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Kynurenine suppresses osteoblastic cell energetics *in vitro* and osteoblast numbers *in vivo*

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Abstract

Aging is a progressive process associated with declining tissue function over time. Kynurenine, an oxidized metabolite of the essential amino acid tryptophan that increases in abundance with age, drives cellular processes of aging and dysfunction in many tissues, and recent work has focused on understanding the pathways involved in the harmful effects of kynurenine on bone. In this study,

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we sought to investigate the effects of controlled kynurenine administration on osteoblast bioenergetics, *in vivo* osteoblast abundance, and marrow fat accumulation. Additionally, as an extension of earlier studies with dietary administration of kynurenine, we investigated the effects of kynurenine on Hdac3 and NCoR1 expression and enzymatic deacetylase activity as potential mechanistic contributors to the effects of kynurenine on osteoblasts. Kynurenine administration suppressed cellular metabolism in osteoblasts at least in part through impaired mitochondrial respiration, and suppressed osteoblastic numbers *in vivo* with no concurrent effects on marrow adiposity. Deleterious effects of kynurenine treatment on osteoblasts were more pronounced in female models as compared to males. However, kynurenine treatment did not inhibit Hdac3's enzymatic deacetylase activity nor its repression of downstream glucocorticoid signaling. As such, future work will be necessary to determine the mechanisms by which increased kynurenine contributes to aging bone bioenergetics. The current study provides novel further support for the idea that kynurenine contributes to impaired osteoblastic function, and suggests that impaired matrix production by kynurenine-affected osteoblasts is attributed in part to impaired osteoblastic bioenergetics. As circulating kynurenine levels increase with age, and human bone density inversely correlates with the serum kynurenine to tryptophan ratio, these mechanisms may have important relevance in the etiology and pathogenesis of osteoporosis in humans.

Keywords

Kynurenine; Bone; Osteoblast; Energy Metabolism; Histone Deacetylase 3; Marrow Fat; Aging

Introduction

Aging is a progressive process broadly characterized by a decline in tissue function over time. The aging process of most living organisms is defined by several hallmarks that include stem cell exhaustion, mitochondrial dysfunction, changes in epigenetic mechanisms, and deregulated nutrient-sensing (1). Similarly, aging bone is marked by altered turnover, loss of tissue density, aberrant substrate utilization at the cellular level, and an accumulation of marrow fat at the expense of bone mass. Kynurenine, an oxidized metabolite of tryptophan, drives cellular processes of aging and dysfunction in many tissues (2,3), and recent work has focused on understanding the pathways involved in the catabolic effects of this oxidized substrate on bone (4).

The kynurenine pathway begins with metabolism of the amino acid tryptophan (5). The least abundant of the essential amino acids, tryptophan may be metabolized in different ways. A small fraction of dietary tryptophan is utilized for protein synthesis when needed—however, it is also susceptible to metabolism by the enzymes tryptophan-2,3-dioxygenase (TDO; in the liver) and indolamine-2,3,-dioxygenase (IDO; in extra-hepatic tissues) once the predominant oxidative pathway has been initiated (6,7). In contrast, about 1% of available tryptophan may be metabolized by the hydroxylation pathway to yield serotonin and associated metabolites (8,9). Tryptophan oxidation may occur as a result of glucocorticoid (GC) activation during inflammatory responses, and it has been reported that upwards of ~95% of tryptophan metabolism in the body occurs through kynurenine production by the GC-responsive tryptophan oxygenase (i.e., TDO) (5,6). The oxidative pathway yields

kynurenine via the intermediate *N*-formylkynurenine, and further conversion steps give rise to numerous metabolites ultimately producing Acetyl CoA and nicotinamide adenine dinucleotide (NAD⁺)/NADH for use in essential biochemical pathways (10).

