Estradiol Protects against Ethanol-Induced Bone Loss by Inhibiting Up-Regulation of Receptor Activator of Nuclear Factor-κB Ligand in Osteoblasts


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ABSTRACT

To investigate the effects of sex hormones on ethanol (EtOH)-induced bone loss, female Sprague-Dawley rats were fed control or EtOH-containing diets (12 g/kg/day) by intragastric infusion. After 3 weeks, rats receiving EtOH had significant decreases in tibial trabecular and total bone mineral density, induction of receptor activator of nuclear factor-κB ligand (RANKL) mRNA expression, and enhanced bone resorption, all of which were prevented by treatment with 17β-estradiol (E2). The addition of progesterone did not enhance the beneficial effect of E2 alone. Consistent with our in vivo findings, EtOH stimulated RANKL mRNA expression in cultured primary osteoblasts, and this expression was blocked by 4-methylpyrazole. Acetaldehyde also induced RANKL expression. Class 1 alcohol dehydrogenase was found to be expressed and EtOH-inducible in cultured osteoblasts, whereas CYP2E1 was undetectable. We found that EtOH induced phosphorylation of extracellular signal-regulated kinase (ERK) and signal transducers and activators of transcription 3 (STAT3). E2 and the mitogen-activated protein kinase inhibitor 2, 2'-amino-3'-methoxyflavone (PD98059) blocked ERK and STAT3 phosphorylation and blocked RANKL induction. Moreover, E2 completely blocked EtOH-induced osteoclastogenesis in a primary osteoblast and osteoclast precursor coculture system. The E2 effects were estrogen receptor-mediated. Therefore, E2 prevents EtOH-induced bone loss by opposing the induction of RANKL mRNA in osteoblasts and ethanol-induced osteoclastogenesis, through opposing effects on sustained ERK signaling.

Chronic ethanol (EtOH) abuse is well known to result in osteoporosis and increased fracture risk in men and women (Chakkalakal, 2005). Moreover, an inverse correlation between EtOH intake and bone mineral density has been reported in both pre- and postmenopausal women (Turner and Sibonga, 2001). The molecular mechanisms whereby EtOH consumption results in osteotoxicity are not well understood. Appropriate bone remodeling depends on a balance between the actions of osteoclasts, which can remove old bone, and osteoblasts, which are responsible for forming new bone (Manolagas, 2000). In the bone marrow, mesenchymal stem cells are capable of differentiating into multiple mature cell types, including osteoblasts (Zuk, 2001). As multifunctional cells, osteoblasts also control the differentiation of osteoclasts. Hematopoietic stem cells develop into osteoclasts in response to the presence of the receptor activator of NF-κB ligand (RANKL) expressed on the surface of osteoclasts, which binds to the protein RANK expressed on the surface of osteoclast precursors. This process is modulated by osteoprotegerin, an endogenous inhibitor of RANK-RANKL signaling (Quinn et al., 2000). After osteoclasts are differentiated, their activity is regulated by a variety of hormones, growth factors, and cytokines (Teitelbaum, 2000). Alcohol abuse may promote bone loss through both inhibition of osteoblastogenesis (Friday and Howard, 1991) and induction

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ABBRIVIATIONS: EtOH, ethanol; RANKL, receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor-κB; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; TEN, total enteral nutrition; UEC, urine ethanol concentration; P, progesterone; E2, 17β-estradiol; IC1, 182,780. Faslodex; pQCT, peripheral quantitative computer tomography; ELISA, enzyme-linked immunosorbent assay; 4-MP, 4-methylpyrazole; PD98059, 2'-amino-3'-methoxyflavone; TRAPase, tartrate-resistant acid phosphatase; RT-PCR, reverse transcription-polymerase chain reaction; OPG, osteoprotegerin; AR, androgen receptor; STAT, signal transducers and activators of transcription; ANOVA, analysis of variance; BMD, bone mineral density; P-, phosphorylated; T-, total; ADH, alcohol dehydrogenase.
of osteoclast differentiation and activation (Cheung et al., 1995). Dai et al. (2000) suggested that EtOH increases RANKL mRNA expression in bone marrow cells resulting in stimulation of osteoclastogenesis and bone resorption mediated via induction of IL-6 (Dai et al., 2000).

