Ethanol Impairs Estrogen Receptor Signaling Resulting in Accelerated Activation of Senescence Pathways, Whereas Estradiol Attenuates the Effects of Ethanol in Osteoblasts

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ABSTRACT: Epidemiological and animal studies have suggested that chronic alcohol consumption is a major risk factor for osteoporosis. Using bone from cycling female rats infused chronically with ethanol (EtOH) in vivo and osteoblastic cells in vitro, we found that EtOH significantly increased estrogen receptor α (ERα) and β (ERβ) mRNA and ERα protein levels. Treatment with 17β-estradiol (E2) in vivo and in vitro interfered with these effects of EtOH on bone and osteoblastic cells. ERα agonist propylpyrazoletriol (PPT) and ERβ agonist diarylpropionitrile (DPN) attenuated EtOH-induced ERα and ERβ gene overexpression, respectively. Similar to the ER antagonist ICI 182780, EtOH blocked nuclear translocation of ERα-ECFP in the presence of E2 in UMR-106 osteoblastic cells. EtOH also downregulated ERE-luc reporter activity. On the other hand, EtOH by itself upregulated some common ERα- and ERβ-mediated genes apparently by an ER-independent pathway. EtOH also transactivated the luciferase activity of the p21 promoter region independent of additional exogenous ERs, activated p21 and p53, and stimulated senescence-associated β-galactosidase activity in rat stromal osteoblasts. E2 treatment attenuated these EtOH actions. We conclude that inhibitory cross-talk between EtOH and E2 in osteoblasts on ERs, p53/p21, and cell senescence provides a pathophysiologic mechanism underlying bone loss and the protective effects of estrogens in alcohol-exposed females.

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Key words: alcohol, estrogen receptor, osteoblast, senescence

INTRODUCTION

CHRONIC ALCOHOL ABUSE has long been recognized as a major risk factor for development of osteoporosis.(1–3) Recent evidence showed binge alcohol exposure can cause bone loss as well.(4) Although the exact mechanism by which ethanol (EtOH) influences bone physiology is not clear, it is likely to involve a combination of both direct and indirect effects on bone cells.(5) EtOH can diffuse into osteoblasts, where alcohol dehydrogenase class 1 (ADH1) is expressed, and be metabolized to acetaldehyde and give rise to reactive oxygen species (ROS) that can exert biological effects.(6) This is considered a direct effect of EtOH. On the other hand, chronic alcohol abuse has been shown to have inflammatory effects in bone marrow, which is known to introduce additional factors that are believed detrimental to the skeleton.(3,7,8) These factors, which include interleukin-1β (IL-1), TNFα, and IL-6, are all secreted by mononuclear cells. (9) EtOH increases RANKL mRNA expression in bone marrow cells, resulting in stimulation of osteoclastogenesis and bone resorption, and this effect was mediated through induction of IL-6.(10) Alcohol consumption also suppresses plasma estrogens in cycling females,(11) suggesting an association between EtOH and reduced estrogen signaling in bone.

Estrogen receptors α (ERα) and β (ERβ) mediate estrogen action in a variety of tissues and cells. Whereas these receptors have overlapping responsibilities, they also have distinct roles in different tissue and cell types.(12) In human osteoblasts where both estrogen receptors are present, genes regulated by estrogen through ERα and/or ERβ show commonalities, but also significant differences.(13) Chronic EtOH ingestion in humans and rats induce an increase in ER expression in liver and breast.(14,15) EtOH-induced increases in mammary ER expression may be associated with an increased risk of breast cancer. However, the significance of EtOH-induced overexpression of ER in other tissues and the consequences for tissue pathology has not been studied. Bone is also recognized as an estrogen sensitive organ, and altered ER signaling in bone cells are known to affect bone quality. Although we have previously predicted that EtOH might alter ER signaling in

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bone cells. Overexpression of ERs in osteoblasts has been linked to activation of p53, p53 is a well-known tumor suppressor gene that maintains the integrity of the genome by its ability to regulate various aspects of cell growth and apoptosis. p53 is also believed to represent a key component of a signal transduction pathway that is stimulated by increased intracellular ROS to trigger apoptosis, and p53 activation also results in ROS generation in the mitochondria. Studies analyzing the role of p53 in different cell types have shown a tissue-specific role for p53 in differentiation of several tissue types. p53 has been shown to regulate osteoblast differentiation, bone formation, and osteoblast-dependent osteoclast differentiation. Activation of p53 in bone tissue has been observed both in aged animals and in sex steroid–deficient gonadectomized mice. Activated p53 has been suggested to be a link to the senescence pathway in bone cells. Moreover, EtOH has recently been reported to induce p53. p21 functions as a downstream effector of p53, and previous studies have implicated p21 involvement in ER signaling. These findings suggest that impaired estrogen signaling in bone cells after EtOH exposure could accelerate senescence.

