Bazedoxifene and conjugated estrogen prevent diet-induced obesity, hepatic steatosis, and type 2 diabetes in mice without impacting the reproductive tract

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IN ADDITION TO THEIR ROLES in sexual development and reproduction, estrogens contribute to the regulation of body weight and body composition and to glucose homeostasis. Insulin sensitivity is greater in women prior to menopause vs. age-matched men (16, 42), in women the risk for weight gain and for the development of type 2 diabetes increases with the decline in the levels of estrogens that occurs at menopause, and menopause favors an increase in total body fat and visceral fat deposition (4). In addition, hormone replacement therapy with conjugated estrogen (CE) and medroxyprogesterone acetate (MPA) in postmenopausal women causes a 21–35% decrease in diabetes occurrence (29, 35). Furthermore, in female rodents and primates, ovariectomy results in impaired insulin sensitiv-

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MATERIALS AND METHODS

Animal model, diet, and treatments. Experiments were performed in female C57BL/6 mice. To design a strategy for the administration of BZA and CE (both from Pfizer) that avoids the disruption of feeding behavior by frequent oral gavage, experiments were initially
performed to establish their systemic delivery. Testing the efficacy of BZA administration by assessing uterotrophic response, chow-fed ovariectomized female mice were given one of the following five treatments by osmotic minipumps placed into the peritoneal cavity: 1) E2 at 10 μg·kg−1·day−1 (Calbiochem), 2) E2 at 1.6 μg/day, 3) BZA at 3 mg·kg−1·day−1, 4) E2 at 10 μg·kg−1·day−1 plus BZA, or 5) E2 at 1.6 μg/day plus BZA. The chosen dose of BZA represents the daily dose previously employed in mice in studies demonstrating BZA antagonism of E2 action on the breast and uterus (10). Following 4 wk of treatment, wet weights were obtained on the entire uterus (both horns). BZA attenuated the uterotrophic response to 1.6 μg/day E2 administration (Fig. 1A). Additional studies using the same overall experimental design showed that treatment with BZA along with 1.6 μg/day E2 yielded uterine weights comparable to those obtained with the administration of vehicle alone (Fig. 1B), which consisted of 43% DMSO, 15% ethanol, and 42% saline. Therefore, effective systemic administration of BZA by osmotic minipump was established.

To prepare CE (Premarin), which is formulated for oral administration, for systemic delivery, cellulose removal was performed by either glass fiber filtration (0.45 μm) or centrifugation (9,000 g). Uterotropic responses to CE post-centrifugation or post-filtration were then evaluated by giving daily intraperitoneal (ip) injections of 5 mg·kg−1·day−1 for 4 days following a 2-wk washout period post-ovariectomy. The dose of 5 mg·kg−1·day−1 is based on the amount of CE prior to cellulose removal, and it mimics previous in vivo use of CE in mice (14). E2 administered in an identical manner at 1.6 μg/day caused a predictable uterotrophic response (Fig. 1C), and CE given systemically either post-centrifugation or post-filtration also invoked a uterotrophic response. Therefore, effective systemic administration of CE was established, and further studies were performed using osmotic minipumps delivering CE postfiltration.

The experiments evaluating the effects of systemically delivered E2 (1.6 μg/day), CE (5 mg·kg−1·day−1), and/or BZA (3 mg·kg−1·day−1) on metabolic parameters were begun at the age of 4 wk, when the mice were housed in pairs and started on a high-fat/high-cholesterol Western diet with 42% of calories derived from fat and 0.2% cholesterol (Teklad Custom Research Diet TD.88137). At 6 wk of age, ovariectomies were performed, and treatments were initiated. Following 6 wk of treatment employing the initially placed osmotic minipump until 8 wk of age), the first minipump was removed, and it was replaced with a second minipump to continue the same treatment for an additional 6 wk. Effective delivery of E2 (1.6 μg/day) via the minipump was evaluated in a parallel experiment that entailed serum sampling just before the 6-wk time point. E2 isolation from serum by ionization in the negative mode (18). Serum E2 was 40 ± 2 pg/ml in the mice administered E2 compared with 8 ± 2 pg/ml in vehicle-treated mice (means ± SE, n = 9/group, P < 0.05). Metabolic end points were evaluated after 10–12 wk of treatment (after 12–14 wk of receipt of the Western diet). This design was chosen because of prior studies demonstrating effects of E2 on the glucose intolerance and insulin resistance that accompany diet-induced obesity at 3 mo of treatment (46). In the various in vivo experiments, the number of mice per study group was from four to nine. The care and use of all study animals was approved by the Institutional Animal Care and Use Committee at UT Southwestern and conducted in accordance with PHS Policy on the Humane Care and Use of Laboratory Animals.