Disruption of sex steroid homeostasis, including a reduction in serum estradiol, is also a reported feature of young female alcoholics (Dorgan et al., 1994). Sex steroids have been reported to protect against bone loss, and it is possible that impaired sex steroid signaling contributes to EtOH-induced bone loss (Turner and Sibonga, 2001). Recent studies suggest that the action of estrogens on bone occurs via effects on both osteoblasts and osteoclasts (Kousteni et al., 2001; Chen et al., 2005). These functions of estrogens are largely mediated through the estrogen receptor (ER) isoforms, ERα and ERβ, and estrogen effects may differ in early and late stages of osteoblast differentiation (Monroe et al., 2003). The inhibitory effect of estrogens on bone resorption has also been suggested to involve regulation of the RANKL-RANK-osteoprotegerin system (Syed and Khosla, 2005). Osteoblasts constitutively express RANKL mRNA, and estrogen has been reported to suppress RANKL expression in human osteoblasts (Bord et al., 2003).

We have previously shown that EtOH-induced bone loss in female rats differs with physiological status (Shankar et al., 2006). In cycling females, EtOH-enhanced bone resorption was associated with increased osteoclast numbers, induction of RANKL in bone marrow and significantly reduced plasma 17β-estradiol (E2) concentrations. In contrast, in pregnant females where plasma E2 was elevated and unaffected by EtOH consumption, EtOH-induced bone loss was lower and was associated with impaired osteoblastogenesis. To test the hypothesis that E2 can prevent EtOH-induced bone resorption by opposing the effects of EtOH on RANKL expression, we have examined the effects of sex steroid supplementation on EtOH-induced bone loss in female rats in vivo, and we have investigated the direct effects of EtOH and E2 on RANKL expression and osteoclastogenesis in vitro, in differentiated primary osteoblasts, and in an osteoblast and osteoclast precursor coculture. We also performed studies to investigate the role of EtOH metabolism and the ERK signaling pathway on RANKL gene expression.

### Materials and Methods

#### Animal Experiments.

Cycling female 250- to 300-g Sprague-Dawley rats (n = 6/group) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences (Little Rock, AR). Rats were surgically implanted with an intragastric cannula as described previously (Ronis et al., 1991; Badger et al., 1993a), and rats were fed by total enteral nutrition (TEN). Liquid diets were formulated to contain the nutrients recommended for rats by the National Research Council. Urine EtOH concentrations (UECs) were monitored daily using a GL5 analyzer fitted with an amperometric oxygen electrode sensor (Analox Instruments Ltd., London, UK) throughout the period of infusion. E2 and progesterone (P) doses were calculated to produce plasma concentrations similar to those observed in pregnant rats (Garland et al., 1987). The TEN animal model has been described previously (Shankar et al., 2006). In brief, diets contained 16% protein, 54% carbohydrate, and 25% fat (corn oil), and EtOH-containing diets were kept isocaloric to the control diets by substituting EtOH for carbohydrate calories. The EtOH dose was 12 g/kg/day. Rats were infused 187 kcal/kg/day for 14 h from 6:00 PM to 8:00 AM during the dark cycle for 3 weeks. Additional groups of control and EtOH-infused animals were supplemented with s.c. E2 (20 µg/kg/day) administered using Alzet osmotic minipumps or E2 + P, suspended in corn oil, given by s.c. injections daily (E2, 20 µg/kg/day, and P, 20 mg/kg/day).

#### Peripheral Quantitative Computerized Tomography.

Tibial bone mineral density was monitored in vivo using peripheral quantitative computer tomography (pQCT) (XCT Research SA, Norland Medical Systems, Fort Atkinson, WI) with software version 5.4 using thresholds of 570 mg/cm³ to distinguish cortical bone and 214 mg/cm³ to distinguish trabecular from cortical and subcortical bone. An initial scan was conducted at the start of the study followed by a scan 3 weeks later. Additional pQCT analyses on tibia were carried out ex vivo following sacrifice.