In the studies presented in this report, we attempted to understand the effect of EtOH treatment in vivo and in vitro on estrogen receptors and ER signaling compared with classic ER ligands, such as 17β-estradiol (E2) and ICI in osteoblasts. We also tried to determine whether EtOH activates p53 and p21 and its promoter and if this led to accelerated senescence in osteoblasts. We present a new paradigm whereby EtOH induces bone loss by impairing ER signaling and the interaction between ER and p21 to determine the fate of osteoblasts. These data also provide a further explanation for why endogenous or exogenous estrogens antagonize the toxic effects of EtOH on bone.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (250–260 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). 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 Science (Little Rock, AR, USA). Rats were surgically implanted with an intragastric cannula as described previously, 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. The TEN animal model has been detailed previously. 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/d. Rats (n = 8/group) were infused 187 kcal/kg3/4/d for 14 h from 6:00 p.m. to 8:00 a.m. during the dark cycle for 4 wk. Additional groups of control and EtOH-infused animals were supplemented with subcutaneous E2 (20 μg/kg/d; Sigma-Aldrich, St Louis, MO, USA) administered using Alzet osmotic minipumps. All rats were weighed every other day, and we found that there are no significant differences among all four groups in body weight in the end of experiment: control, 278.1 ± 1.8 g; EtOH, 273.8 ± 2.1 g; E2, 278.1 ± 2.3 g; E2 + EtOH, 280.2 ± 3.7 g.

Cell culture

Control cycling female rat bone marrow cells were harvested from femurs according to methods described previously. To have stromal osteoblasts for treatment, bone marrow cells were seeded at a density of 3 × 106 cells/well in six-well cell culture plates in the presence of MEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Hyclone Laboratories, Logan, UT, USA) and 1 mM of ascorbyl-2-phosphate (Sigma-Aldrich), 4 mM L-glutamine, and 100 U/ml of each penicillin and streptomycin (Sigma-Aldrich), conditions known to drive osteoblast differentiation. One half the cell culture medium was changed every 5 days, and after 20 days, mature osteoblasts were developed for treatment. Neonatal rat calvarial osteoblastic cells were isolated from untreated 4-day-old rat pups by sequential collagenase digestion using a method described previously. Rat calvarial osteoblastic cells and the rat osteoblast-like cell line UMR-106 (ATCC, Rockville, MD, USA) were cultured in αMEM supplemented with 10% FBS. When cells were ready to be treated, culture medium was saturated with O2 and CO2 in an incubator for 2 h and plates were sealed during EtOH treatment; these treatment procedures were detailed previously.

Real-time RT-PCR analysis

Rat tibial bone RNA and osteoblastic cell RNA were extracted using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer’s recommendation followed by DNase digestion and column clean-up using Qiagen minicolumns. Briefly, at the time of death, the right tibia was taken, and bone marrow cells were flushed with Eagle’s MEM + Hanks’ salts after cleaning the surrounding connective tissue. Tibial bones were frozen in liquid nitrogen. Tibial bone was placed in 1000 μL TRI Reagent and homogenized using a polytron-aggregate (Kinematica). One hundred microliters of 1-bromo-3-chloropropane (BCP) was added, and the mixture was centrifuged for 15 min at speed of 16,000 rpm and 4°C. Four hundred fifty microliters supernatant was taken, and an equal volume of isopropanol was added and centrifuged for additional 15 min (16,000 rpm, 4°C). After washing the RNA pellet with 75% ethanol, isolated RNA was resuspended in RNase-free water. Treated cells from 6-well plates were washed with twice with PBS, and 1000 μL TRI Reagent was added into each well. Cells were scraped into a 1.5-mL Eppendorf tube. RNA preparation was identical to that of isolation of RNA from bone tissue. Reverse
transcription was carried out using an iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA). Real-time RT-PCR was carried out using SYBR Green and an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers for rat ERα and ERβ, PTH-like hormone (PTHLH), bone morphogenetic protein 6 (BMP6), phosphodiesterase 3B (PDE3B), Bel2-associated transcription factor (BTF), Autotaxin, and stromal antigen 2 (STAG2) were designed using Primer Express software 2.0.0 (Applied Biosystems), and all primer sequences used in this study are listed in Table 1.

### Western blotting

Tibia bone tissue proteins and in vitro cellular proteins were extracted using a cell lysis buffer as described previously. Phosphorylation of p53 and total p53 in bone tissue and in vitro osteoblasts was assessed by Western immunoblotting using goat polyclonal antibody recognizing phosphorylated p53 (Santa Cruz Biotechnology) and rabbit polyclonal antibody recognizing total p53 (Cell Signaling), followed by incubation with either an anti-goat or an anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz). The status of p21, ERα, and GAPDH protein in bone tissue and in vitro osteoblasts were analyzed by immunoblotting, using rabbit polyclonal antibodies recognizing p21 and ERα (Santa Cruz) and a mouse monoclonal antibody recognizing GAPDH (Santa Cruz), followed by incubation with either an anti-rabbit or an anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz) and SuperSignal West Pico chemiluminescent substrate (Pierce). Quantification of the intensity of the bands in the autoradiograms was performed using a VersaDoc imaging system (Bio-Rad).

