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Binge Alcohol-Induced Bone Damage is Accompanied by Differential Expression of Bone Remodeling-Related Genes in Rat Vertebral Bone

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Abstract

Binge alcohol-related bone damage is prevented by concurrent administration of bisphosphonates, suggesting an activation of bone resorption with patterned alcohol exposure. Although chronic alcohol abuse is known to cause osteopenia, little is known about the effects of binge drinking on bone metabolism. We examined the effects of binge alcohol exposure on the relationship between bone damage and modulation of bone remodeling-specific gene expression profiles. Our hypothesis was that bone damage observed in young adult rats after binge alcohol exposure is associated with differential expression of bone remodeling-related gene expression. We further hypothesized that this differential gene expression specific to bone remodeling (bone resorption or formation related) would be influenced by the duration of binge alcohol exposure. Binge alcohol (3 g/kg, i.p.) was administered on 3 consecutive days each week, for 1 or 4 weeks, to adult male rats. Matched control animals were injected with an equal volume of isotonic saline. Lumbar vertebrae, L4-5, were analyzed for the presence of bone damage by quantitative computed tomography and compressive strength analysis. Total RNA was isolated from an adjacent vertebrae (L3), and whole transcriptome gene expression data were obtained for each sample. The expression levels of a subset of bone formation and resorption-associated differentially expressed genes were validated by quantitative reverse transcriptase–polymerase chain reaction. Bone loss was not observed after 1 week of treatment but was observed after four binge alcohol cycles with a 23% decrease in cancellous bone mineral density and 17% decrease in vertebral compressive strength compared with control values ($P < 0.05$). We observed that the duration of binge alcohol treatment influenced the modulation of expression profiles for genes that regulate the bone formation process. The expression of key bone formation-related marker genes such as osteocalcin and alkaline phosphatase were significantly reduced ($P < 0.05$) after acute binge alcohol exposure, and expression of regulators of osteoblast activity such as bone morphogenetic proteins and parathyroid hormone receptor displayed significantly ($P < 0.05$) decreased differential expression. The expression of sclerostin, a key canonical Wnt inhibitory protein, was significantly increased after acute binge alcohol treatment. The expression of important regulators of osteoclast maturation and activity such as NF-$\kappa\beta$ (nuclear factor $\kappa\beta$) ligand (RANKL) and interleukin-6 were significantly increased ($P < 0.05$) by binge alcohol, and osteoprotegerin levels were significantly decreased ($P < 0.05$) in vertebral bone. These results show that expression patterns of several key bone remodeling genes are significantly perturbed by binge alcohol treatment, suggesting that perturbation of gene expression associated with bone remodeling may be one mechanism contributing to the disruption of bone mass homeostasis and subsequent bone loss observed after binge alcohol exposure in rodents.

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Keywords

Bone turnover; Remodeling; Cytokines; RANK; RANKL; OPG; Osteoblasts; Osteoclasts; Osteocytes

Introduction

Alcohol abuse is a well-known risk factor for both osteopenia and osteoporosis [1]. Alcohol-related bone loss is most likely caused by imbalances created in the bone remodeling cycle related to alcohol's depressive effects on bone formation activity [2–4] and activation of bone resorption [5–8]. The specific molecular and cellular targets of alcohol responsible for this imbalance in bone remodeling are not well defined. Although inherited polymorphisms at specific gene loci have been identified as associated with a higher risk for developing postmenopausal osteoporosis [9], little is known about how gene expression patterns are modulated during bone loss, or how expression patterns differ in bone compromised by age, hormone insufficiency, or excessive alcohol consumption. Changes in bone-specific gene expression after acute or chronic alcohol exposure have been examined for only a few bone matrix target genes such as type 1 collagen, osteocalcin, and alkaline phosphatase, with transient increases in osteocalcin expression noted both after acute [10] and chronic alcohol treatment [4] and lasting changes in collagen 1 expression seen after chronic alcohol exposure [4]. The significance of these findings with respect to alcohol-related changes in bone formation is not clear. By means of transcriptome analysis, which we performed to understand the genomic response of bone tissue to alcohol exposure in a rodent model, we recently identified two pathways that regulate the bone remodeling process: integrin signaling, vital for osteoclastic bone resorption [11], and the canonical Wnt signaling pathway that regulates mesenchymal stem cell differentiation and promotes osteogenesis [12] as potential novel targets of alcohol in bone [13]. These data provide a link between the observed effects of alcohol exposure on bone remodeling and specific molecular pathways that are disrupted by alcohol.

Our laboratory uses a rodent model of binge alcohol exposure to achieve blood alcohol levels (BALs) consistent with those observed in heavy human binge drinking and chronic alcohol abuse [14] to test the effects of these high BALs on bone metabolism. We have demonstrated significant decreases in bone mineral density (BMD) and bone strength that occur in adult male, female, and ovariectomized rats exposed to repeated binge cycles [8,15,16]. We have also demonstrated that binge alcohol-related bone loss can be prevented by concurrent administration of aminobisphosphonates, suggesting that increased bone resorption is responsible for at least part of the bone loss observed after binge alcohol administration in rodents [8].