#### Serum E2, P, and Bone Markers.

Radioimmunoassays were performed for serum E2 and P using kits according to the manufacturer’s recommendation (DiGregorio et al., 2001). Cells were seeded at a density of 3 × 10⁵ cells per well in six-well cell culture plates in the presence of minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 4 mM l-glutamine, and 100 U/ml each of penicillin and streptomycin (Sigma-Aldrich), conditions known to drive osteoblast differentiation. After 2 days, one-half of nonadherent cells in each well was collected and frozen in liquid nitrogen. The nonadherent cell fraction, considered to contain osteoclast precursors (Chen et al., 2005), was used in later osteoclast differentiation cocultures (described below). Adherent cells were cultured further, and one-half of the medium was replaced every 5 days. After 20 to 25 days, osteoblasts were fully differentiated (DiGregorio et al., 2001). Fetal bovine serum in the cell culture medium was then reduced to 2%, and the differentiated primary osteoblasts were treated with EtOH and/or acetaldehyde and E2 for 12 to 72 h in the presence or absence of the ER inhibitor ICI 182,780 (Faslodex), the ADH class I inhibitor 4-methylpyrazole (4-MP), or the ERK phosphorylation inhibitor PD98059. Plates were sealed with Parafilm and tape to prevent evaporation of EtOH from the wells. The concentration of EtOH in culture medium was assayed according to the methods described above.

#### In Vitro Osteoclastogenesis in Osteoblast and Osteoclast Precursor Cocultures.

To study osteoclast formation in vitro, frozen nonadherent cells prepared as described above containing osteoclast precursors were added into each well of differentiated primary osteoblasts with density of 1 × 10⁵ cells per well in the presence of 20 nM 1α,25-dihydroxyvitamin D₃ (Sigma-Aldrich), and TRAP enzyme assay kit (Rat LAPS) from Nordic Biosciences Diagnostic.

#### Ex Vivo Osteoblast Cell Cultures.

Bone marrow cells were harvested from untreated femurs of 250- to 300-g cycling female rats according to methods described previously (DiGregorio et al., 2001). Serum osteocalcin, a marker of bone formation, was detected using an ELISA assay (Rat-MID osteocalcin ELISA; Nordic Biosciences Diagnostic, Herlev, Denmark). Serum procollagen cross-links, a marker of bone resorption, were detected using an ELISA assay kit (Rat LAPS) from Nordic Biosciences Diagnostic.
culture plate wells, and the final ethanol/dimethyl sulfoxide concentration caused by addition of reagents was less than 0.01%.

Reverse Transcription-Polymerase Chain Reaction and Real-Time RT-PCR. Tibial bone marrow RNA and osteoblast RNA were extracted using TRI Reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's recommendation followed by DNase digestion and column cleanup using QIAGEN mini columns. Reverse transcription was carried out using an iScript kit from Bio-Rad (Hercules, CA). Real-time RT-PCR was carried out using SYBR Green and an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers for rat RANKL, osteoprotegerin (OPG), osteocalcin, ERα and ERβ, and the androgen receptor (AR) were designed using Primer Express software 2.0.0 (Applied Biosystems), and all primer sequences used in this study are listed in Table 1. To check ADH1, CYP2E1, and sex-steroid receptor expression in the primary osteoblast cultures, RNA was taken from those cells, and cDNA was synthesized using the kit described above. RT-PCR was performed under the following conditions to amplify the ER and androgen receptor genes: 58°C annealing temperature and 35 cycles.

Western Blotting. Cellular proteins were extracted using a cell lysis buffer as described previously (Chen et al., 2005). Phosphorylation of ERK1/2 and STAT3 in osteoblasts was assessed by Western immunoblotting using phosphospecific anti-mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to ERK1/2 and STAT3. Anti-rabbit polyclonal antibodies recognizing total ERK2 and total STAT3 (Santa Cruz Biotechnology, Inc.) were used to normalize the quantitation of phospho-ERK1/2 and phospho-STAT3 expression.

Data and Statistical Analyses. Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Student Newman-Keuls post hoc analysis were used for multiple pairwise comparisons of the groups except for analysis of serum sex steroids and bone turnover makers. The latter data were skewed from a normal distribution and were analyzed by Kruskal-Wallis nonparametric comparison of treatment pairs using SPSS (SPSS Inc., Chicago, IL). Values were considered statistically significant at P < 0.05.

