to be measured [abbreviated according to Parfitt et al. (32)]: trabecular area (Tb.Ar); tissue area (T.Ar); trabecular perimeter (Tb.Pm); resorption perimeter (Rs.Pm); double-labeled surface (dL.Pm); single-labeled surface (sL.Pm); and inter label width (Ir.L.Wi). Only secondary spongiosa in the proximal tibial metaphysis was measured in a representative region 1 mm distal to the epiphyseal growth plate and extending 3 mm distally. The following indices were then calculated according to Li et al (33): BV/TV (Tb.Ar/T.Ar, %); trabecular width, Tb.Wi [(2000/1.199) x (Tb.Ar/Tb.Pm), μm]; trabecular number, Tb.N [(1.199/2) x (Tb.Pm/T.Ar), n/mm], trabecular separation, Tb.Sp [(2000/1.199) x (T.Ar-Tb.Ar)/Tb.Pm, μm], mineralizing surface, MS [(dL.Pm + sL.Pm/2)/Tb.Pm x 100, %], mineral apposition rate, MAR (Ir.L.Wi/ interval, μm/d), bone formation rate, BFR/B.Ar (MAR x MS/ Tb.Ar x 365 x 100, %/y); and resorption surface Rs.S (Rs.Pm/Tb.Pm x 100, %). Bone formation rate per unit area is considered equivalent to the rate of bone turnover (34).

Cellular Histomorphometry

Serial sections were cut at 5μm on a microtome (Shandon Historange, Pittsburgh, USA) and alternately stained with Masson's trichrome, or tartrate resistant acid phosphatase (TRAP) counterstained with methyl green (0.2%). TRAP staining was used as a specific histological marker of osteoclasts. Since this enzyme is also expressed in other cells of the macrophage lineage and non-hematopoietic cell types such as selected chondrocytes (35), TRAP positive cells were confirmed as osteoclastic with the aid of a serial section stained with Masson's trichrome. Osteoclast number (Oc.N) and trabecular perimeter (Tb.Pm) were measured in the proximal tibial metaphysis at a magnification of X 800 and osteoclast surface (OcS) was calculated (Oc.N/Tb.Pm, %).

Statistical analysis

All data were analyzed using StatView (version 5.0; SAS Institute Inc., Cary, North Carolina, USA, 1992-1999) using a two-way ANOVA and significant differences determined post-hoc using Fisher's PLSD. Significance was accepted at $P < 0.05$. Probability levels between 0.05 and 0.1 were classified as marginal if the difference between means was greater than twice the SEM and non significant at $P > 0.1$.

RESULTS

Confirmation of Ovariectomy

Ovariectomy in rats was confirmed by the presence of atrophied uterine horns and a significantly reduced wet and dry uterine mass ($P < 0.0001$). On average, OVX rats had a fresh uterine mass of 0.290 g and a dry uterine mass of 0.054g. In contrast, SHAM rats were associated with fresh and dry uterine masses of 0.996g and 0.170g, respectively. The mass of uteri from SHAM rats was not significantly different to basal control rats. Five rats displayed unusually high uterine masses and were excluded from further analysis.

Bone Histomorphometry

The pattern of bone loss observed following OVX was highly consistent with previous studies of similar duration (36). Ovariectomy in adult rats resulted in an almost complete ablation of trabecular bone in the proximal tibial metaphysis. Ninety-eight days following OVX, bone volume (BV/TV), Tb.Wi and Tb.N were significantly reduced ($P < 0.0001$) and Tb.Sp was significantly increased ($P < 0.0001$) compared to SHAM (Table 1). Indicative of an increase in bone turnover, bone formation rate (BFR/BS and BFR/B.Ar) ($P < 0.0001$), MS and MAR ($P < 0.05$) were also significantly elevated (Table 2). Relative to SHAM, resorption surface showed a biphasic response with a significant increase from baseline to 70 days ($P < 0.05$), and a decrease between 70 and 98 days; a pattern that has previously been reported (36). At T1, OVX rats displayed a significantly greater OcS compared to SHAM controls ($P < 0.0001$), with no difference at T2 (Fig 1), when remodeling parameters would be approaching a new equilibrium, but on a significantly smaller bone volume.