The goal of the current investigation was to identify changes in expression profiles for bone formation and bone resorption-related genes that may help identify the actual effector proteins responsible for alcohol-induced changes in bone remodeling. Our hypothesis was that bone damage observed in young adult rats after binge alcohol exposure is associated with significant differential expression of bone remodeling-related genes. We further hypothesized that specific bone remodeling gene expression profiles would be influenced by the duration of binge alcohol exposure. We report here on the results of a comprehensive examination of bone remodeling-related gene expression after acute and chronic binge alcohol exposure of young adult rats exhibiting changes in bone integrity parameters consistent with alcohol-related bone damage.

Materials and Methods

Binge Alcohol Model

This investigation received full approval from the Loyola University Institutional Animal Care and Use Committee. Adult male Sprague Dawley rats were obtained at 16 weeks of age (375–399 g range; Harlan, Indianapolis, IN) and acclimated to the laboratory environment for 1 week. Prior to the start of the experiment, animals were randomly assigned to one of the following treatment groups with 12 animals per group, chosen on the basis of a power analysis performed with preliminary gene array data generated in our laboratory (data not shown). Animals receiving 1 week of treatment were saline-treated control and binge alcohol-treated animals. Animals receiving 4 weeks of treatment were saline-treated control and binge alcohol-treated animals. These groups are referred to as acute or chronic binge alcohol treatment groups, respectively, throughout the text on the basis of the number of weeks of alcohol treatment received (1 week = acute, 4 weeks = chronic). Animals were housed in pairs, with paired animals assigned to the same treatment group. Animals were weighed before the initiation of treatment and weekly throughout the study period. Animals were allowed free access to both food and water throughout the study.

Alcohol administration was by a single daily i.p. injection of a 20% (vol/vol) ethanol/saline solution at a dose of 3 g/kg. This dose was chosen to achieve peak BALs of approximately 300 mg/dl [17]. Control animals were given an i.p. injection of an equal volume of sterile isotonic saline at the time of alcohol group injections. Alcohol or saline injections were given starting at 9:00 AM, for 3 consecutive days each week. No i.p. injections were given during the remaining 4 days of each week.

Twenty-four hours after their last saline or alcohol injection, rats were rendered unconscious by CO_2 inhalation and killed by decapitation. Bone samples (lumbar vertebrae) were removed from each animal, dissected free of all soft tissue, and either snap frozen in liquid nitrogen and stored at −80°C for subsequent molecular analysis, or wrapped in saline-soaked gauze and stored at −20°C for BMD and biomechanical analysis.

Blood Ethanol Determination

BALs were determined by NAD^+ reduction assay (Sigma Diagnostics, St. Louis, MO). Peak BAL was measured in four rats that were not included in any of the experimental protocol groups. These animals were killed by decapitation 60 minutes after a single i.p. injection of 20% (vol/vol) ethanol/saline solution. The timing of peak BAL after i.p. injection was estimated on the basis of a previous study that evaluated the pharmacokinetic profile of a 3 g/kg i.p. alcohol injection in rats [17].

Bone Mineral Density Measurements

Cancellous and cortical BMD of the fourth and fifth lumbar vertebrae from each animal were determined by quantitative computed tomography (pQCT) with a Norland Stratec bone densitometer (Orthometrix, Inc., White Plains, NY). Each intact vertebra was positioned uniformly on a support so that the instrument-scanning plane was perpendicular to the longitudinal axis of the vertebral body. Scout views were used to focus the pQCT analysis on the body of each vertebral segment and to determine the midpoint of each vertebral body. Midpoint analysis was performed to ensure that each sample was analyzed at the exact same point to minimize variation due to density differences based on sampling area. Three consecutive measurements were performed at a resolution of 70 μm/voxel 1 mm apart from this point. By means of a predetermined peel algorithm, the cancellous area of each vertebral segment was defined as 45% of the total bone cross-sectional area of each measured plane of the vertebral body. The remaining fraction was defined as cortical bone. The instrument was

set to use the threshold contour mode (soft-tissue threshold set at 220 mg/cm^3) and a concentric peel algorithm. Scans were made at 50 kV and 0.3 mÅ.

Biomechanical Structural Properties of Lumbar Vertebrae

Compressive strength tests were performed on the L4 and L5 vertebral bodies from each rat with an Instron materials testing machine (model 5544, Canton, MA). The vertebral end plates were potted in bone cement by using a previously described method that resulted in two parallel loading surfaces necessary to perform a uniform compression test on individual rodent vertebrae, as previously described [8]. The specimens were prepared so that the posterior elements of the vertebra did not contact the loading platforms. Compression testing was performed at a cross-head speed of 0.5 mm/min to eliminate any strain rate effects. A 100-kg load cell was used to monitor the compressive load, and a precision sensor was used to measure the axial deformation of the specimen. The load-deformation data were analyzed to obtain the compressive strength of the vertebrae, defined as the maximum load sustained before failure.