Results

Effects of Gonadal Steroid Supplementation on EtOH-Induced Bone Loss in Vivo. Experimental animals were fed by diets with or without EtOH, and they were administered E2 or P for 3 weeks. Mean UECs in EtOH-treated groups ranged from 121.8 to 256.1 mg/dl. UECs reflect serum EtOH concentrations (Badger et al., 1993b), and they are similar to those observed in human alcoholics (Wadstein and Skude, 1979). Mean trabecular bone mineral density (BMD) and total BMD (Fig. 1) were decreased by EtOH (P < 0.05). The decreased BMD resulting from EtOH treatment was normalized by E2 treatment (P < 0.05). P had no additional significant effects relative to E2 alone on restoring EtOH-induced bone loss. E2 treatment produced serum concentrations similar to those observed in pregnant rats (Table 2). C, ex vivo total BMD (slice 2); D, ex vivo trabecular BMD (slice 2). White bars represent the control groups, and black bars represent the EtOH groups. EtOH induced bone loss. E2 treatment produced serum concentrations similar to those observed in pregnant rats (Garland et al., 1987), and no effects were observed on serum P. E2 + P treatment increased serum P concentrations of cycling rats to a level similar to that in pregnant rats (Table 2). EtOH treatment increased serum P concentrations compared with controls and even more so following P treatment (P < 0.05), but no significant effects were observed on serum E2.
The bone resorption marker RatLAPS was altered by EtOH and sex steroid treatments \((P < 0.05; \text{Table 2})\). RatLAPS was higher in the EtOH-treated group compared with E2 treated groups treated with E2 or E2 \(+ P\) \((P < 0.05)\). EtOH and E2 reduced serum concentrations of the bone formation marker osteocalcin \((P = 0.07\) and \(P < 0.05\), respectively). Supplementation with E2 or E2 \(+ P\) was unable to reverse the EtOH-associated reduction in the serum levels of osteocalcin.

**EtOH and Sex Steroid Effects on RANKL mRNA Expression in Bone Marrow in Vivo.** RANKL is located on the surface of osteoblasts and is a critical signaling molecule that promotes and supports osteoclast differentiation and activation. RANKL mRNA levels in bone marrow were greater in the EtOH-treated group than the control group \((P < 0.05)\), and this was completely blocked by in vivo supplementation with either E2 alone or E2 \(+ P\) \((P < 0.05)\) (Fig. 2A). Osteoprotegerin is an endogenous inhibitor of RANKL signaling which modulates the bone resorption process. Osteoprotegerin mRNA levels were not altered by EtOH, E2, or E2 \(+ P\) (Fig. 2B). Osteocalcin mRNA levels were significantly decreased in EtOH-treated groups, but E2 or E2 \(+ P\) did not reverse these effects (Fig. 2C).

**EtOH Induces RANKL mRNA Expression in Differentiated Primary Osteoblasts in Vitro.** We performed in vitro studies to establish a cellular mechanism for our in vivo findings. Bone marrow cells were taken from the femur of untreated cycling 250- to 300-g Sprague-Dawley female rats. Differentiated osteoblasts were generated after 20 days in culture according to previous methods (DiGregorio et al., 2001). Primary osteoblasts treated with 25 or 50 mM EtOH for different times had a dose-responsive increase in RANKL mRNA expression as measured by real-time RT-PCR \((P < 0.05)\) (Fig. 3A). RANKL mRNA expression was highest at 12 to 24 h following the beginning of treatment and declined thereafter \((P < 0.05)\). RT-PCR analysis revealed that mRNA encoding ADH1, the main EtOH-metabolizing enzyme, was detected in osteoblasts and quantitation by real-time RT-PCR confirmed that this mRNA was inducible by EtOH \((P < 0.05)\) (Fig. 3B). The decline in RANKL mRNA expression followed induction of ADH1 and the disappearance of EtOH in the culture medium as a result of metabolism (Fig. 3C). An EtOH concentration of 50 mM in vitro is equal to 230 mg/dl, and it was similar to that attained in our study in vivo. RT-PCR analysis demonstrated that the other important enzyme involved in EtOH oxidation, CYP2E1, was not detectable in either EtOH-treated or EtOH-nontreated differentiated primary osteoblasts even after 30 cycles of amplification (Fig. 3D).