### DNA constructs and luciferase activity assays

ERα transciptional activity was determined by measuring the EtOH- and estradiol-stimulated, ERα-mediated activation of the estrogen-responsive reporter plasmid ERE-TK-Luc. The ERE-TK-Luc reporter and wildtype ERα plasmid used in this report were published previously. Human p21 promoter pGL2-p21-Luc, containing the p21 promoter-luciferase reporter fusion and two p53-binding consensus sites, was kindly provided by Dr Lieberman. ERE-TK-Luc and pGL2-p21-Luc plasmids were transfected into UMR-106 cells with or without ERα co-transfection using Lipofectamine 2000 (Invitrogen). Transfected cells were treated with E2, ICI 182780 (Faslodex) and EtOH for 24 h, and lysates were harvested for luciferase assays. The experiment was performed according to protocols from the manufacturer (Promega). Luciferase activity was measured on a MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA, USA).

#### Transient transfection and subcellular localization of ERα

Using 24-well plates, UMR-106 cells were transiently transfected using Lipofectamine 2000 (Invitrogen), with full-length wildtype ERα inserted into the appropriate ECFP vector (Clontech), along with red fluorescent protein (pDs 1Red-N1; Clontech, Palo Alto, CA, USA) targeted to the nucleus (nRFP). These two constructs were detailed previously. Transfected cells were cultured for 24 h. Subsequently, cells were serum-starved by culturing in the presence of 2% BSA for 4 h and treated with vehicle, 50 mM EtOH, 10⁻⁹ M E2, and 10⁻⁹ M ICI 182780 for 2 h. The cells showing either nuclear or cytoplasmic accumulation of ERα were directly visualized using a fluorescence microscopy with connection to a camera.

#### Senescence-associated β-galactosidase activity and staining

Fully differentiated rat bone marrow stromal osteoblasts in 6- and 24-well plates were treated with E2 for 30 min before adding 50 mM EtOH, and plates were well sealed for 24 and 48 h. The senescence-associated β-galactosidase activity assay, performed using the β-galactosidase enzyme assay kit (Promega), measured the absorbance at 420 nm according to the manufacturer’s instruction. Cell β-galactosidase staining was also performed according to a method published previously. Senescent cells were identified as blue-stained cells by standard light microscopy.

#### Statistical analyses

Data are expressed as means ± SD. One-way and two-way ANOVA followed by Student-Newman-Keuls post-hoc analysis was used to compare the treatment groups. Values were considered statistically significant at p < 0.05.

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### Table 1. Real-Time RT-PCR Primer Sequences

| Gene     | Forward primer | Reverse primer |
|----------|----------------|----------------|
| ERα      | TCTGACAATCGACGCCAGAAT | ACAGCACAGTACGGCTCTTCCT |
| ERβ      | TGTGCCAGCCCTGTTACTGTC | CATGACCAAACGCCGTAATG |
| PTHLH    | GACAAGGGGCAATCCATCCA | GACACCTCCGAGGCTCTGGA |
| BMP6     | TGGATCATGACGCCACAAAAG | GACACCTCCGAGGCTCTGGA |
| PDE3B    | GATTTCCTGGGACTGGGACCTG | TGCGAGCTTATTTAGCACTGTA |
| BTF      | ACCAATTATCGAATGCTGACA | GACGCTTCAAGCTAGCGAGT |
| Autotaxin| TCGTGGATTATGGCCCAACCTT | GGGCTTCAAGCTAGCGAGT |
| STAG2    | TCGAGAAAAGGTGACCAATTG | TGACCGAATAGGCAATCC |
| IIS      | CCTGTAATATTGAATGCTCCACTTT | ATACGCTATTGGAGCTGGAATTACC |
| GAPDH    | TGAGGTGACCGCATCTCTTT | TGGTAACCCAGGCCTCCGATA |

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RESULTS

EtOH stimulates estrogen receptor overexpression and activation of p53 and p21 in bone tissue

Using pQCT, we found that, in the EtOH-infused group, trabecular BMD was significantly lower compared with the control total enteral nutrition group (control: 234.6 ± 11 versus EtOH: 182.7 ± 8.2 mg/cm³, n = 7; p < 0.05). E2 treatment (E2: 300.0 ± 11.2 mg/cm³, n = 7) increased trabecular BMD (p < 0.05 versus control) and reversed the effect of EtOH on tibial trabecular BMD (E2 + EtOH: 284.8 ± 8.3 mg/cm³, n = 7; p < 0.05 versus EtOH). These results were similar to those we have previously published. Using RNA extracted from tibial bone, real-time PCR was carried out for estrogen receptor subtypes α and β (ERα, ERβ). EtOH and E2 treatment both upregulated ERα and ERβ gene expression (p < 0.05), although EtOH had a larger effect (Fig. 1A). Interestingly, in the E2 + EtOH group, E2 attenuated EtOH effects on both ERα and ERβ gene expression. Similarly, this EtOH and E2 interaction was observed when ERα protein levels were evaluated using Western blot (Fig. 1B). Because previous evidence indicated that EtOH can increase p53 expression in heart tissue, we measured p53 and its phosphorylation status along with p21 levels. Both total and phosphorylated p53 were increased in the EtOH group, and E2 attenuated EtOH effects on both ERα and ERβ gene expression. Similarly, this EtOH and E2 interaction was observed when ERα protein levels were evaluated using Western blot (Fig. 1B).