Statistical Analysis of Bone Damage Parameters

Statistical analysis was performed by SPSS software (SPSS Inc., Chicago, IL). Analysis of statistical significance for BMD and vertebral strength data was performed by one-way analysis of variance and Tukey's honestly significant difference multiple-comparison procedure. Significance was noted at $P \leq 0.05$. Multiple regression analyses were used to determine the relative contributions of body weight vs. treatment condition on the effects observed in the dependent variables (BMD, strength) we studied. These analyses were performed in a two-step hierarchical fashion. Information about treatment groups was carried by three dummy coded dichotomies to account for the four treatment conditions. All regression analyses were conducted in a similar fashion. First, the dependent variable was regressed upon posttreatment weight. Next, the treatment group dichotomies were added to the equation. The adjusted R^2 was measured in each step, and the change in this statistic was tested for significance. The change in adjusted R^2 indicated the percentage of variance that was explained by treatment group after accounting for the variability due to weight.

RNA Isolation

Before RNA isolation, the L3 lumbar vertebral body of each frozen sample was excised from the intact vertebral segment with a Dremel rotary saw. Samples were prepared for RNA extraction by first pulverizing each bone sample individually with a Freezer Mill (Spex CertiPrep, NJ). RNA was extracted from the powdered bone samples with the RiboPure RNA isolation kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. RNA samples were quantified by spectroscopy (NanoDrop Technologies, Wilmington, DE) and visualized for purity and integrity with the Agilent 2100 Bioanalyzer (Santa Clara, CA) before gene array analysis.

Gene Expression Array Analysis

Twelve biological replicate samples per treatment group were used for analysis. Gene expression array analysis was performed with the Applied Biosystems 1700 Gene Expression Array System (Foster City, CA). The ABI rat genome survey microarray contains probes representing a complete, annotated, and manually curated set of approximately 27,000 rat genes from both the public and Celera databases. Analysis was performed according to the manufacturer's protocol with the Functional Genomics Core Laboratory at the University of Chicago. Briefly, digoxigenin-UTP-labeled cRNA was generated and linearly amplified from 2 μg of total RNA using the ABI chemiluminescent RT-IVT labeling kit. Arrays were prehybridized for 1 h at 55°C in hybridization buffer with blocking reagent. Ten micrograms of labeled cRNA was incubated in fragmentation buffer for 30 min at 600 and subsequently

hybridized to each microarray in 0.5-ml hybridization buffer for 16 h. Chemiluminescent detection and image acquisition were performed by the ABI chemiluminescence detection kit and an ABI 1700 microarray analyzer. Primary image analysis was performed by the ABI microarray analyzer software package.

Array Data Analysis

Data analysis was performed by the GeneSpring array analysis software package (GeneSpring GX 7.31, Agilent Corporation, Carmel, IN). Before analysis, a qualified data set was obtained by performing bilevel quality control. First, at the replicate level, principal component analysis and hierarchical cluster analysis were performed to identify outlier samples from within each biological replicate group that fell distal to cohort replicates comprising each treatment group. Second, filtering at the gene level was performed, removing those genes with signal to noise ratios (S/N) below 3.0 in 6 of 12 arrays per group. This quality analysis resulted in the removal of outlier arrays from the experiment and produced a qualified gene list of 12,655 genes (out of 27,000 total genes) used for all subsequent analyses. Statistical comparisons were performed by Wilcoxon–Mann–Whitney nonparametric analysis with the Benjamini and Hochberg false discovery rate multiple testing correction.

Validation of Gene Array Data

Quantitative real-time RT-PCR (qRT-PCR) was performed on a select subset of relevant genes that showed differential expression by gene array analysis. A preliminary experiment was performed to select an appropriate endogenous control gene for RT-PCR analysis (β2-microglobulin, B2M) as previously described [13]. Genes selected for validation included osteocalcin (Bglap2), alkaline phosphatase (Alp1), collagen-type 1α2 (Col1a2), bone morphogenetic protein 2 (Bmp2), parathyroid hormone receptor (Pthr1), receptor activator for NF-$\kappa\beta$ (nuclear factor $\kappa\beta$) ligand (RANKL), osteoprotegerin (OPG), and interleukin-6 (IL-6). Validation was performed (n = 8/group) with the 7500 Fast Real-Time RT-PCR Gene Expression System (ABI). Each cDNA (20 ng) sample was amplified in duplicate using gene-specific and control (B2M) TaqMan Gene Expression Assay primer/probes sets. Data were analyzed by SDS software 1.4 by the $2^{-\Delta\Delta CT}$ relative quantification method [18].

Results

General Observations

All animals gained weight during the treatment period. No differences were observed in body weight between acute (1 week) binge alcohol- and saline-treated animals. After chronic (4 week) binge exposure, alcohol-treated rats showed an approximate 4% increase in body weight compared with their baseline weight (baseline 380 ± 15 g, final 393 ± 18 g), vs. an 11% increase in the respective saline control group (baseline 377 ± 15 g, final 412 ± 12 g). This difference in posttreatment weight between control and chronic binge alcohol-treated animals was significant ($P < 0.05$). A once-daily alcohol treatment protocol was used to avoid alcohol withdrawal symptoms that can occur when high doses of alcohol are administered twice daily [19]. All alcohol-treated animals were monitored daily throughout the study period, and no behavioral symptoms of alcohol withdrawal [20] were observed during the 4-day period each week when alcohol was not administered. Rats exhibited approximately 1–2 h of acute alcohol intoxication immediately after each i.p. injection. Peak BALs, measured 1 h after i.p. alcohol injection in four alcohol-naive animals not included in subsequent experiments, averaged 280 mg/dl, consistent with published reports of peak BALs in rats after administration of 3 g/kg i.p. alcohol [17]. Necropsy performed on each animal revealed no apparent internal injuries from i.p. alcohol injections. Abdominal organs of alcohol-treated animals seemed normal at gross inspection.