To test whether EtOH had direct effects on induction of RANKL expression in osteoblasts or whether it needed to be metabolized to exert its action, we pretreated the cells with the ADH1 inhibitor 4-MP and measured the RANKL mRNA expression 24 h after exposure to EtOH (Fig. 4A). We found that 4-MP at concentration of 100 \(\mu\)M was able to completely block the EtOH-induced RANKL mRNA expression in the osteoblast culture system \((P < 0.05)\). These data indicated that the induction of RANKL mRNA expression requires EtOH metabolism in the osteoblast and is consistent with our additional finding that the EtOH metabolite acetaldehyde was also able to induce RANKL mRNA expression \((P > 0.05)\) in the primary osteoblast culture system at concentrations attainable in tissues following EtOH consumption in vivo (Eriksson, 2001) (Fig. 4B).

**Sustained ERK Signaling Is Required for EtOH-Mediated Induction of RANKL mRNA Expression in Osteoblasts.** Mature osteoblasts were treated with 50 mM EtOH, and cell lysates were collected at different time points as indicated in Fig. 5. Western blotting was conducted to...
determine the pattern of phosphorylation of ERK and its downstream target STAT3. The latter is known to be required for induction of RANKL in osteoblasts (O’Brien et al., 1999). We observed an increase in ERK phosphorylation following EtOH treatment for 12 h, and this was sustained through 48 h of treatment (Fig. 5). Phosphorylation of STAT3 mirrored ERK activation at each time point (Fig. 5). PD98059, a mitogen-activated protein kinase inhibitor, inhibited the increase in ERK phosphorylation produced by EtOH treatment (Fig. 6A) and completely reversed the EtOH-induced increase in RANKL mRNA expression in response to EtOH (P < 0.05) (Fig. 6B).

**Fig. 3.** RANKL and ADH1 mRNA expression in mature primary osteoblasts. A, RANKL mRNA concentrations in response to different doses of EtOH (25 and 50 mM) and different times (0–72 h) determined by real-time RT-PCR. B, ADH1 mRNA concentrations in response to different doses of EtOH (25 and 50 mM) and different times (0–72 h) determined by real-time RT-PCR. C, EtOH concentrations in the cell culture medium from six-well cell culture plates 0 to 96 h after cells were treated with 50 mM EtOH. D, RT-PCR analysis of ADH1, CYP2E1, and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in rat liver and in differentiated osteoblasts treated with or without 50 mM EtOH for 48 h in vitro. Data are expressed as mean ± S.E.M. for triplicate assays and individual gene expression in real-time RT-PCR analysis was normalized to the expression level of 18S. ∗, P < 0.05 versus control.

**Fig. 4.** A, inhibition of EtOH-induced RANKL mRNA expression in osteoblasts by 4-MP. Osteoblasts were pretreated with 100 μM 4-MP for 30 min and then treated with or without 50 mM EtOH for 24 h. B, induction of RANKL mRNA in osteoblasts by acetaldehyde. Osteoblasts were treated with 0.1 to 2.5 mM acetaldehyde for 24 h. RANKL mRNA expression was measured by real-time RT-PCR. Data are expressed as mean ± S.E.M. for triplicate assays and RANKL mRNA expression was normalized to the expression level of 18S. ∗, P < 0.05 versus untreated control cells.

**Fig. 5.** Estrogen Receptors Mediate the Effects of E2 on RANKL mRNA Expression. In the presence of E2, both ERK and STAT3 phosphorylation induced by EtOH were attenuated (Fig. 5). In addition, E2 treatment of differentiated osteoblasts in vitro suppressed RANKL mRNA expression at doses of 0.1 and 1 nM (P < 0.05) and inhibited EtOH-induced RANKL mRNA expression at doses of 1 and 10 nM (P < 0.05) (Fig. 7A). RT-PCR analysis demonstrated the presence of mRNA for ERα and ERβ and the AR in differentiated primary osteoblasts (Fig. 7B). The estrogen receptor antagonist ICI 182,780 blocked the suppressive effects of E2 on RANKL mRNA expression, and ICI 182,780 also completely reversed the inhibitory effects of E2 on EtOH-induced RANKL mRNA expression (P < 0.05) (Fig. 7C).