E2 and EtOH regulate common, ERα-, and ERβ-specific mediated genes in bone

Prompted by the above data that EtOH upregulates ERα and ERβ gene expression in bone, we next studied EtOH and E2 cross-talk through ERα and ERβ. The following six genes were selected: PTHLH, BMP6, PDE3B, BTF, Autotaxin, and STAG2. According to data published by Stossi et al., PTHLH and BMP6 are considered...
common genes mediated through ERα and ERβ, PDE3B and BTF are mediated specifically through ERα, whereas Autotaxin and STAG2 are mediated through ERβ specifically. Using RNA extracted from tibial bone and real-time PCR analysis, we found that EtOH and E2 alone upregulated all six genes with no specificities for either ERα or ERβ. Interestingly, in the EtOH + E2 group, all six gene expressions were lower (p < 0.05) compared with EtOH or E2 alone, especially Autotaxin and STAG2, which were lowered back to control level (Fig. 2).

E2 interacts with EtOH to regulate ERα and ERβ gene expression in in vitro osteoblastic cell cultures

To further confirm the interaction of E2 and EtOH on ERα and ERβ gene expression specifically in osteoblasts, we used stromal osteoblasts derived from bone marrow cells, calvarial osteoblasts isolated from neonatal calvaria, and the UMR-106 osteoblastic cell line. In the first set of experiments, mature stromal osteoblasts derived from bone marrow were treated with EtOH using 50 and 100 mM, with or without 30-min 10^{-8} M E2 pretreatment. Twenty-four hours later, RNA was extracted, and real-time PCR and ERα and ERβ gene expression were studied. EtOH induced ERα and ERβ gene expressions in a concentration-dependent fashion. In agreement with the data we obtained in vivo, E2 induced both ERα and ERβ gene overexpression. As we expected, E2 did not show an additive effect but rather attenuated EtOH's effect on both ERα and ERβ gene expression at two different EtOH concentrations (Figs. 3A and 3B). Similarly, in the second set of experiments, concentration-dependent responses of ER gene expression to EtOH were observed using isolated neonatal calvarial osteoblasts, and E2 was also able to attenuate 50 mM EtOH-induced expression of both ER genes (Figs. 3C and 3D). To test the hypothesis that the action of EtOH on ER gene expression is non-receptor specific, we used ERα specific agonists PPT and ERβ specific agonist DPN to treat UMR-106 osteoblastic cells with or without EtOH. We found that PPT and DPN were able to attenuate EtOH-induced ERα and ERβ gene expression specifically (Figs. 3E and 3F). This suggested that EtOH stimulates ER gene expression in a nonselective fashion but that ERα- and ERβ-specific pathways are involved in feedback regulation of each receptor type. To test the hypothesis that EtOH activates some common ER-regulated genes because of activated oxidative stress in osteoblasts, we used UMR-106 osteoblastic cells and treated the cells with N-acetyl cysteine (NAC), which is a well-known antioxidant chemical together with EtOH. We found that pretreatment with 10 mM NAC not only attenuated EtOH-induced ERα and ERβ gene expression but also completely blocked EtOH-activated ER-regulated (common, ERα specific and ERβ specific) gene expression in those osteoblasts (Figs. 3G–3K).

Differential effects of EtOH and E2 on ERE and p21 promoter reporter in UMR-106 osteoblastic cells

Based on the above observations that ER genes and p21 expression are induced by EtOH, we next compared the
effect of EtOH, E2, and ICI on the estrogen-responsive reporter and the p21 promoter. ERE-TK-Luc or the human pGL2-p21-Luc was transfected into UMR-106 osteoblastic cells, with or without co-transfection of wildtype ERα. Twenty-four hours later after transfection, cells were treated with EtOH 50 mM, E2 10^{-9} M, ICI 10^{-7} M, and a combination of E2 + EtOH and E2 + ICI (with E2 10^{-9} M and ICI 10^{-7} M 30-min pretreatment before adding EtOH) for an additional 24 h. Luciferase activity was measured. All data were corrected for renilla activity and relative to control treated with or without ERα, respectively. *Significantly greater and **significantly lower than control vehicle treated cells in 24-well plate quadruplicates at p < 0.05. #Significantly different when EtOH and E2 + EtOH were compared at p < 0.05 as determined by two-way ANOVA followed by Student-Newman-Keuls posthoc analysis for multiple pairwise comparisons.

**FIG. 4.** E2, but not ICI, alters EtOH-induced regulation of ERE-TK-Luc and p21 promoter activities in osteoblasts. (A and B) Rat UMR-106 osteoblastic cells in 24-plate quadruplicates were transfected with ERE-TK-Luc plasmid with or without co-transfection of wildtype ERα. (C and D) Rat UMR-106 osteoblastic cells in 24-plate quadruplicates were transfected with pGL2-p21-Luc plasmid with or without co-transfection of wildtype ERα. Twenty-four hours later after transfection, cells were treated with EtOH 50 mM, E2 10^{-9} M, ICI 10^{-7} M, and a combination of E2 + EtOH and E2 + ICI (with E2 10^{-9} M and ICI 10^{-7} M 30-min pretreatment before adding EtOH) for an additional 24 h. Luciferase activity was measured. All data were corrected for renilla activity and relative to control treated with or without ERα, respectively. *Significantly greater and **significantly lower than control vehicle treated cells in 24-well plate quadruplicates at p < 0.05. #Significantly different when EtOH and E2 + EtOH were compared at p < 0.05 as determined by two-way ANOVA followed by Student-Newman-Keuls posthoc analysis for multiple pairwise comparisons.