Effects of Binge Alcohol on Vertebral Bone Mineral Density and Strength

After alcohol or saline treatment and before gene array analysis, bone integrity was quantified by standard assays used previously in our laboratory [8]. Consecutive lumbar vertebrae (L4-5) dissected from each animal were analyzed for BMD and compressive strength to failure. The next adjacent lumbar segment (L3) was used for the molecular studies described in the following section. These data are summarized in Table 1. An acute binge alcohol treatment cycle did not significantly decrease vertebral cancellous or cortical BMD, or compressive strength compared with matched saline control values. In contrast, chronic binge alcohol treatment cycles significantly decreased vertebral cancellous BMD ($P < 0.05$) and compressive strength values ($P < 0.05$) compared with matched saline-control animals. The size of vertebral bodies, determined by cross-sectional area measurements obtained during pQCT analysis, was equivalent across treatment groups (data not shown), suggesting that the decreased BMD and vertebral compressive strength values observed after binge alcohol treatment were the result of altered mineral and biomechanical properties of the vertebrae and were not due to treatment-related differences in vertebral body area.

Because a significant effect of chronic binge alcohol treatment was observed on final posttreatment weight gain compared with control animals, we performed multiple regression analysis to determine the relative contributions of body weight vs. treatment condition (i.e., binge alcohol) on the significant effects observed in the dependent variables we studied (BMD, strength). This analysis revealed that posttreatment weight accounted for an adjusted 1% of the variance in vertebral trabecular BMD and was not statistically significant ($F_{1,21} = 1.22$, $P = .282$). Treatment group, however, accounted for an additional 39% of the variance and was significant ($F_{3,18} = 5.50$, $P = .007$). Likewise, posttreatment weight accounted for none of the variance in vertebral compressive strength and treatment group accounted for 25% of the variance observed ($F_{3,16} = 3.10$, $P = .05$).

Effects of Binge Alcohol on Bone Formation-Related Gene Expression

We observed a number of bone formation-related gene expression profiles that were differentially expressed after acute or chronic binge alcohol exposure. These results, which are summarized in Table 2, demonstrate relative expression level changes for genes after acute or chronic binge alcohol as a percentage of respective control level with associated *P* values. Examples include the expression levels of two well-characterized markers of bone formation, alkaline phosphatase and osteocalcin, both significantly decreased after acute binge exposure ($P = 0.015$, and $P = 0.003$, respectively). Other genes with alcohol-related significantly decreased expression include three members of the bone morphogenetic family of proteins (BMP2, -4, -5) and the receptor for BMP2 (BMP2r). Expression of several bone-related collagen genes represented on the array were also significantly decreased by alcohol. Other genes of interest included in the bone formation list displaying significantly changed expression levels are a number of different growth factors and angiogenesis factors, Pthr1, and the canonical Wnt inhibitor sclerostin (SOST).

Effects of Binge Alcohol on Resorption-Related Gene Expression Profiles

Bone resorption gene expression profiles were also assayed for differential expression after binge alcohol exposure. These results are summarized in Table 3. We observed differential expression for two regulators of osteoclastogenesis, RANKL and OPG. RANKL levels were elevated by acute alcohol exposure and tended to normalize after chronic binge alcohol. OPG mRNA levels were observed as decreased after chronic alcohol exposure, and qRT-PCR verified that the differential expression patterns observed for both OPG and RANKL were significant ($P < 0.05$). Other bone resorption-related genes showing significant differential expression include interleukin-6 (IL-6), oncostatin M (OSM), and the matrix metalloproteinase enzymes Mmp8 and Mmp13.

Confirmation of Gene Expression Data

Real-time quantitative RT-PCR (qRT-PCR) analysis was used to confirm the direction and magnitude of change in mRNA levels in selected genes differentially expressed by array analysis. A preliminary experiment identified an appropriate endogenous control gene β2-microglobulin (B2M), chosen because it displayed little variation in message levels across treatment groups (data not shown). Genes selected for validation included those from both bone formation- and bone resorption-related categories displaying differential expression after acute or chronic binge alcohol treatment relative to control. Gene-specific expression data obtained by both gene array and qRT-PCR analysis are displayed on a shared axis as a line graph. There was excellent overall correlation between gene array and qRT-PCR data with respect to the direction and magnitude of gene expression levels across treatment groups for each selected gene (Fig. 1).

Discussion

Although the bone remodeling cycle has long been thought to be the primary target for the deleterious effects of alcohol on bone integrity, a comprehensive examination of the effects of alcohol on bone remodeling-related gene expression has not been previously performed. We report here on the results of such an investigation and discuss the potential effect on bone of our finding that binge alcohol exposure causes significant differential expression of selected osteoblast or osteoclast-expressed genes with documented roles in bone remodeling.