**E2 Inhibits EtOH-Induced Osteoclast Differentiation In Vitro.** In osteoblast and osteoclast precursor coculture studies, previously collected nonadherent bone marrow cells containing osteoclast precursors were added to fully differentiated osteoblasts at the start of EtOH treatment. EtOH or acetaldehyde treatment significantly increased differentiated osteoclast numbers (Fig. 8, C and E). E2 (1 nM) reduced osteoclast numbers by itself (P < 0.05) (Fig. 8, B and G), and it completely blocked the osteoclastogenic effects of EtOH or acetaldehyde (P < 0.05) (Fig. 8, D, F, and G).

**Discussion**

It has been known for many years that EtOH abuse can cause a variety of harmful effects on hematopoiesis (Heermans, 1998; Prakash et al., 2001). Toxic effects of EtOH in
the bone marrow result in bone loss and therefore lead to increased risks of osteoporosis and bone fracture (Sampson, 1998; Prakash et al., 2001; Chakkalakal, 2005). Chronic EtOH intake that produces serum EtOH concentrations found in alcoholics (Wadstein and Skude, 1979) will cause bone loss as a result of both inhibited osteoblastogenesis and stimulated osteoclastogenesis and increased bone resorption. The mechanistic basis of EtOH effects on either osteoblastogenesis or osteoclastogenesis has not yet been detailed. In contrast, that estrogens can prevent bone loss resulting from sex-steroid deficiency has been broadly accepted, and recently the molecular mechanism through which this occurs has been characterized in some aspects (Riggs et al., 2002). Consistent with our earlier in vivo studies in cycling and pregnant female rats (Shankar et al., 2006), the data presented here suggest that the mechanism of EtOH-induced bone loss depends on endocrine status. Because pregnancy increases circulating sex steroid concentrations, estrogens were suspected to be the deciding factor in determining the mechanistic difference of EtOH-induced bone loss in these two groups of animals (Shankar et al., 2006). In the current study, we have shown for the first time that EtOH effects on bone in E2-supplemented cycling female rats were similar to that observed in pregnant rats (Garland et al., 1987). Analyses of serum markers for bone turnover were consistent with our previous observations (Shankar et al., 2006). E2 seemed to significantly suppress EtOH-induced bone resorption, and molecular marker studies from bone marrow suggested that it did so by blocking the EtOH-induced expression of RANKL in osteoblasts. It seems that the protective effects of E2 on EtOH-induced loss of bone density are mainly due to its inhibitory effects on osteoclastogenesis or osteoclast activity because BMD was restored by E2 treatment even though the bone formation marker osteocalcin remained suppressed. The effects of E2 and EtOH on osteocalcin were complex. Although no further decrease on osteocalcin mRNA was observed, plasma concentrations were lower following combined treatment. This may reflect post-transcriptional effects or effects on osteocalcin secretion. The increase of serum P concentrations with the combination of E2 and EtOH-treated rats may be due to impaired P metabolism or clearance (Sugano et al., 1995; Hidestrand et al., 2005).

Experiments using an in vitro cell culture model of differentiated primary osteoblasts and osteoblast and osteoclast precursor cocultures further characterized the cellular and molecular mechanisms underlying the opposing effects of EtOH and E2 on bone. We have demonstrated for the first time that E2 acting through the estrogen receptor can inhibit the induction of the osteoclast differentiation factor RANKL and directly interfere with EtOH-induced osteoclastogenesis. However, the signal transduction mechanisms underlying
In contrast, no expression of CYP2E1 mRNA was detected in differentiated osteoblasts. The ADH class I inhibitor 4-MP was able to abolish EtOH-induced RANKL expression in osteoblasts, whereas the primary EtOH metabolite acetaldehyde was also able to stimulate RANKL mRNA expression. These data suggest that EtOH metabolism to acetaldehyde in osteoblasts is required to produce increases in RANKL expression.