**FIG. 5.** Cellular localization of ERα in UMR-106 cells. UMR-106 osteoblastic cells were grown in a 24-well plate, transfected with an ERα-ECFP expression plasmid (0.4 μg/well) together with nRFP nuclear anchor protein (0.1 μg/well), and treated with vehicle, 50 mM EtOH, 10^{-9} M E2, and 10^{-7} M ICI for 2 h before viewing (×20 lens) directly under a fluorescent microscopy connected with a camera. In EtOH and E2 combination treatment (E2 + EtOH), E2 was added 30 min before EtOH treatment. In the EtOH, E2 + EtOH, and ICI-treated cells, ERα-ECFP was found throughout the cell, whereas in E2-treated cells, ERα-ECFP translocated into the nucleus. Pictures were taken under fluorescent microscopy (×20) from representative areas from each treatment well.
Cellular localization of ERα in UMR-106 cells

To explore the differences of the effects of EtOH and E2 on estrogen receptor in bone cells, we compared the cellular distribution of ERα-ECFP expression plasmid together with Nuc-ERFP in UMR-106 osteoblastic cells by fluorescent microscopy. After ERα-ECFP was transfected into a cell, ERα was distributed mainly in the cytoplasm of control treated cells. The addition of E2 resulted in rapid nuclear translocation of ERα, whereas ERα from cells treated with EtOH or ICI remained in the cytoplasm (Fig. 5). Like ICI, we found that the majority of ERα-ECFP was in the cytosol after addition of EtOH to E2-treated cells for 2 h (Fig. 5).

Senescence-associated β-galactosidase activity is increased in EtOH-treated osteoblasts

Previous reports have strikingly shown that an activating mutation of p53 causes early onset of aging-associated phenotypes in mice. Our above data showed that EtOH induces p53 and p21 in bone. This further prompted us to examine senescence-associated β-galactosidase activity in osteoblasts treated with EtOH and E2. Mature stromal osteoblasts were exposed to EtOH and E2 for 24 and 48 h, and p53 was identified by Western blotting. Increased phosphorylation of p53 by EtOH in cell lysates collected at 24 and 48 h. E2, however, attenuated the effects of EtOH. The numbers represent the ratio of intensity of the band of targeted protein over control GAPDH for a single treatment. Bars represent means ± SD. *p < 0.05 vs. control vehicle treated cells in 6-well plate triplicates. **Significantly different EtOH vs. E2 + EtOH (p < 0.05 as determined by two-way ANOVA followed by Student-Newman-Keuls posthoc analysis for multiple pairwise comparisons).

FIG. 6. EtOH induces ERα, activates p53 and p21, and increases senescence-associated β-galactosidase activities in bone marrow stromal osteoblasts. Mature stromal osteoblasts derived from bone marrow cells in the presence of osteogenic medium were treated with EtOH, E2, and E2 + EtOH for 24 and 48 h. (A and B) Senescence-associated β-galactosidase enzyme activity measured from cell lysates collected 24 and 48 h after treatment. EtOH increased enzyme activity at 24 and 48 h; however, E2 blocked or attenuated the effects of EtOH at either 24 or 48 h. (C–F) Senescence-associated β-galactosidase cell staining was performed after 48-h treatment. Blue stained cells showed positive for senescence-associated β-galactosidase activity. (G and H) Western blots showed increased ERα, p53, and p21 and increased phosphorylation of p53 by EtOH in cell lysates collected at 24 and 48 h. E2, however, attenuated the effects of EtOH. The numbers represent the ratio of intensity of the band of targeted protein over control GAPDH for a single treatment. Bars represent means ± SD. *p < 0.05 vs. control vehicle treated cells in 6-well plate triplicates. **Significantly different EtOH vs. E2 + EtOH (p < 0.05 as determined by two-way ANOVA followed by Student-Newman-Keuls posthoc analysis for multiple pairwise comparisons).