Bone formation has long been believed to be the primary target of alcohol's deleterious effects on bone metabolism [21]. The expression of alkaline phosphatase and osteocalcin, both well-validated markers for bone formation activity, were significantly decreased after acute binge alcohol treatment in this study, suggesting that bone formation activity is depressed after binge alcohol exposure. Our data support previously reported effects of alcohol on bone marrow osteocalcin mRNA levels [22] but contrast with other studies demonstrating transiently increased osteocalcin levels after a single alcohol injection in female rats [10] or chronic alcohol feeding in adolescent male animals [4]. Although differences in animal age and sex may account for these differences, a more likely explanation is that the binge-patterned high BALs achieved in the current study are more damaging to bone than lower BALs and cause a significant depression of bone formation activity not observed in previous studies. A recent study demonstrating that intermittent high-dose alcohol exposure by vapor inhalation also has significant detrimental effects on osteoblast function backs this supposition [23]. The acute or lower chronic alcohol doses given to rats in the studies mentioned above may indeed be leading to a positive effect on osteoblast function, as suggested by the osteocalcin data presented in these studies. The mechanism underlying this positive effect of alcohol on markers of osteoblast function is currently not known, but this observation is supported by human longitudinal studies on factors modulating bone mass, which also show a positive effect of low-dose alcohol consumption on age-related maintenance of bone mass [24].

The bone morphogenetic (BMP) family of proteins have well-documented anabolic effects on bone [25]. We observed a general decrease in BMP gene expression after chronic binge alcohol treatment, with levels of BMP2, -4, and -5 and the receptor for BMP2 (BMP2r) significantly decreased. Decreased BMP mRNA expression has not been previously associated with alcohol-induced bone loss; it suggests that the decreases in bone formation observed after alcohol exposure [10] may be due in part to decreased BMP-mediated signaling in osteoblasts. This result, coupled with decreases in the expression of several bone collagen genes and other bone formation-related genes observed here, suggests that one way that alcohol may affect bone formation is to cause a general decrease in osteoblast mRNA synthesis or the stability of transcripts required for bone formation-related protein synthesis. This mechanism could account for the decreased bone formation activity observed after alcohol exposure [21].

Interestingly, Pthr1 expression levels were decreased by more than 40% after both acute and chronic binge exposure, suggesting that parathyroid hormone (PTH) signaling in osteoblasts may also be compromised by alcohol exposure. We previously demonstrated that binge alcohol-induced bone loss in rats was mitigated by high-dose intermittent PTH receptor administration [15]. Taken together, these data suggest that exogenous intermittent PTH administration may be required to stimulate PTH-mediated osteoblast activity in alcohol-exposed bone.

We recently discovered that the canonical Wnt signaling pathway, which plays a critical role in bone formation [12], may be a bone-specific target of binge alcohol exposure in adult rats [13]. Analysis of our data found that the steady-state level of sclerostin mRNA, a Wnt inhibitor produced only by osteocytes [26,27], is significantly increased after acute binge treatment. This is an intriguing finding because it suggests that alcohol exposure could also be targeting the osteocyte, causing these cells to increase production of sclerostin and effectively turning off canonical Wnt signaling in bone. This would have detrimental consequences on maintenance of bone mass as a result of decreases in the maturation of osteoblast precursors and subsequent bone formation, controlled by canonical Wnt target gene activity. Sost has also been shown to antagonize some BMP-mediated bone anabolic activity, which could further depress bone formation [26,27].

Although osteoblasts and bone formation are generally accepted as the primary targets of alcohol in bone tissue, targeting of osteoclast regulation and bone resorption as a mechanism underlying alcohol's deleterious effects on bone is less accepted. Recently published reports support a role for increased bone resorption after both chronic and binge alcohol exposure. Zhang and coworkers [7] demonstrated increased bone resorption in mice after chronic alcohol exposure and determined that OPG, a soluble decoy receptor for RANKL produced by osteoblasts that functions as a modulator of bone resorptive activity, could block increased bone resorption induced by chronic alcohol exposure. Our laboratory demonstrated increased bone resorption in young adult male rats by measuring serum deoxypyrodinoline levels after exposure to repeated binge alcohol cycles [8]. Treatment with the antiresorptive bisphosphonate risedronate prevented not only binge alcohol-induced increases in serum deoxypyrodinoline, but also decreases in cancellous BMD and compressive strength associated with binge alcohol treatment. Chen and coworkers recently demonstrated increased bone resorption and RANKL mRNA expression after chronic alcohol feeding in female rats and in primary cultured bone cells [22], suggesting that an increase in osteoblast RANKL expression may be the mechanism responsible for increased bone resorptive activity associated with alcohol.