DFU Treatment

Ninety-eight days after OVX, rats treated with DFU, the COX-2 inhibitor, maintained significantly

greater BV/TV than VEH controls ($P < 0.001$, Table 1), but lower than SHAM controls ($P < 0.05$). Trabecular width was significantly greater and Tb.Sp was significantly lower than VEH for both doses of DFU ($P < 0.001$), and not significantly different to SHAM values at T2 ($P < 0.001$). Trabecular number was significantly higher with DFU treatment compared to VEH ($P < 0.05$) at T2, but still lower than SHAM ($P < 0.01$). Rats treated with DFU also had lower bone formation rates ($P < 0.0001$) at T2 compared to VEH. Both doses of DFU maintained BFR/BS and BFR/B.Ar at SHAM levels following OVX, indicating an effective suppression of accelerated bone turnover. Mineralizing surface was marginally lower in rats treated with 2.0mg.kg^{-1} of DFU compared to VEH at T2 ($P < 0.08$). At 2.0 mg.kg^{-1}, MAR was significantly greater than VEH at T1; but, by T2, MAR was significantly lower than VEH for both doses and no different to SHAM (Table 2).

At 70 days, DFU had effectively reduced indices of bone resorption. Resorption surface was significantly lower in DFU groups compared to VEH ($P < 0.05$), and not significantly different to SHAM animals. Rats treated with the 2.0 mg.kg^{-1} DFU also had marginally lower OcS ($P = 0.08$) compared to VEH at T1. The lower dose of DFU had relatively little effect on these parameters, with OcS remaining significantly higher than VEH and SHAM animals ($P < 0.05$)(Fig 1).

KH064 Treatment

With the exception of OcS, the effects of DFU and KH064, the sPLA$_2$-IIa inhibitor, on static and dynamic remodeling indices in the rat were very similar. When compared to VEH at 98 days, BV/TV was two-fold greater in rats treated with KH064 ($P < 0.08$); and Tb.Wi was significantly greater ($P < 0.001$), being maintained no differently to SHAM values (Table 1). KH064 at 0.4 mg.kg^{-1} marginally elevated Tb.N above VEH levels, ($P < 0.08$), and Tb.Sp was significantly reduced with 4.0 mg.kg^{-1} of KH064 compared to VEH ($P < 0.001$). Although DFU and KH064 treatment preserved a significant proportion of BV/TV and Tb.N, treatment was not sufficient to restore the early loss of bone that occurred during the non-treatment period post-OVX. These variables were maintained at levels greater than VEH groups, but less than SHAM animals,

implying that the loss of bone occurred soon after surgery. These early trabecular modifications were not improved by treatment with DFU or KH064, indicating that the COX-2 and $sPLA_2$ inhibitors are essentially anti-catabolic (37), being able to maintain existing trabecular architecture, but unable to build or improve already weakened or lost bone.

At T1, indices of bone formation rate were significantly higher in rats treated with the higher dose of KH064 compared to VEH ($P < 0.05$). This is a manifestation of the early inhibition of resorptive activity by KH064, giving rise to a bone formation phase earlier than that seen in VEH groups. In contrast, both BFR/BS ($P < 0.05$) and BFR/B.Ar ($P < 0.0001$) were significantly lower at T2 with both doses of KH064 treatment, such that there was no significant difference to SHAM levels. Only rats treated with the higher dose of KH064 had significantly elevated MS at T1 ($P < 0.001$), and MAR was significantly higher following treatment, but significantly lower at T2 when compared to VEH ($P < 0.05$)(Table 2). At both time-points, KH064 treatment maintained MAR at SHAM levels.