DISCUSSION

In human alcoholics and in experimental animal studies, EtOH has been shown to cause a variety of tissue damage. For example, EtOH-induced liver damage resulting from overproduction of reactive oxygen species (ROS) has been well documented. We have previously developed a female rat model in which EtOH is infused overnight as part of a system of TEN to mimic consumption patterns and blood EtOH concentrations observed in alcoholics. Using this model, we showed that bone loss occurred after chronic EtOH infusion independent of nutritional status. In addition, we showed that EtOH treatment reduces estradiol in cycling females and disrupts estrogen or ER signaling in bone. In this report for the first time, we showed that EtOH also directly stimulates overexpression of ERs in bone in vivo and in osteoblasts in vitro and that this can be reversed by ER agonist treatment. In general, ERs are ligand-inducible transcription factors. When
bound to E2, ERs activate the expression of genes that have estrogen responsive elements (ERE)s in their promoter regions. EtOH, which is not a ligand of ER, stimulated both receptors to be overexpressed but also prevented E2-stimulated ERα translocation to the nucleus and downregulated ERE-luc reporter activity in osteoblasts. These data suggest that the ability of EtOH to up-regulate ER expression is a feedback consequence of impaired ER signaling. In agreement with our present data, a previous study reported that ER concentration is increased in hepatocytes after EtOH stimulation and suggested that EtOH administration could be considered as a form of chemical castration. However, these data are in contrast to previously published data in human breast cancer cell lines in which EtOH and E2 synergistically activated ERE-luc reporter activity. This may suggest tissue-specific interactions between EtOH and ER signaling.

Bone tissue is known to express both ERs, unlike other tissues such as uterus, liver, ovary, and prostate, where one of the two ERs predominates. We attempted to find differences between ERα- and ERβ-regulated gene expression in osteoblasts in response to EtOH stimulation. By far, the most extensive previous evidence on ER-specific signaling in osteoblasts has been recently published by Stossi et al. They showed that, in human osteoblastic cells, BMP6 and PTHLH are ERα and ERβ common, PDE3B and BTF are ERα specific, and ENPP2 and STAG2 are ERβ-specific genes in response to E2. We found that E2 induced expression of all of these genes but that the combination of E2 and EtOH attenuated this response. These data are also consistent with the hypothesis that EtOH impairs ER-mediated signaling in osteoblasts.

An interesting finding in this study was that the p21 promoter in osteoblasts is activated by EtOH in the presence or absence of exogenous ERα and that p21 protein expression was significantly increased. This was accompanied by increased phosphorylation of p53. The biological significance of these findings is that p53 and p21 have significantly been found to be accumulated in alcoholic tissues and associated with cancer occurrence. In normal physiologic situations, in breast cancer cells, ER and p53 exert opposing effects on cellular proliferation. Recent evidence has shown that ER may directly bind to p53, leading to downregulation of transcriptional activation by p53. There is a lack of extensive evidence on the relationship between ER and p53 in osteoblasts and how estrogen regulates p53 is also controversial. β-galactosidase is well established as a biomarker to identify senescent cells in culture and aging skin in vivo. The increase in p21 expression and p53 activity after EtOH treatment was associated with higher levels of SA-β-galactosidase activity. This suggests that the senescence pathway is activated by EtOH in these osteoblasts. Our data showed that co-treatment of EtOH-treated osteoblasts with E2 resulted in a reversal of effects on p21 promoter activation, p21 overexpression, and p53 phosphorylation and inhibition of EtOH-induced senescence. These data are consistent with our previous studies that have shown that E2 treatment can prevent EtOH-induced bone loss in female rats and suggest negative cross-talk between ER-mediated signaling and EtOH-stimulated cell signaling.

In this regard, the one surprising finding of this study was that the six ER-regulated genes we chose to study—BMP6, PTHLH, PDE3B, BTF, ENPP2, and STAG2—were all expressed at higher levels after EtOH treatment alone in addition to being induced by E2 but that the combination of EtOH and E2 led to reduced expression. The most likely explanation for this apparently contradictory finding is that EtOH induces these genes through an ER-independent pathway probably involving increased production of ROS in osteoblasts and that negative cross-talk of E2 on EtOH signaling and of EtOH on ER signaling explains the effects of EtOH + E2. Another less likely possibility is that, although EtOH inhibits ER-mediated signaling through ERE elements, it could stimulate some ER-mediated signaling pathways through indirect effects on ER interactions with transcription factors at other elements such as AP1 or Sp1 sites. In this regard, it should be pointed out that, as with p21 in this study, in our previous studies with RANKL in osteoblasts, E2 treatment either has no effect or suppresses RANKL mRNA, EtOH induces RANKL mRNA expression, and the combination of E2 and EtOH prevents this induction. In addition, osteoblasts treated with the antioxidant NAC together
with EtOH showed that NAC completely blocked EtOH-induced gene expression. These observations further suggested that ROS is involved in the negative cross-talk of E2 on EtOH signaling in osteoblasts. However, whether the putative ER membrane (versus nuclear) pathway is responsible for this effect clearly needs to be investigated in future studies. Moreover, G protein–coupled receptors, which have currently been shown to directly bind estrogen, may be the other possible mechanism for the effect of EtOH in osteoblasts.

From our data, as summarized in Fig. 7, we believe that EtOH-triggered accelerated osteoblastic cell senescence results from activation of p53 and p21. This action of EtOH and negative cross-talk between EtOH signaling and ER-mediated pathways contributes to osteoporotic bone loss in chronic alcohol drinkers and protective effects of estrogens on EtOH-induced bone loss.