Dietary tryptophan and its metabolites exhibit differing contributions to whole-body physiology. In bone, the naturally occurring L-isomers of tryptophan and other aromatic amino acids have roles in promoting the osteogenesis of BMSC by increasing alkaline phosphatase activity as well as upregulating the expression of osteoblastic genes such as runt-related transcription factor 2 (Runx2) and osteocalcin (11,12). Additionally, tryptophan catabolism—in particular, the activation of the kynurenine pathway—mediates osteoblastogenesis in interferon gamma (IFN γ)-treated human mesenchymal stem cells through the upregulated expression and activity of IDO1 (13). However, an excess of oxidized tryptophan metabolites such as kynurenine can combat the pro-osteogenic effects of the unaltered amino acid precursors and negatively impact bone mesenchymal stem cell differentiation and bone microarchitecture in various disease models (4,14,15). Assessments of human bone marrow aspirates revealed that aging-associated elevated kynurenine in the bone is inversely associated with total femur BMD while positively correlated with the circulation of bone resorption markers (e.g., TRAP-5b and RANKL) and increased risk of hip fragility fracture (16). Consistently, increased circulating kynurenine with aging is recognized as a contributor to the pathogenesis of osteoporotic bone, and current research is focused on understanding the mechanisms involved. Chronic administration of dietary kynurenine exacerbated the aged bone phenotype of low bone mass and high marrow fat in young mice, similar to that of old mice on a standard diet (4,17). Moreover, kynurenine treatment reduced the expression of the epigenetic regulator histone deacetylase 3 (Hdac3) and its cofactor nuclear receptor corepressor 1 (NCoR1) in bone marrow stromal cells (BMSC)—a trend consistent with aged osteoblasts and the cellular profile of osteoporotic bone in both mice and humans (18-20). Despite these findings, there are still uncertainties regarding the mechanisms by which kynurenine impairs osteogenesis and promotes marrow fat accumulation.

The aging-associated changes in the lineage commitment of BMSC are influenced, at least in part, by shifts in metabolic potential at the cellular level. Under normal conditions, BMSC rely on glycolysis to meet energy demands while the occurrence of mitochondrial respiration increases dramatically upon differentiation to osteoblasts or adipocytes (21-23). The oxidative metabolic pathway utilized in differentiated cells yields high amounts of ATP, but also generates more reactive oxygen species (ROS) that may impair cellular function. Tan et al. (24) have shown that, in addition to reduced osteogenic potential, aged BMSC accumulate increased intracellular ROS. This finding is consistent with the bioenergetic profile of adipocytes, where ROS increased with adipogenic differentiation of BMSC and even mediates lineage commitment to adipocytes (25-27). The bioenergetics of bone-resident cells also depends on substrate availability, whereas diseases of nutrient-sensing or nutrient availability contribute to the pathogenesis of osteoporosis (28-31). Glucose has a substantial effect on osteoblast energy metabolism and bone biology, but the role of fatty acid oxidation for ATP production in bone is not well-characterized (32,33). Ongoing work is dedicated to understanding the shifts in substrate utilization and energy metabolism by

osteoblasts, and further investigation is necessary to determine how kynurenine affects the bioenergetics of BMSC-derived cell types.

In this study, we sought to investigate the effects of controlled kynurenine administration on osteoblast bioenergetics, *in vivo* osteoblast abundance, and marrow fat accumulation. Additionally, as an extension of earlier studies with dietary administration of kynurenine, we investigated the effects of kynurenine on Hdac3 and NCoR1 expression and enzymatic deacetylase activity.

Materials and Methods

Animals and in vivo treatments

All animal experiments were conducted under guidelines provided by the National Institute of Health (NIH) and protocols approved by the Augusta University Institutional Animal Care and Use Committee. For the *in vitro* kynurenine experiments, young (3 month old) male and female C57BL/6 mice were obtained from The Jackson Laboratory (strain #00064; n=4 per sex). *In vivo* kynurenine injections were administered to adult (6-8 months old) male and female C57BL/6 mice (n=5 per sex) obtained from the NIA rodent colony, where L-Kynurenine sulfate salt (Sigma-Aldrich #K3750, St. Louis, MO, USA) was dissolved into PBS and administered by intraperitoneal (i.p.) injection at a dosage of 10 mg/kg bodyweight/day, 5 days per week for 4 weeks. Vehicle (phosphate buffered saline, PBS) injections were administered as controls for all *in vivo* studies (n=5 per sex). All animals were euthanized by carbon dioxide asphyxiation followed by thoracotomy. One tibia per adult mouse was collected at sacrifice for histology.