Compared to VEH, resorption surface was marginally lower in rats treated with the lower dose of KH064 ($P < 0.08$) and significantly reduced by the higher dose ($P < 0.05$) at T1 (Fig 1). In contrast, resorption surface was significantly higher with KH064 treatment at T2 compared to VEH ($P < 0.001$). This seemingly counter-intuitive observation is due to the almost complete ablation of trabecular bone in the tibial metaphysis of vehicle-treated animals. The higher BV/TV in treated animals continues to provide bone surfaces for remodeling, and a measurable extent of RsS. Compared to VEH, treatment with either dose of KH064 significantly reduced relative osteoclast surface at T1 ($P < 0.001$), with no differences observed when compared to SHAM values. There was no significant treatment effect at T2.

Table 1: Static histomorphometric indices of bone remodeling in the proximal tibial metaphysis of adult rats

	Timepoint (Days Post-Surgery)							
	0	**14**	**70 (T1)**			**98 (T2)**		
	Baseline	*OVX Control*	*VEH*	*Low dose*	*High dose*	*VEH*	*Low dose*	*High dose*
BV/TV (%)	19.50							
SHAM	(2.19)		14.22 (0.99)			16.03 (1.6)		
OVX[C]		18.10 (1.85)	5.55 (0.89)[A]			2.12 (0.69)[A]		
COX-2 Inhibitor				7.56 (1.34)	8.42 (1.72)		6.43 (1.12)[AE]	11.37 (1.57)[AB]
$sPLA_2$ Inhibitor				8.14 (2.13)	6.11 (0.96)		6.40 (1.43)[AE]	6.30 (1.52)[AE]
Tb.Wi (µm)	48.10							
SHAM	(1.73)		46.19 (1.19)			48.80 (2.56)		
OVX[C]		42.79 (1.81)	43.76 (2.26)			32.06 (5.21)[A]		
COX-2 Inhibitor				43.26 (1.74)	41.6 (1.83)		43.75 (2.71)[B]	51.85 (5.09)[B]
$sPLA_2$ Inhibitor				44.27 (6.44)	39.49 (1.30)		47.92 (3.04)[B]	48.64 (1.57)[B]
Tb.N (#/mm)	4.02							
SHAM	(0.36)		3.25 (0.27)			3.36 (0.20)		
OVX[C]		4.19 (0.34)	1.27 (0.19)[A]			0.52 (0.12)[A]		
COX-2 Inhibitor				1.75 (0.30)	1.93 (0.34)		1.47 (0.25)[AB]	2.16 (0.26)[AB]
$sPLA_2$ Inhibitor				1.61 (0.27)	1.52 (0.22)		1.30 (0.31)[AE]	1.23 (0.26)
Tb.Sp (µm)	216.53							
SHAM	(24.49)		284.34 (33.31)			258.56 (17.87)		
OVX[C]		209.86 (24.23)	884.80 (128.40)			3115.07 (831.2)[A]		
COX-2 Inhibitor				720.16 (163.40)	636.47 (134.10)		910.19 (254.10)[B]	494.18 (67.95)[B]
$sPLA_2$ Inhibitor				861.69 (247.20)	1132.74 (554.60)		2106.80 (1110.00)	1434.99 (632.90)[AB]
Rs.S (%)	10.14							
SHAM[C]	(1.24)		9.31 (1.23)			6.84 (0.65)		
OVX[C]		10.27 (0.88)	13.69 (1.60)[A]			3.41 (1.27)		
COX-2 Inhibitor				10.40 (2.08)[E]	10.07 (1.60)[B]		5.07 (0.55)	5.58 (0.71)
$sPLA_2$ Inhibitor				10.39 (1.08)[E]	9.09 (1.31)[B]		11.59 (1.96)[AB]	9.96 (1.42)[B]
Oc.S (n/mm)	3.413							
SHAM	(0.645)		4.167 (0.708)			2.564 (0.595)		
OVX		5.126 (0.842)	7.188 (1.180)[A]			1.840 (0.748)		
COX-2 Inhibitor				6.536 (1.041)[A]	4.951 (1.251)[AE]		5.372 (1.720)[AB]	2.247 (0.812)
$sPLA_2$ Inhibitor				2.675 (0.927)[B]	2.824 (0.778)[B]		2.869 (0.507)	2.552 (0.806)