We observed differential expression of two key regulators of osteoclast maturation, RANKL and OPG. The RANKL-OPG system is widely recognized as vital to osteoclast differentiation and resorption activity [28,29]. This observation suggests a molecular mechanism whereby alcohol treatment could stimulate osteoclast differentiation and activation through increasing RANKL-mediated signaling and decrease the attenuating effect of OPG, leading to increased bone resorption. Because both RANKL and OPG are made by osteoblasts, these data support the concept that osteoblast-mediated bone formation and resorption regulatory activities may be the key targets of alcohol in bone. The expression of RANK, the receptor for RANKL found on osteoclast cells, shows decreased expression after a 4-week alcohol binge, possibly representing the activation of a negative feedback loop modulating an alcohol-related increase in osteoclast activity. Although increased RANKL expression observed in this study is in agreement with previous reports [22], our observation that OPG mRNA levels are depressed by bingelike alcohol exposure is novel and supports the concept that intermittent binge alcohol exposure may be especially disruptive to osteoblast function. Recent evidence suggesting that OPG expression is regulated by Wnt/β-catenin signaling [30] suggests that the effect of alcohol

on OPG expression that we demonstrate here is controlled through the alcohol-related targeting of the canonical Wnt pathway that we have previously demonstrated [13].

IL-6 and OSM are inflammatory cytokines that stimulate bone resorptive activity [31] by increasing expression of RANKL and decreasing OPG expression [32]. Our results demonstrate significantly increased IL-6 and OSM expression levels after chronic binge alcohol, suggesting that binge alcohol stimulation of inflammatory cytokines could be responsible for the observed modulation of RANKL and OPG levels, although increased RANKL expression precedes the increases in IL-6 and OSM observed here.

The effect of chronic binge alcohol treatment on final posttreatment animal weight is a limitation to the current study that could affect the interpretation or relevance of our results. We addressed this concern, as described in Results, by statistical regression analysis, which revealed no significant contribution of weight to the observed decreases in vertebral BMD and strength, and which revealed highly significant treatment (i.e., alcohol)-related effects on bone integrity. This analysis strongly suggests that alcohol treatment has a damaging effect on bone in this experimental paradigm and that it is likely either directly or indirectly responsible for the changes in bone remodeling-related gene expression presented here. The fact that binge alcohol-exposed rats gained weight throughout the study period suggests that animals were in good overall health and were consuming enough food during the study period. Although not measured in this study, no significant differences in weekly food intake were observed between control and binge alcohol-treated adult animals in a previous investigation [15]. A direct effect of alcohol exposure on weight gain was observed in a previous study by our laboratory in animals exposed to chronic alcohol administration using the Lieber-DeCarli liquid diet, with control animals given a liquid diet matched to the caloric intake of their alcohol-fed counterparts, eliminating the potential confounding of reduced caloric intake by alcohol-exposed animals [33].

In summary, analysis of bone formation- and resorption-specific gene expression profiles after binge alcohol treatment demonstrates a general trend of decreased differential expression for several bone formation-related genes and the modulation of important regulators of bone resorption. This information adds to our understanding of the mechanisms underlying alcohol-induced bone loss by demonstrating that on a molecular level, alcohol targets the expression of genes with well-defined roles in bone remodeling. Therapeutic agents targeting bone remodeling have found clinical utility in the treatment of other metabolic bone diseases such as osteoporosis; this study demonstrates that current and future therapeutic agents targeting bone turnover should be useful in treating or preventing alcohol-related bone loss. This information may also be useful in designing the next generation of drugs that target the unique aspects of alcoholic bone disease.

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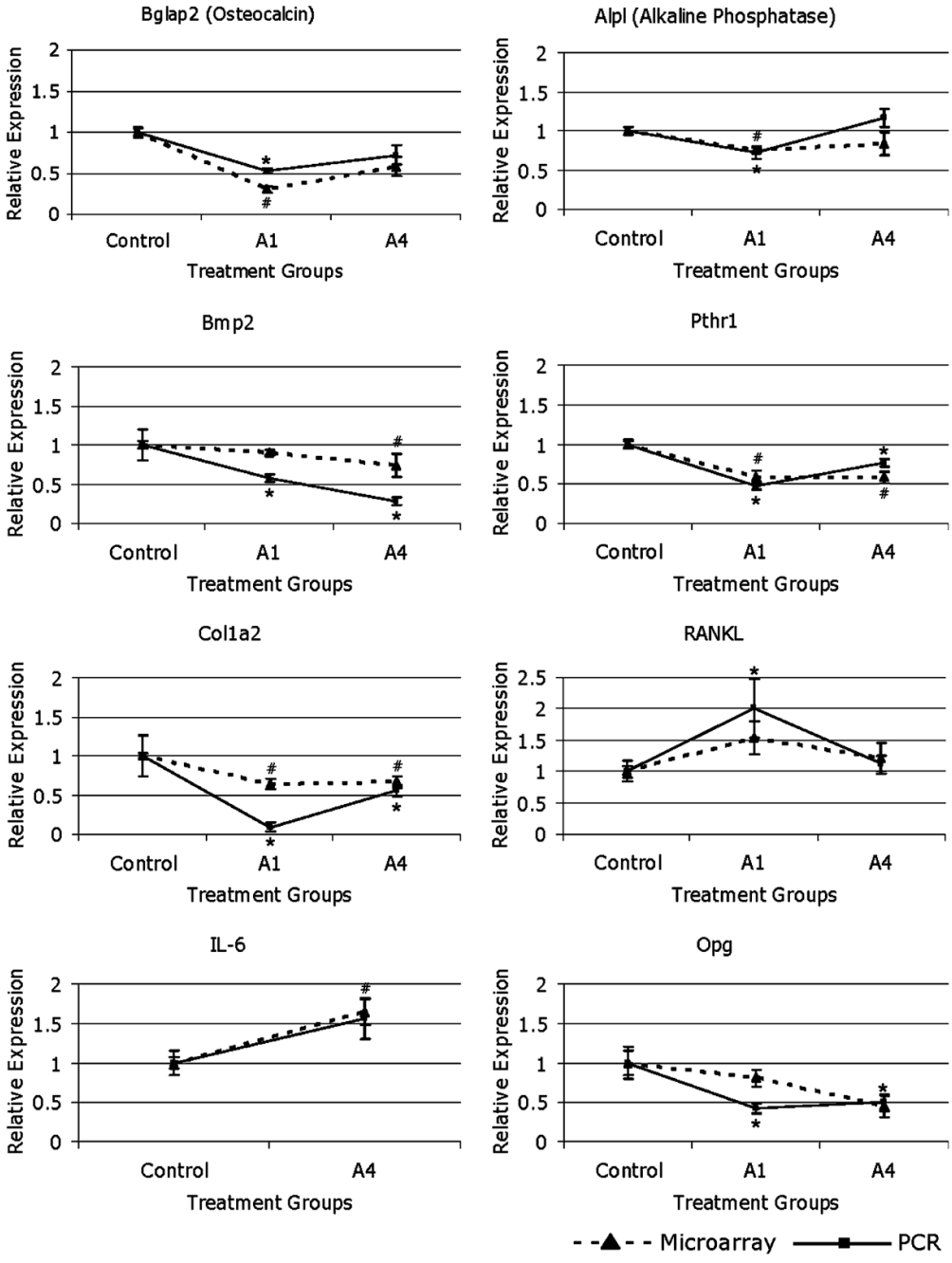