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REFERENCES

1. Chen JR, Heley RL, Hidestrander M, Shankar K, Lui X, Lumpkin CK, Simpson PM, Badger TM, Ronis MJ 2006 Estradiol protects against ethanol-induced bone loss by inhibiting up-regulation of receptor activator of nuclear factor-kappaB ligand in osteoblasts. J Pharmacol Exp Ther 319:1182–1190.
2. Shankar K, Hidestrander M, Haley RL, Skinner RA, Hogue W, Jo CH, Simpson PM, Lumpkin CK Jr, Aronson J, Badger TM, Ronis MJ 2006 Different molecular mechanisms underlie ethanol-induced bone loss in cycling and pregnant rats. Endocrinology 147:166–178.
3. Shankar K, Hidestrander M, Liu X, Chen JR, Haley RL, Perrien DS, Skinner RA, Lumpkin CK Jr, Badger TM, Ronis MJ 2008 Chronic ethanol consumption inhibits postnatal and analastic bone rebuilding in female rats. J Bone Miner Res 23:538–549.
4. Calluci JJ, Juknelis D, Patwardhan A, Wezeman FH 2006 Binge alcohol treatment increased vertebral bone loss following ovariectomy: Compensation by intermittent parathyroid hormone. Alcohol Clin Exp Res 30:665–672.
5. Chakkalakal DA 2005 Alcohol-induced bone loss and deficienct bone repair. Alcohol Clin Exp Res 29:2077–2090.
6. Chen JR, Shankar K, Nagarajan S, Badger TM, Ronis MJ 2008 Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. J Pharmacol Exp Ther 324:50–59.
7. Perrien DS, Wahl EC, Hogue WR, Feige U, Aronson J, Ronis MJ, Badger TM, Lumpkin CK Jr 2004 IL-1 and TNF antagonists prevent inhibition of fracture healing by ethanol in rats. Toxicol Sci 82:656–660.
8. Wahl EC, Aronson J, Liu L, Liu Z, Perrien DS, Skinner RA, Badger TM, Ronis MJ, Lumpkin CK Jr 2007 Chronic ethanol exposure inhibits distraction osteogenesis in a mouse model: Role of the TNF signaling axis. Toxicol Appl Pharmacol 220:302–310.
9. Kimble RB 1997 Alcohol, cytokines, and estrogen in the control of bone remodeling. Alcohol Clin Exp Res 21:385–391.
10. Dai J, Lin D, Zhang J, Habib P, Smith P, Murtha J, Fu Z, Yao Z, Qi Y, Keller ET 2000 Chronic alcohol ingestion induces osteolastogenesis and bone loss through IL-6 in mice. J Clin Invest 106:887–895.
11. Gavaler JS 1995 Alcohol effects on hormone levels in normal postmenopausal women and in postmenopausal women with alcohol-induced cirrhosis. Recent Dev Alcohol 12:199–208.
12. Matthews J, Gustafsson JA 2003 Estrogen signaling: A subtle balance between ER alpha and ER beta. Mol Interv 3:281–293.
13. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS 2004 Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: Distinct and common target genes for these receptors. Endocrinology 145:3473–3486.
14. Colantuoni A, Emanuele MA, Kovacs EJ, Villa E, Van Thiel DH 2002 Hepatic estrogen receptors and alcohol intake. Mol Cell Endocrinol 193:101–104.
15. Fan S, Meng Q, Gao B, Grossman J, Yadegari M, Goldberg JD, Rosen EM 2000 Alcohol stimulates estrogen receptor signaling in human breast cancer cell lines. Cancer Res 60:5635–5639.
16. Harmston WR, Taddayon P, Kolman K, Chandar N 2005 Effect of overexpression of estrogen receptors in osteoblasts. In Vitro Cell Dev Biol Anim 41:264–271.
17. Bargonetti J, Manfredi JJ 2002 Multiple roles of the tumor suppressor p53. Curr Opin Oncol 14:86–91.
18. Migliazzo E, Giorgio M, Mele S, Pellicci G, Reboli P, Pandolfi PP, Lanfrancone L, Pellici PG 1999 The p53-h65shc adaptor protein controls oxidative stress response and life span in mammals. Nature 402:309–313.
19. Trinei M, Giorgio M, Cicalèse A, Barozzi S, Ventura A, Migliazzo E, Milia E, Padura IM, Raker VA, Maccarana M, Petronilli V, Minucci S, Bernardi P, Lanfrancone L, Pellici PG 2002 A p53-p66shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative-stress induced apoptosis. Oncogene 21:3872–3878.
20. Giorgio M, Migliazzo E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcacci M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pellici PG 2005 Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 122:221–233.
21. Soddu S, Blandino G, Scardigli R, Martinelli R, Rizzo MG, Crescenzi M, Sacchi A 1996 Interference with p53 protein inhibits hematopoietic and muscle differentiation. Mol Cell Biol 16:487–495.
22. Porrello A, Cerone MA, Coen S, Gurtner A, Fontemaggi G, Cimino L, Piaggio G, Sacchi A, Soddu S 2000 p53 regulates myogenesis by triggering the differentiation activity of pRb. J Cell Biol 151:1295–1304.
23. Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karasey G, de Crombrugghe B, Ye H, Li B 2006 p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoelastogenesis, and bone remodeling. J Cell Biol 172:115–125.