Values are mean (SEM) for percentage trabecular area (Tb.Ar/T.Ar), trabecular width (Tb.Wi), trabecular number (Tb.N), trabecular separation (Tb.Sp), resorption surface (Rs.S/TS) and osteoclast surface (Oc.S) measured in the rat proximal tibial metaphysis in sham-operated (SHAM); ovariectomised (OVX); Vehicle-treated (VEH); and in rats treated with COX-2 inhibitor (DFU, 2.0 or 0.02mg/kg/d) or $sPLA_2$-IIA inhibitor (KH064, 4.0 or 0.04mg/kg/d). Untreated rats were euthanized at the time of surgery (Baseline) and 14 days post-surgery (OVX Control). Treated rats were euthanized 70days (T1) or 98days (T2) post-surgery. [A]P<0.05 vs SHAM; [B]P<0.05 vs VEH; [C]P<0.01 significant change with timepoint; [E]P<0.08 vs VEH.

Table 2: Dynamic histomorphometric indices of bone remodeling in the proximal tibial metaphysis of adult rats

	Timepoint (Days Post-Surgery)							
	0	**14**	**70 (T1)**			**98 (T2)**		
	Baseline	*OVX Control*	*VEH*	*Low dose*	*High dose*	*VEH*	*Low dose*	*High dose*
MS (%)	5.94							
SHAM	(0.97)		7.98 (1.42)			7.58 (1.24)		
OVX[C]		3.98 (0.72)	11.97 (2.04)			19.41 (3.71)[A]		
COX-2 Inhibitor				14.67 (2.92)	16.55 (1.96)		13.89 (1.74)	11.06 (1.52)[E]
$sPLA_2$ Inhibitor				11.57 (1.15)	26.25 (9.20)[AB]		15.08 (1.53)	15.60 (2.09)
MAR (µm/d)	1.25							
SHAM[C]	(0.08)		1.06 (0.07)			1.11 (0.07)		
OVX[C]		0.7 (0.07)[D]	1.03 (0.06)			1.43 (0.19)[A]		
COX-2 Inhibitor				1.16 (0.16)	1.33 (0.11)[B]		1.13 (0.11)[E]	1.07 (0.07)[B]
$sPLA_2$ Inhibitor				1.05 (0.07)	1.32 (0.13)[B]		1.25 (0.14)	1.14 (0.10)[B]
BFR/BS ($\mu m^2/\mu m^3/y$)	27.71							
SHAM	(5.13)		33.50 (7.33)			31.44 (6.08)		
OVX[C]		10.90 (2.49)	46.63 (9.55)			122.47 (32.75)[A]		
COX-2 Inhibitor				73.00 (20.75)	84.01 (14.34)		59.91 (11.79)[B]	44.65 (7.55)[B]
$sPLA_2$ Inhibitor				45.80 (6.68)	118.47 (30.77)[AB]		68.52 (9.28)[B]	67.58 (12.05)[B]
BFR/B.Ar (%/y)	100.88							
SHAM	(21.68)		134.58 (31.29)			113.31 (22.32)		
OVX[C]		42.05 (8.98)	183.36 (37.98)			745.83 (221.3)[A]		
COX-2 Inhibitor				306.34 (93.36)	360.84 (77.77)		248.63 (59.99)[B]	155.40 (32.64)[B]
$sPLA_2$ Inhibitor				210.06 (48.40)	491.06 (107.10)[AB]		250.84 (18.18)[B]	238.50 (43.05)[B]

Values are mean (SEM) for mineralising surface (MS), mineral apposition rate (MAR), bone formation rate at the bone surface (BFR/BS), and bone turnover (BFR/B.Ar) measured in the rat proximal tibial metaphysis in sham-operated (SHAM); ovariectomised (OVX); Vehicle-treated (VEH); and in rats treated with COX-2 inhibitor (DFU, 2.0 or 0.02mg/kg/d) or $sPLA_2$-IIA inhibitor (KH064, 4.0 or 0.04mg/kg/d). Untreated rats were euthanized at the time of surgery (Baseline) and 14 days post-surgery (OVX Control). Treated rats were euthanized 70days (T1) or 98days (T2) post-surgery. [A]P<0.05 vs SHAM; [B]P<0.05 vs VEH; [C]P<0.01 significant change with timepoint; [E]P<0.08 vs VEH.