Histology

Bone tissues were fixed in 10% neutral buffered formalin then decalcified in 15% Ethylenediaminetetraacetic acid (EDTA) for two weeks before paraffin embedding and sectioning using a Leica Microtome (7 μ m sections; Leica Biosystems, Buffalo Grove, IL, USA). Hematoxylin and eosin (H&E) stains were applied to the sections for imaging, and histological images were captured using an Olympus IX70 inverted microscope (Olympus Corporation, Center Valley, PA, USA) with a camera attachment. Bone marrow adipocyte area fraction (Ad.Ar/M.Ar), marrow adipocyte density (N.Ad/M.Ar), average adipocyte area (Ad.Ar/N.Ad), osteoblast number (N.Ob/BS), and osteoblast surface (Ob.S/BS) were quantified with Bioquant Osteo software.

BMSC Culture

Primary bone marrow stromal cells (BMSC) were harvested from young male and female C57BL/6 long bones (femora, tibiae, humeri) by syringe flush using basal cell culture medium consisting of α MEM (Gibco #12561-072; Thermo Fisher Scientific, Waltham, MA, USA) + 20% fetal bovine serum (FBS; Atlanta Biologicals #S11150H, Flowery Branch, GA, USA) + 1% antibiotic-antimycotic (Gibco #15240-062) + 1% MEM non-essential amino acids (Gibco #11140-050). Osteogenic induction (to obtain BMSC-derived osteoblasts; BMSC-OB) began at seeding (Day 0) using basal cell culture medium supplemented with ascorbic acid (50 μ g/ml; Sigma-Aldrich #A4544) and β -

glycerophosphate (10 mM; Sigma-Aldrich #G9422). Kynurenine treatment *in vitro* was performed by including L-Kynurenine (dissolved in water; Sigma-Aldrich #K8625) in the cell culture medium at a final concentration of 100 μ M. Culture medium for primary cells was changed every 3 days until cell harvest.

Gene Expression

To study mRNA transcript expression levels, BMSC-derived osteoblasts were harvested on day 7 of osteogenic induction with TRIzol reagent (Invitrogen #15596-018; Thermo Fisher Scientific) followed by RNA isolation. RNA quality was assessed by absorbance at 260 and 280 nm (NanoDrop, Thermo Fisher Scientific). RNA (2000 ng) was reverse-transcribed from three biological replicates per group (where each replicate represented an independent cell culture vessel) into cDNA using the Superscript™ III First-Strand Synthesis System (Invitrogen #18080-051) and a programmable thermal cycler (Bio-Rad). Quantitative polymerase chain reaction (qPCR) amplification and measurements were performed by combining cDNA (37.5 ng cDNA per 15 μ L reaction volume reaction, run in triplicate) with primers for genes of interest (Table 1) and PerfeCTa SYBR Green Fastmix (QuantaBio #95072-012; VWR, Radnor, PA, USA) reagent, and procedures were run in the Bio-Rad CFX-Connect Real-Time System. Comparative threshold cycle (2^{-CT} ; (20)) methods were used to quantify gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene (internal control) for data normalization.

Cellular Bioenergetics (Seahorse XF)

BMSC-derived osteoblasts were seeded into 96-well tissue culture-treated Seahorse extracellular flux (XF) 96 microplates (Agilent Technologies, Santa Clara, CA, USA) at a density of 15,000 cells/well and allowed two days to adhere. Seahorse Cellular Energy Phenotype (Agilent #103325-100) assays were run at 37°C on cells incubating in pH 7.4 assay medium (Seahorse XF base medium + 10mM D-glucose + 1 mM sodium pyruvate + 2 mM GlutaMAX supplement [Gibco #35050-061]) in a Seahorse XFe96 analyzer. During the assay, cells were treated with a combination of oligomycin (an inhibitor of mitochondrial ATP production) and FCCP (a mitochondrial membrane depolarizing agent) to induce a stressed metabolic state. Raw values for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized to nucleic acid content for each well using the CyQUANT Cell Proliferation Assay on cell homogenates (Invitrogen #C7026). The fluorescence intensity of the nucleic acid-bound dye was read at Ex/Em = 480/520 nm on the BioTek Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Hdac3 Activity Assay