Our present study also showed that EtOH-induced RANKL mRNA expression is downstream of the phosphorylation of ERK and STAT3. These data further suggest that EtOH has its effects on bone remodeling as a result of sustained activation of protein kinases. It is becoming clear that mitogen-activated protein kinase molecules, including ERK, are involved in the signal transduction of a variety of cellular responses, including proliferation, differentiation, survival, and execution of inflammatory responses (Cross et al., 2000; Aroor and Shukla, 2004). In agreement with our observation of sustained increases in ERK phosphorylation in osteoblasts, EtOH has been reported to cause gradual and sustained activation of ERK in both primary rat hepatocytes and liver in vivo and this prolonged activation of ERK was shown to play a prominent role in cell cycle arrest (Cross et al., 2000). It is well known that E₂ can acutely activate ERK in osteocytes in vitro (Chen et al., 2005). However, this is a rapid and transient effect occurring over a time scale of minutes followed by a return to base values after 2 h. We present data in the current study that chronic in vitro E₂ treatment attenuates EtOH-induced ERK phosphorylation over a period of 12 to 48 h. This suggests that ERK may play an important role in modulation of both EtOH and E₂ signaling in osteoblasts. Sustained ERK activation in response to EtOH exposure in vivo activates nuclear transcription factors and alters gene expression within the liver, leading to the development of steatosis and inflammation in the early stages of EtOH-induced liver injury (Nagy, 2004). We speculate that some actions of EtOH in bone marrow may be analogous to the effects of EtOH in the liver.

We hypothesize that signaling convergence of EtOH and E₂ on regulation of RANKL mRNA expression in osteoblasts affects osteoclast differentiation perhaps as a result of opposing actions on the ERK signaling pathway. However, E₂ not only suppresses RANKL expression in osteoblasts (Bord et al., 2003) but also down-regulates osteoclastogenesis by decreasing the responsiveness of osteoclast precursors to RANKL (Srivastava et al., 2001). Another possibility to account for the EtOH and E₂ interaction observed in osteoblasts at the cellular level is that they may both act on the oxidative stress signaling in bone cells. In alcohol dehydrogenase metabolism of EtOH, the major byproduct is acetaldehyde, which causes oxygen radical generation and lipid peroxidation (Lieber, 1993; Eriksson, 2001). Reactive oxygen species are able to induce RANKL expression in mouse osteoblasts, and the induction of RANKL by reactive oxygen species may be via ERK (Bai et al., 2005). Conversely, estrogens have been described to have antioxidant effects on bone cells (Lean et al., 2003). Therefore, investigation of the interaction of estrogens and EtOH on RANKL expression and ERK signaling in bone osteoblasts in the presence and absence of antioxidants is a priority for future research.

In conclusion, the observations reported in this article demonstrate that the female sex hormone E₂ prevents EtOH-
induced bone loss by opposing the induction of RANKL mRNA in osteoblasts and therefore interferes with osteoclastogenesis. This strongly suggests the existence of negative cross-talk between EtOH and estrogen signaling pathways in bone cells consistent with our previous data showing protection from EtOH-induced bone loss in pregnant relative to cycling rats (Shankar et al., 2006). To reach our ultimate goal of understanding the mechanism of EtOH-induced bone loss and how sex hormones prevent this loss, further studies on signaling pathways involving those two variables are needed. We present data here that support our hypothesis that estrogens can antagonize the toxic effects of EtOH on bone, and we have begun to characterize the cellular and molecular mechanisms by which these effects are mediated.

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References


Fig. 8. E2 inhibition of EtOH- and acetaldehyde-induced osteoclastogenesis in osteoblast/osteoclast precursor cocultures in vitro. Multinuclear osteoclasts were identified by TRAPase staining. A, control well. B, 1 nM E2-treated well. C, 50 nM EtOH-treated well. D, cells from a well treated with combination of E2 and EtOH. E, 50 nM acetaldehyde-treated well. F, E2 plus acetaldehyde-treated well. G, TRAPase-positive osteoclast-like cells counted in each well by microscopy. All pictures were taken under microscopy with 10× magnification. Data are presented as mean ± S.D. for triplicate assays. Means with different letters differ significantly from each other at p < 0.05. a < b < c < d as determined by one-way ANOVA followed by Student-Newman-Keuls posthoc analysis for multiple pairwise comparisons.