DISCUSSION

Ovariectomy produces a state very similar to postmenopausal bone loss. Both states are associated with an increase in remodeling activation and the appearance of overactive osteoclasts, which contribute to an increase in the depth of resorption (36, 38). Seventy days post-OVX we observed significant reductions in the bone volume of the proximal tibial metaphysis, associated with a decrease in trabecular number and increases in resorption and osteoclast surfaces. We found that specific inhibitors of human COX-2 and human $sPLA_2$-IIa enzymes effectively inhibited OVX-induced bone loss in rats by conserving trabecular bone mass and architecture through reduced bone remodeling and decreased resorptive activity. These data in rats support epidemiological evidence for a bone sparing effect of NSAIDs in postmenopausal women (26), and reinforce the critical role of COX-2-dependent prostaglandins in activated states of remodeling (3). Moreover, the inhibitor of $sPLA_2$-IIa significantly prevented OVX-induced rises in osteoclast number, suggesting an important role for $sPLA_2$-IIa in osteoclastogenesis, independent of the COX-2 metabolic pathway.

DFU treatment preserved approximately 58% of the trabecular bone in the proximal tibia, compared to the residual 10% in VEH groups. Naproxen treatment (a non-specific COX inhibitor) has also been shown to maintain cancellous bone volume in OVX rats at about 65% after 6 weeks (27). Lane et al. (27) hypothesized that Naproxen may slow the progression of estrogen-dependent bone loss without affecting or ameliorating the final bone mass. However, over the substantially longer duration of treatment herein, our study does not support that conclusion. Instead our study provides evidence for maintenance of trabecular mass above VEH controls with COX-2 inhibition at 98 days post-OVX. The reduction of both formation and resorption indices at this time, and the return of bone formation rate to SHAM control levels, indicates that the period of rapid bone loss has ended and a new steady state of bone mass has been achieved. This signifies that while treatment continues, the preservation of trabecular architecture with DFU treatment will be retained.

The arrangement of the trabecular struts and their contiguity is more important than trabecular volume or density in determining the stiffness of trabecular bone (39-41), suggesting that structural stiffness is spatially dependent. An increase in both connectivity and mass increases trabecular strength more effectively than an increase in trabecular thickness alone (40, 41). Therefore, even though DFU and KH064 treatment could not restore the early loss of bone, maintenance of trabecular width and separation indicates preservation of connectivity, which is more important than mass conservation in determining the strength of the bone (39, 42). For example, when matched for age, sex, race, menopausal status and bone volume, postmenopausal patients with fracture have significantly lower trabecular plate density but significantly higher trabecular thickness compared to those without fracture (43). Hence, the structural integrity of the cancellous bone may become rapidly compromised when bone volume falls below a critical value (42). Therefore the effect of DFU and KH064 in preventing structural dissolution may decrease the risk of fracture by conserving the structural strength of the trabecular bone matrix.

In rats treated with the human $sPLA_2$-IIa inhibitor, trabecular number and trabecular separation showed only small improvements above VEH rats, whilst COX-2 inhibition resulted in a significantly higher Tb.N compared to VEH and Tb.Sp was maintained at SHAM levels. These small differences between rats treated with DFU and KH064 may be explained by the redundancy of $sPLA_2$-IIa. Surprisingly $sPLA_2$-IIa knockout mice display no abnormality in inflammatory function, suggesting that other PLA_2 classes and subtypes may compensate for the lack of $sPLA_2$-IIa, at least in the mouse (44). When osteoblastic cells are stimulated by TNF-α and ET-1, for example, other phospholipases (such as PLC and PLD) can also liberate AA independent of PKC activation (45-47). While cytosolic PLA_2 inhibitors and COX-2 inhibitors completely abolish PGE_2 production induced by IL-1α in osteoblasts *in vitro*, $sPLA_2$ inhibitors only partially affect PGE_2 synthesis *in vitro* (48). The multiplicity of PLA_2 enzymes may account for differences in DFU and KH064 treatment. It is unlikely that $sPLA_2$-IIa is the only phospholipase involved in the progression of OVX-induced bone loss in the rat.