Osteoblastic cultures were harvested by cell scraping and pelleted in PBS. Cell pellets were incubated in hypotonic buffer solution (20 mM Tris-HCl pH 7.4, 10mM NaCl, 3 mM MgCl₂) on ice, followed by lysis with 10% NP-40 detergent (Thermo Fisher #85124; Thermo Fisher Scientific, Waltham, MA, USA). The cytoplasmic fraction of protein was removed after centrifugation, and the nuclear protein pellets were re-suspended in a solution of cell extraction buffer (Invitrogen #FNN0011; Life Technologies, Carlsbad, CA, USA), protease inhibitor cocktail (Sigma-Aldrich #P-2714), and 0.3M phenylmethylsulfonyl

fluoride (PMSF; Thermo Fisher #36978) and incubated on ice for 30 minutes to complete lysis. The nuclear protein was isolated by centrifugation at 14,000g for 30 minutes, after which the supernatant was saved for analysis. All nuclear protein lysates were quantified prior to the Hdac3 activity assay using the Bio-Rad DC™ Protein Assay System (Bio-Rad Laboratories, Hercules, CA, USA). Hdac3 activity was measured using reagents and protocols provided with the Hdac3 Activity Assay Kit (Sigma-Aldrich #EPI004). Nuclear protein was loaded into 96-well plates at 2 µg per well, in duplicate, with one replicate of the sample receiving the Hdac3 inhibitor trichostatin A (TSA) as a negative control. Fluorescence of the deacetylated substrate was measured at Ex/Em = 380/500 nm using the BioTek Synergy HT Multi-Mode Microplate Reader.

Transcription Assays

For *in vitro* transcriptional activity experiments, immortalized murine mesenchymal lineage C2C12 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin (Gibco #15140-122; Thermo Fisher Scientific). C2C12 cells were seeded into 12-well plates at a density of 20,000 cells/well and allowed one day to adhere prior to the start of experiments. Each well was transfected with 200 ng of a promoter construct for the mouse mammary tumor virus carrying a luciferase tag (MMTV-Luc), 10 ng of *Renilla*-luciferase control promoter, and 600 ng of effector plasmid of the following types: pcDNA3 control effector plasmid, Hdac3 plasmid (300 ng Hdac3 + 300 ng pcDNA3), NCoR1 plasmid (300 ng NCoR1 + 300 ng pcDNA3), or Hdac3 + NCoR1 (300 ng Hdac3 + 300 ng NCoR1). Media changes were administered the next day to begin drug treatments of 100 µM L-Kynurenine (Sigma-Aldrich #K8625) or 10⁻⁷ M Dexamethasone (Sigma-Aldrich #D1756). Following a 24-hour drug treatment, cell lysates were harvested with lysis buffer and transcription assays were performed using the Promega Dual-Luciferase Reporter Assay System (Promega #E1960; Promega Corporation, Madison, WI, USA). The luminescence of the Firefly and *Renilla* luciferases was measured using the BioTek Synergy HT Multi-Mode Microplate Reader.

Statistical Analyses

All *in vitro* experiments were performed at least three times. Differences between two groups were calculated using Student's t-tests, and statistical significance was reported with a 95% confidence interval (p<0.05). To examine the simultaneous effects of two independent variables (e.g., kynurenine treatment and sex, or Hdac3 and NCoR1 transfections) on properties of interest, groups were compared by two-factor analysis of variance (ANOVA) with interaction using JMP Pro 14 statistical analysis software. Data from *in vitro* studies are plotted as the mean ± SEM of three biological replicates. Data from *in vivo* studies are represented in box plots showing the median, quartiles, and outlier fences for each dataset, where outlier fences represent first quartile – 1.5*(interquartile range) and third quartile + 1.5*(interquartile range); each data point shown represents one mouse (n=5 per group). Synergistic effects were reported for interaction p-values of p<0.05. When significant interactions were detected, groups were compared by Tukey's HSD posthoc testing.