24. Almeida M, Han L, Martin-Millan M, Plotkin LI, Stewart SA, Roberson PK, Kousteni S, O’Brien CA, Bellido T, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC 2007 Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. J Biol Chem 282:27285–27297.
25. Chu J, Tong M, de la Monte SM 2007 Chronic ethanol exposure causes mitochondrial dysfunction and oxidative stress in immature central nervous system neurons. Acta Neuropathol 113:659–673.
26. Jankila A, Eriksson PC, Eklund K, Sarviharju M, Häkkinen M, Mäki T 2005 Effect of chronic ethanol ingestion and gender on heart left ventricular p53 gene expression. Alcohol Clin Exp Res 29:1368–1373.
27. Abukheide AM, Vitolo MI, Argandi P, De Marzo AM, Karakas B, Konishi H, Gustin JP, Lauring J, Garay JP, Pendleton C, Konishi Y, Blair BG, Brenner K, Garrett-Mayer E, Carraway H, Bachman KE, Park BH 2008 Tamoxifen-stimulated growth
of breast cancer due to p21 loss. Proc Natl Acad Sci USA 105:288–293.
28. Ronis MJ, Lumpkin CK, Ingelman-Sundberg M, Badger TM 1991 Effects of short-term ethanol and nutrition on the hepatic microsomal monooxygenase system in a model utilizing total enteral nutrition in the rat. Alcohol Clin Exp Res 15:693–699.
29. Badger TM, Crouch J, Irby D, Hakkar R, Shahare M 1993 Episodic excitation of ethanol during chronic intragastric ethanol infusion in the male rat: Continuous vs. cyclical ethanol and nutrient infusions. J Pharmacol Exp Ther 264:938–943.
30. Di Gregorio GB, Yamamoto M, Ali AA, Abe E, Roberson P, Manolagas SC, Jilka RL 2001 Attenuation of the self-renewal of transit-amplifying osteoblast progenitors in the murine bone marrow by 17 beta-estradiol. J Clin Invest 107:803–812.
31. Wong GL, Cohn DV 1975 Target cells in bone for parathyroid hormone and calcitonin are different: Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Proc Natl Acad Sci USA 72:3167–3171.
32. Kousteni S, Bellido T, Plotkin LI, O’Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: Dissociation from transcriptional activity. Cell 104:719–730.
33. Yin Y, Zhu A, Jin YJ, Liu YX, Zhang X, Hopkins KM, Lieberman HB 2004 Human RAD9 checkpoint control/proapoptotic protein can activate transcription of p21. Proc Natl Acad Sci USA 101:8864–8869.
34. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J 1995 A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367.
35. Jänkkäi H, Eklund KK, Kokkonen JO, Kovanen PT, Linstedt KA, Härkönén M, Mäki T 2001 Ethanol infusion increases ANP and p21 gene expression in isolated perfused rat heart. Biochem Biophys Res Commun 281:328–333.
36. Tyner SD, Venkatachalam S, Choi J, Jones S, Ghebranious N, Igelmman H, Lu X, Soron G, Cooper B, Brayton C, Hec Park S, Thompson T, Karsenty G, Bradley A, Donehower LA 2002 p53 mutant mice that display early ageing-associated phenotypes. Nature 418:45–53.
37. Thakur V, Pritchard MT, McMullen MR, Wang Q, Nagy LE 2000 Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: Role of increased reactive oxygen in LPS-stimulated ERK1/2 activation and TNF-alpha production. J Leukoc Biol 79:1348–1356.
38. Chung KW 1990 Effects of chronic ethanol intake on aromatization of androgens and concentration of estrogen and androgen receptors in rat liver. Toxicology 62:285–295.
39. Kuiper GG, Shughrue PJ, Merchenthaler I, Gustafsson JA 1998 The estrogen receptor beta subtype: A novel mediator of estrogen action in neuroendocrine systems. Front Neuroendocrinol 19:253–286.
40. Yokoyama A, Omori T, Tanaka Y, Yokoyama T, Sugihara H, Mizukami T, Matsushita S, Higuchi S, Maruyama K, Ishii H, Hibi T 2007 p53 Protein accumulation, cancer multiplicity, and aldehyde dehydrogenase-2 genotype in Japanese alcoholic men with early esophageal squamous cell carcinoma. Cancer Lett 247:243–252.
41. Sayeed A, Konduri SD, Liu W, Bansal S, Li F, Das GM 2007 Estrogen receptor alpha inhibits p53-mediated transcriptional repression: Implications for the regulation of apoptosis. Cancer Res 67:7746–7755.
42. Bovenkerk S, Lanciloti N, Chandar N 2003 Induction of p53 expression and function by estrogen in osteoblasts. Calcif Tissue Int 73:274–280.
43. Gustafsson JA 2000 An update on estrogen receptors. Semin Perinatol 24:66–69.
44. Barkhem T, Haldosén LA, Gustafsson JA, Nilsson S 2002 Gene expression in HepG2 cells: Complex regulation through crosstalk between the estrogen receptor alpha, an estrogen-responsive element, and the activator protein 1 response element. Mol Pharmacol 61:1273–1283.
45. Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S 2002 Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 275:5379–5387.
46. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER 2005 A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625–1630.

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