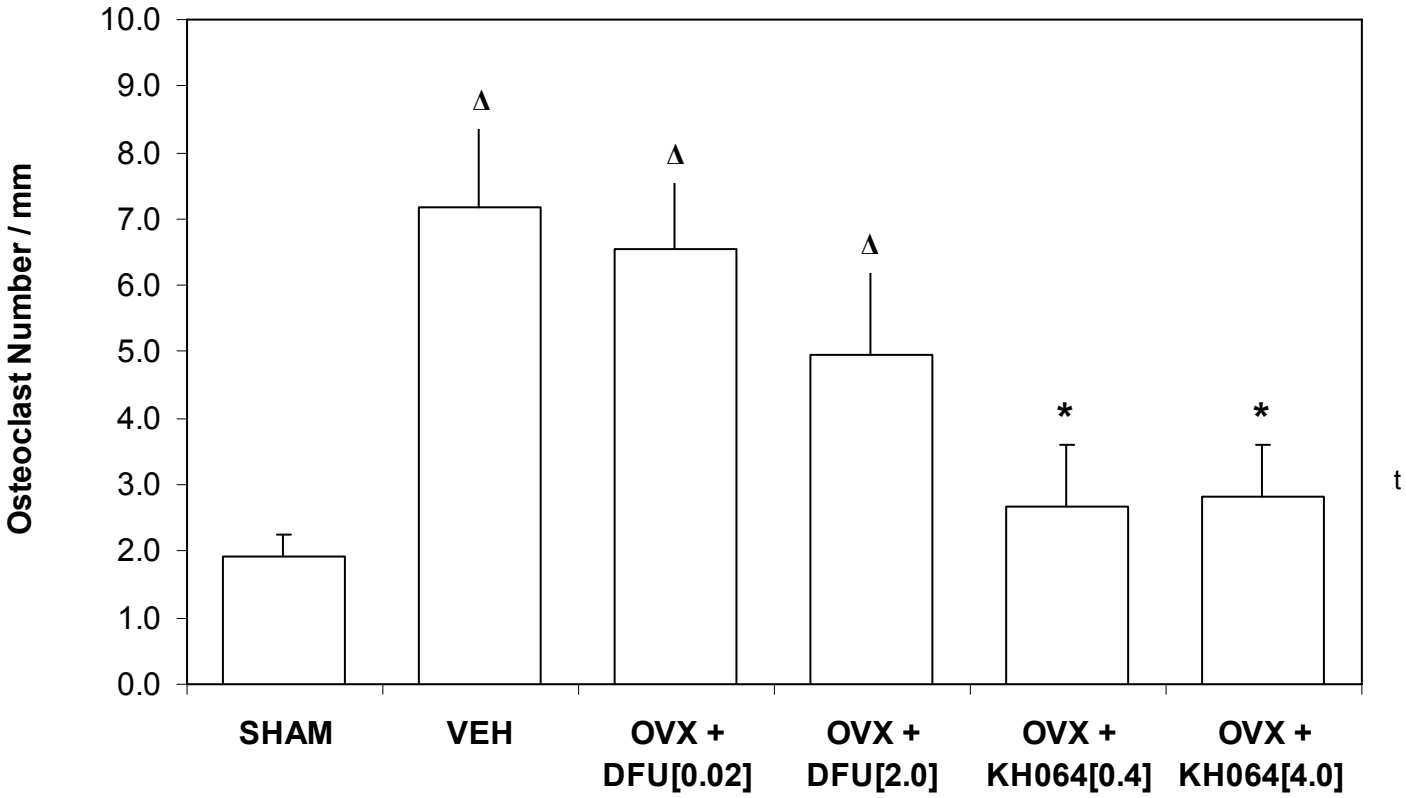

Figure 1. Effect of inhibition of COX-2 (DFU) and sPLA$_2$-IIa (KH064) on OVX-induced changes in osteoclast surface (Oc.N/Tb.Pm) in the proximal tibial metaphysis of the adult rat, 70 (T1) and 90 (T2) days post-surgery. Osteoclast number (Oc.N) was determined by TRAP and Masson's Trichrome staining. Rats were either sham-operated and treated with vehicle (SHAM); or ovariectomized and administered vehicle (VEH), DFU 0.02mg/kg/ml (DFU[0.02]) and 2.0mg/kg/ml (DFU[2.0]), or KH064 at 0.4mg/kg/ml (KH064[0.4]) and 4.0mg/kg/ml (KH064[4.0]). Values are the mean ± SE of 8 rats. ***P*<0.05 and **P*<0.08, when compared to VEH. Δ*P*<0.05, when compared to SHAM.

Both doses of KH064 were associated with a significantly reduced osteoclast surface; whereas DFU failed to effect a significant change. Because $sPLA_2$ is upstream of COX, it may play a more important hierarchical role in osteoclastogenesis *in vivo* than COX-2. Unlike $sPLA_2$, COX-2 does not act directly as a trans-cellular signal, and its activity is limited to the cells in which it is expressed. The observed decrease in resorption surface, despite little change in osteoclast number with DFU, is consistent with the inhibition of COX-2 in mature osteoclasts, but a relatively smaller effect on osteoclastogenesis. Therefore, the modulation in osteoclast numbers following OVX may be the result of the action of leukotrienes and/or PAF that do not require COX activity for synthesis. The effectiveness of the $sPLA_2$ inhibitor in producing a complete blockade of OVX-induced increases in relative osteoclast number may be explained by the relationship between phospholipases, leukotrienes and PAF. Both leukotrienes (49) and PAF (50) have demonstrated resorptive activity and could be responsible for the change in osteoclast function following OVX. The inhibition of $sPLA_2$ may have reduced the substrate