Results

In vitro kynurenine treatment suppressed BMSC-OB oxidative phosphorylation.

Analysis of oxidative phosphorylation activity via Seahorse XF assays revealed that female and male BMSC-derived osteoblasts treated with kynurenine demonstrated a strong trend for blunted oxygen consumption rate (OCR) under both baseline and stressed conditions. Specifically, female BMSC-OB demonstrated a 15.2% reduction in OCR under baseline conditions and a 26.3% reduction in OCR under stressed conditions ($p=0.064$ and $p=0.030$, respectively) (Fig 1A). Likewise, male BMSC-OB demonstrated a 9.7% reduction in OCR under baseline conditions and an 11.7% reduction in OCR under stressed conditions ($p=0.084$ and $p=0.056$, respectively) (Fig 1B). ECAR was not affected by kynurenine under baseline conditions in either male or female BMSC-OB ($p>0.733$) (Fig 1 A-B). Under stressed conditions, ECAR tended to be lower in kynurenine-treated female cells ($p=0.079$), but was not affected in male cells ($p=0.700$). Metabolic potential represents the ability to increase energy production in response to stress to meet energy demands. Interestingly, female BMSC-OB treated with kynurenine demonstrated a trend for impaired metabolic potential (OCR: -15.5% , $p=0.089$; ECAR: -25.0% , $p=0.009$), whereas this trend was not observed in male BMSC-OB ($p>0.670$).

In vivo kynurenine administration via injection blunted osteoblastic bone formation.

Dietary administration of kynurenine on a low-protein diet background for 8 weeks, or intraperitoneal injection of kynurenine for 10 days, suppressed dynamic indices of bone formation activity (e.g., mineral apposition rate, bone formation rate)(4). Given the *in vitro* effects observed on osteoblastic cell energetics, we also wished to determine whether kynurenine treatment would affect osteoblast numbers *in vivo*. Daily intraperitoneal injections of kynurenine (10mg/kg body mass) for 4 weeks significantly reduced osteoblast number and osteoblast surface ($p=0.009$ and $p=0.002$, respectively), but a synergistic interaction effect was observed between drug treatment and sex ($p=0.039$), where the suppression of osteoblast number was more substantial in female mice than in males (Fig 2A-B).

In vivo kynurenine administration via injection did not affect marrow adiposity.

In our previous studies, dietary administration of kynurenine on a low-protein background for 8 weeks significantly increased bone marrow adiposity (4). In the current study, however, daily intraperitoneal injections of kynurenine for 4 weeks did not significantly impact marrow adiposity (Fig 3A, $p=0.477$), marrow adipocyte density (Fig 3B, $p=0.860$), or marrow adipocyte size (Fig 3C, $p=0.713$). Marrow adiposity did show sex-specific differences, with female mice demonstrating greater values for marrow adiposity and marrow adipocyte density as compared to males ($p<0.0001$, Fig 3A-B). No interaction effects between sex and kynurenine treatment were detected ($p>0.186$).

In vitro kynurenine treatment did not suppress osteoblastic Hdac3 enzymatic activity.

Dietary administration of kynurenine on a low-protein background for 8 weeks suppressed BMSC-OB expression of the epigenetic enzyme Hdac3 and its important co-factor, NCoR1