Fig. 1.

Validation of microarray data for select genes by qRT-PCR. Osteocalcin (Bglap2), alkaline phosphatase (Alpl), bone morphogenetic protein 2 (Bmp2), Pthr1, pro-alpha-2(1) collagen (Col1a2), receptor activator of NF-$\kappa\beta$ ligand (RANKL), interleukin-6 (IL-6), and osteoprotegerin (Opg). For each gene, both microarray (*triangles*) and PCR (*square*) data are shown relative to the control group; $n = 8$/group. Data are shown as mean ± SEM. $^*P < 0.05$ for qRT-PCR data when compared with control, Student's *t*-test. $\#P < 0.05$ for microarray data when compared with control, Wilcoxon–Mann–Whitney nonparametric analysis with Benjamini and Hochberg multiple testing correction

Table 1

Treatment effects on bone integrity parameters

Treatment group	Cancellous BMD (mg/cm^3)	Cortical BMD (mg/cm^3)	Compressive strength (*N*)
Saline control (C1)	345.1 ± 5.8	752.2 ± 6.8	226.6 ± 20.4
Acute binge alcohol (A1)	345.8 ± 12.2	757.9 ± 15.6	243.3 ± 20.7
Saline control (C4)	361.2 ± 13.0	714.4 ± 13.5	306.4 ± 14.7
Chronic binge alcohol (A4)	279.7 ± 4.2*	650.1 ± 16.9	253.3 ± 7.3*

Vertebral cancellous BMD, cortical BMD, and compressive strength data are shown as the mean ± SEM. C1, 1-week control group; A1, 1-week alcohol group; C4, 4-week control group; A4, 4-week alcohol group; n = 12/group

* $P < 0.05$, C4 vs. A4, by one-way analysis of variance with Tukey's post hoc test