requirements for leukotriene and PAF synthesis, resulting in a decrease in OVX-associated resorptive activity. In contrast to COX-2, $sPLA_2$ is important in both PG and leukotriene metabolism, with AA acting as the common substrate. COX-2 inhibitors have the limited capacity of affecting the PG biosynthesis pathway only, whilst $sPLA_2$ inhibitors can modulate PG, leukotriene and lysophospholipid metabolism. The inhibition of leukotriene production by KH064 may account for reduced osteoclast numbers following OVX, in contrast to the limited effect of DFU treatment.

In conclusion, treatment with inhibitors of human COX-2, or human $sPLA_2$-IIa, maintain trabecular integrity and reduce bone turnover in rats to prevent the deterioration in bone architecture associated with estrogen loss. DFU and KH064 were effective inhibitors of OVX-induced bone loss in rats by conserving trabecular bone mass and architecture by maintaining interconnectivity through reduced bone remodeling and decreased resorptive activity. These data support epidemiological evidence in humans for a bone sparing effect of COX-2 inhibition under conditions of increased bone turnover related to aging or estrogen loss. In contrast to DFU, KH064

significantly prevented OVX-induced rises in osteoclast number in rats, which suggests that $sPLA_2$-IIa has an important role in osteoclastogenesis, one that is not dependent on the COX-2 metabolic pathway. Through inhibition of prostaglandin, leukotriene and possibly the PAF signaling pathways, $sPLA_2$ inhibition can prevent rises in osteoclast activity resulting in a significant maintenance of trabecular architecture in the proximal tibial metaphysis of the rat following estrogen removal. These results may be important in dissecting the roles of individual eicosanoids in the maintenance and remodeling of human bone.

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