when primary BMSC-OB from the treated mice were cultured *in vitro* (4). To determine whether these changes could be attributed to the direct effects of kynurenine on BMSC-OB, and whether those changes may drive the observed effects of kynurenine on osteoblastic cell energetics (Fig 1), we conducted *in vitro* experiments in the presence or absence of kynurenine (100 μ M) and quantified metrics of Hdac3 enzymatic activity and expression. Surprisingly, *in vitro* kynurenine treatment did not affect Hdac3 enzymatic activity (Fig 4A), nor Hdac3 or NCoR1 mRNA expression (Fig 4B). Hdac3 suppresses activation of the glucocorticoid-responsive MMTV-luciferase reporter (20); since we previously found that kynurenine treatment decreased Hdac3 expression, we sought to determine whether Hdac3's repression of the MMTV-luciferase reporter construct would be blunted in the presence of kynurenine. Hdac3 repressed the MMTV-luciferase reporter as previously described (20), and transfection of Hdac3's cofactor NCoR1 interacted with Hdac3 to synergistically further suppress this metric of glucocorticoid signaling activity (Fig 4C-D). Treatment of BMSC-OB for 24 hours with 100 μ M kynurenine did not affect any of these outcomes (Fig 4C-D). Kynurenine treatment did tend to down-regulate expression of the osteoblastic gene Osterix (SP7), but only in female cultures (-59% , $p=0.080$) (Fig 4B).

Discussion

In this study, we sought to investigate the effects of controlled kynurenine administration on marrow fat accumulation and osteoblast bioenergetics, and to investigate the direct effects of kynurenine on Hdac3 and NCoR1 expression and deacetylase activity in these models via *in vitro* treatment experiments as a possible contributor to the observed phenotypic changes. Our data demonstrate that elevated kynurenine suppressed cellular metabolism in osteoblasts at least in part through impaired mitochondrial respiration, and suppressed osteoblastic numbers *in vivo* with no concurrent effects on marrow adiposity. However, *in vitro* kynurenine treatment did not inhibit Hdac3's enzymatic deacetylase activity nor its repression of downstream glucocorticoid signaling in this study. As such, future work will be necessary to determine the mechanisms by which increased kynurenine contributes to changes in aging bone energy metabolism.

Bone loss from kynurenine treatment has previously been attributed to decreased osteoblastic bone formation activity and increased osteoclastic bone resorption (4). Moreover, BMSC-OB from kynurenine-treated mice demonstrated a gene expression signature for enhanced lipid storage, potentially suggestive of impaired cellular bioenergetics (4). In the current study, we tested this hypothesis directly and found that *in vitro* kynurenine treatment blunted the ability of BMSC-OB to utilize mitochondrial respiration to generate energy, as demonstrated by lower values for oxygen consumption rate (OCR) in kynurenine-treated cells under both baseline and stressed conditions. Unlike undifferentiated BMSC, which rely predominantly on glycolysis to meet energy demands, osteoblasts depend heavily on mitochondrial respiration to produce the ATP necessary for energetically expensive bone matrix production (21-23). The impaired ability to up-regulate oxidative phosphorylation to generate ATP under conditions of cellular stress in kynurenine-treated cultures may contribute to the reduced bone formation activity we observed in the current study and previously (4). This mechanism would be consistent with our previous observations that *in vitro* kynurenine treatment blunted the ability of BMSC-OB cultures to

produce a calcified matrix, and suppressed their expression of Runx2 (comparable to the mild suppression of Osx we observed in female BMSC-OB) (4).

The current study provides preliminary support for the idea that the effects of kynurenine on osteoblasts may be sexually dimorphic. In particular, kynurenine-induced suppression of osteoblastic metabolic potential in Seahorse assays and Osterix mRNA levels in analyses of gene expression approached significance in female cultures but not in males ($p=0.089$ female vs. $p=0.939$ male for OCR metabolic potential, $p=0.009$ for female vs. $p=0.670$ for male ECAR metabolic potential, $p=0.080$ for female vs. $p=0.878$ for Osx mRNA expression), and *in vivo* kynurenine treatment suppressed osteoblast number more substantially in female mice than in male mice. At present, the biological mechanisms behind this trend for sexual dimorphism are unknown. Previous studies report differences in the levels of kynurenine and tryptophan between men and women, although some disagreement exists between studies. For example, it has been reported that serum levels of kynurenine and tryptophan are both lower in women than in men (34,35), but it has also been reported that women have higher serum levels of free tryptophan, lower levels of total tryptophan, and no differences in levels of kynurenine as compared to men (36,37). Of interest, human bone mineral density is inversely proportional to the circulating kynurenine to tryptophan ratio (38), and osteoporosis patients exhibit lower circulating levels of tryptophan as compared to controls (39). One possibility is that BMSC and BMSC-OB from females, who may be chronically exposed to lower levels of tryptophan and/or kynurenine *in vivo* than males, are more sensitive to the deleterious effects of increased kynurenine on cellular function. However, as some of our results did not quite achieve statistical significance, and the reported sex-specific difference in kynurenine levels between males and females show some disagreement in the literature (34-37), it is not possible to make a definitive conclusion at present. Future studies encompassing a wider range of dosages and treatment regimens will be needed to conclusively establish whether kynurenine's effects on bone and osteoblasts are sexually dimorphic.