Table 2

Bone formation-related gene expression

		Treatment			
		Acute binge alcohol		Chronic binge alcohol	
Common name	**Description**	**% Control**	***P* value**	**% Control**	***P* value**
Bone formation markers					
Alpl	Alkaline Phosphatase	76.2 ± 4.3	0.015	83.2 ± 13.9	0.371
Bglap	Osteocalcin	30.8 ± 4.6	0.003	58.5 ± 13.6	0.081
Bone morphogenetic proteins					
Bmp2	Bone morphogenetic protein 2	90.2 ± 4.1	0.189	73.8 ± 11.9	0.046
Bmpr2	Bone morphogenic protein receptor II	108.8 ± 6.2	0.205	55.7 ± 4.8	0.005
Bmp4	Bone morphogenetic protein 4	135.6 ± 29.2	0.055	56.8 ± 8.8	0.006
Bmp5	Bone morphogenetic protein 5	86.1 ± 5.5	0.095	56.1 ± 9.7	0.012
Collagens					
Col1a2	Pro-alpha-2(1) collagen	64.1 ± 7.2	0.013	66.4 ± 7.9	0.006
Col2a1	Procollagen, type II, alpha 1	39.2 ± 7.0	0.011	46.3 ± 13.8	0.014
Col4a1	Procollagen, type IV, alpha 1	47.4 ± 5.1	0.004	53.7 ± 6.8	0.005
Col4a2	Procollagen, type IV, alpha 2	48.7 ± 4.2	0.003	53.6 ± 6.9	0.003
Col4a3 bp	Procollagen, type IV, alpha 3	114.5 ± 5.3	0.102	72.6 ± 6.0	0.014
Col4a4	Procollagen, type IV, alpha 4	52.9 ± 4.4	0.004	87.7 ± 13.4	0.671
Col5a1	Collagen, type V, alpha 1	41.9 ± 3.7	0.003	36.9 ± 3.8	0.003
Col6a2	Procollagen, type VI, alpha 2	65.9 ± 5.3	0.015	61.9 ± 3.8	0.004
Col9a1	Procollagen, type IX, alpha 1	40.9 ± 9.8	0.020	56.3 ± 15.6	0.104
Col9a3	Procollagen, type IX, alpha 3	53.3 ± 8.3	0.029	57.0 ± 20.7	0.173
Col10a1	Collagen alpha 1 type X	52.3 ± 11.7	0.047	61.4 ± 12.9	0.092
Col15a1	Procollagen, type XV	81.4 ± 6.0	0.055	55.6 ± 7.3	0.004
Col18a1	Collagen, type XVIII, alpha 1	68.2 ± 4.3	0.015	65.5 ± 4.8	0.014
Growth factors					
Fgfrl	Fibroblast growth factor receptor 1	72.2 ± 6.3	0.041	43.5 ± 4.6	0.003
Fgfr2	Fibroblast growth factor receptor 2	73.8 ± 5.2	0.015	52.2 ± 5.0	0.003
Fgfr3	Fibroblast growth factor receptor 3	69.0 ± 12.6	0.129	66.9 ± 3.4	0.026
Igf1	Insulin like growth factor 1	58.6 ± 3.6	0.003	60.0 ± 4.6	0.003

		Treatment			
		Acute binge alcohol		Chronic binge alcohol	
Common name	**Description**	**% Control**	***P* value**	**% Control**	***P* value**
Igf2	Insulin like growth factor 2	85.2 ± 7.2	0.275	45.5 ± 6.5	0.003
Pdgfa	Platelet derived growth factor, alpha	78.3 ± 3.8	0.020	55.6 ± 6.5	0.003
Tgfbl	Transforming growth factor, beta 1	64.5 ± 4.7	0.009	78.5 ± 4.5	0.026
Tgfb2	Transforming growth factor, beta 2	66.1 ± 10.8	0.034	46.0 ± 6.0	0.003
Tgfb3	Transforming growth factor, beta 3	56.3 ± 7.3	0.015	40.8 ± 3.6	0.003
Vegf	Vascular endothelial growth factor	82.5 ± 4.6	0.038	77.6 ± 6.9	0.026
Vegfc	Vascular endothelial growth factor C	146.1 ± 13.1	0.038	99.3 ± 23.6	0.933
Other bone formation genes					
Sost	Sclerostin	357.3 ± 71.3	0.013	107.2 ± 30.9	0.929
Pthr1	Parathyroid hormone receptor 1	59.1 ± 2.9	0.003	58.1 ± 5.6	0.003

Differential expression values and associated significance are shown for bone formation-associated gene expression. Each gene is listed by common name, description and expression value (±SEM) as a percentage of respective control for the following treatment groups: 1-week alcohol group, and 4-week alcohol group. Statistical comparisons were performed by Wilcoxon–Mann–Whitney nonparametric analysis with the Benjamini and Hochberg false discovery rate multiple testing correction

Table 3

Bone resorption-related gene expression

		Treatment			
		Acute binge alcohol		Chronic binge alcohol	
Common name	**Description**	**% Control**	***P* value**	**% Control**	***P* value**
RANK-RANKL signaling					
RANKL	Receptor activator of NF-κB ligand	154.7 ± 27.4	0.317[a]	120.3 ± 22.5	0.513
OPG	Osteoprotegerin	80.6 ± 9.8	0.511	44.6 ± 17.2	0.128[a]
Matrix metalloproteinases					
Mmp2	Matrix metallopeptidase 2	70.6 ± 7.0	0.034	58.6 ± 4.4	0.003
Mmp8	Neutrophil collagenease	144.1 ± 9.9	0.013	105.1 ± 15.9	0.809
Mmp9	Matrix metalloproteinase 9	87.0 ± 5.4	0.205	102.1 ± 11.6	0.767
Mmp13	Collagenase	148.3 ± 18.1	0.047	144.7 ± 15.6	0.038
Cytokines					
II6	Interleukin 6	120.5 ± 22.2	0.683	164.1 ± 15.3	0.050
Osm	Oncostatin M	92.5 ± 7.2	0.653	134.5 ± 12.6	0.045
Tnfa	Tumor necrosis factor superfamily, member 2	124.1 ± 18.7	0.591	185.2 ± 27.1	0.050

Differential expression values and associated significance are shown for bone resorption-associated gene expression. Each gene is listed by common name, description and expression value (±SEM) as a percentage of respective control for the following treatment groups: 1-week alcohol group; and 4-week alcohol group. Statistical comparisons were performed by Wilcoxon–Mann–Whitney nonparametric analysis with the Benjamini and Hochberg false discovery rate multiple testing correction

[a] $P < 0.05$ via qRT-PCR validation