In our previous studies, dietary administration of kynurenine on a low-protein background for 8 weeks or short-term injection (10 days) of kynurenine suppressed BMSC-OB expression of the epigenetic enzyme Hdac3 and its important co-factor, NCoR1 when primary BMSC-OB from the mice treated *in vivo* were cultured *in vitro* (4). We were surprised that direct *in vitro* treatment of wildtype BMSC-OB with kynurenine in the current study did not affect Hdac3 expression or activity. However, the drug dosage and cell population chosen for *in vitro* studies here may have attributed to these disparate results. In recent work (currently under review), we found that *in vitro* treatment of purified BMSC (isolated by negative immunodepletion against CD11b and CD45R/B220, CD11c, and plasmacytoid dendritic cell antigen (PDCA)-1 followed by positive immunoselection against stem cell antigen (Sca)-1) with 200 μM kynurenine reliably suppressed expression of Hdac3 at the mRNA and protein level. This high 200 μM dose of kynurenine, rather than lower dosages, was also required to significantly suppress osteogenic differentiation as measured by alizarin red production. In the current study, we chose to study the effects of 100 μM *in vitro* kynurenine treatment on BMSC-OB to best match and compare against our earlier work (4,40) and to more closely reflect the endogenous KYN levels in the culture media of different cell types that ranges from 5 to 60 μM (41,42). Taken together, our current results

suggest that the ability of kynurenine to suppress Hdac3 expression and activity in osteoblasts may be mediated by its earlier influence on BMSC behavior and osteogenic commitment, but additional studies will be needed to test this hypothesis. Regarding our *in vivo* results, it is also possible that differences in circulating kynurenine levels induced by dietary administration, short-term intraperitoneal injection, and longer-term intraperitoneal injections (as studied here) differ, explaining some of the disparate results obtained; in future studies, it will be important to quantify serum kynurenine levels following treatment to directly relate observed phenotypes to circulating kynurenine abundance. Moreover, it will be important to repeat these studies in older mice, as circulating kynurenine levels increase with age, and aged mice may respond differently (e.g., demonstrate sensitization) to further increases in serum kynurenine.

In conclusion, the current study provides novel further support for the idea that kynurenine contributes to impaired osteoblastic function, and suggests that impaired matrix production by kynurenine-affected osteoblasts is attributed in part to impaired osteoblastic bioenergetics. As circulating kynurenine levels increase with age (4), and human bone density inversely correlates with the serum kynurenine to tryptophan ratio (38), these mechanisms may have important relevance in the etiology and pathogenesis of osteoporosis in humans.

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Highlights

- Kynurenine, an oxidized metabolite of tryptophan, increases in abundance with age
- Previous work showed that kynurenine treatment impaired osteoblastic bone formation
- In this study, kynurenine decreased cellular metabolism in osteoblasts in vitro
- Kynurenine treatment also decreased osteoblast numbers in vivo
- The harmful effects of kynurenine were more pronounced in females than males

Figure 1: Kynurenine decreases metabolic activity in BMSC-OB.

A-B) BMSC from A) female and B) male mice were isolated and differentiated into osteoblasts for 14 days, after which mitochondrial respiration and glycolytic activity were assessed via measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively, under baseline and stress conditions via the Seahorse Cellular Energy Phenotype assay. Metabolic potential was calculated as the percentage change in OCR or ECAR between baseline and stressed conditions using the manufacturer's software. P-values shown represent statistical comparisons between kynurenine (yellow) and vehicle (white)-treated cells. VEH: vehicle, KYN: kynurenine (100 μ M).

Figure 2: *In vivo* kynurenine treatment decreased osteoblast numbers.

Adult female and male mice were administered kynurenine (10 mg/kg bodyweight/day, 5 days per week) via intraperitoneal injection for 4 weeks. Osteoblast number (N.Ob/BS) and osteoblast surface (Ob.S/BS) were quantified on H&E stained frontal sections from one tibia per mouse. Letters above each bar represent groupwise-comparisons via Tukey's HSD post-hoc test; bars that share similar letters are not significantly different from one another ($p > 0.05$). Kynurenine treatment significantly suppressed osteoblast number only in female mice, evidenced by a significant interaction effect between sex and drug treatment variables ($p = 0.039$) and significant differences via posthoc testing. A similar pattern was suggested for osteoblast surface, but the trend did not achieve statistical significance ($p = 0.098$). Box plots show the median, quartiles, and outlier fences for each dataset, where outlier fences represent first quartile $- 1.5 \times$ (interquartile range) and third quartile $+ 1.5 \times$ (interquartile range); each data point shown represents one mouse.

Figure 3: *In vivo* kynurenine treatment did not alter marrow fat.

Adult female and male mice were administered kynurenine (10 mg/kg bodyweight/day, 5 days per week) via intraperitoneal injection for 4 weeks. A) Marrow adiposity (Ad.Ar/M.Ar), B) marrow adipocyte density (N.Ad/M.Ar), and C) average marrow adipocyte size were quantified on H&E stained frontal sections from one tibia per mouse. Female mice demonstrated higher marrow fat content than males ($p < 0.0001$), but kynurenine treatment did not affect any of these properties in either sex ($p > 0.477$). Box plots show the median, quartiles, and outlier fences for each dataset, where outlier fences represent first quartile $- 1.5 \times (\text{interquartile range})$ and third quartile $+ 1.5 \times (\text{interquartile range})$; each data point shown represents one mouse.

Figure 4: Kynurenine treatment did not affect Hdac3 expression or enzymatic activity.

A-B) BMSC from female and male mice were isolated and differentiated into osteoblasts. Hdac3 enzymatic activity (A) was assessed with a commercially available kit after 14 days of osteoblastic differentiation. VEH: vehicle, KYN: kynurenine (100 μ M). Gene expression was assessed after 7 days of osteoblastic differentiation. P-values shown represent statistical comparisons between kynurenine (yellow) and vehicle (white)-treated cells within each sex. C) The mouse mammary tumor virus (MMTV)-luciferase reporter construct (20), which is sensitive to glucocorticoid signaling, was transfected into mesenchymal lineage C2C12 cells. The effects of Hdac3, NCoR1, dexamethasone (Dex; 100 nM), and kynurenine (100 μ M) were assessed via transfection or treatment with these reagents as described in the text. Hdac3 and NCoR1 synergistically repressed activity of the MMTV-Luciferase promoter under all drug treatment conditions investigated (interaction $p < 0.0192$).

Table 1.

Oligonucleotide primer sequences for RT-qPCR. Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Osx, osterix-1; Hdac3, histone deacetylase 3; NCoR1, nuclear receptor corepressor 1.

Gene	Sequence (5' → 3')
Gapdh	Fwd: GGGAAGCCCATCACCATCT
	Rev: GCCTCACCCCATTTGATGTT
Osx	Fwd: GGAGGTTTCACTCCATTCCA
	Rev: TAGAAGGAGCAGGGGACAGA
Hdac3	Fwd: GCATTGAGGACATGGGGAA
	Rev: TTTCGGACAGTGTAGCCACC
NCoR1	Fwd: TTATCGGAGCCACCTACCCA
	Rev: CAGGTAAGCAGCAGCAGGA