**Evaluations of adiposity, energy homeostasis, and activity.** Fat mass and lean body mass were determined by NMR (Minispec NMR Analyzer, Bruker). Between 10 and 11 wk of treatment following acclimatization for 6 days, indirect calorimetry and locomotion data were simultaneously measured over a 4-day period in the Mouse Metabolic Phenotyping Core at UT Southwestern, using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments) (51).

**Adipose and liver tissue analyses.** At the time of euthanasia, subcutaneous white adipose tissue (WAT) was quantified by determining the weight of the inguinal fat pad, and visceral WAT was quantified by determining the weight of the uterine or mesenteric fat pad. The abundance of brown adipose tissue (BAT) was also assessed by harvesting and weighing the intrascapular fat pad. Adipose tissue
histology was assessed by hematoxylin and eosin staining of sections of inguinal WAT following fixation with 4% paraformaldehyde. Images were captured using a NIKON TE2000-E microscope and Photometric Coolpix HQ2 CCD camera, and adipocyte area was measured using NIH ImageJ software. More than 500 cells were measured per image, and three images were evaluated per mouse. Liver histology was also evaluated in hematoxylin-eosin-stained sections after paraformaldehyde fixation, and liver triglyceride content was measured by the Folch method (19). Weighed tissue samples were homogenized in methanol-choloform, and after overnight extraction, 0.7% sodium chloride was added. The aqueous layer was aspirated, and duplicate aliquots of the chloroform-lipid layer were dried under nitrogen gas. The lipid was reconstituted in isopropyl alcohol and assayed for TG spectrophotometrically with enzymatic reagents from Thermo DMA (Arlington, TX).

Glucose and insulin homeostasis. For glucose tolerance tests (GTT), mice were fasted for 4–6 h and injected ip with D-glucose (1 g/kg body wt). Tail vein blood samples were obtained at the indicated times for plasma glucose measurement by glucometer (ONE TOUCH Ultra2, Johnson & Johnson). Fasting plasma insulin concentrations were determined by ELISA (Crystal Chem) (51). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated to assess changes in insulin sensitivity (6, 37). Pyruvate tolerance tests (PTT) entailed determinations of plasma glucose following ip injection of pyruvate (2 g/kg body wt).

Statistical analysis. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) and Tukey’s post hoc testing. For parameters assessed both over time and between treatment groups, two-way ANOVA and Tukey’s post hoc testing were performed, with significance accepted at the 0.05 level of probability.

RESULTS

Impact on body weight and composition, energy expenditure, food intake, and activity. To determine how BZA in combination with CE or alone impacts body weight and composition, ovariectomized female mice on Western diet were treated with vehicle, either agent alone, or the combination for 10–12 wk. Treatment with E2 was performed to serve as a positive control. Whereas starting weights were identical in the five study groups, the weight increase observed in the vehicle-treated group at 6 wk of treatment was attenuated by E2, CE, BZA, or CE + BZA to equal degree (Fig. 2A). All four treatments also blunted the weight increase over the ensuing 5 wk. Quantification of body fat at 11 wk of treatment revealed that E2, CE, BZA, or CE + BZA lessened the adiposity caused by the Western diet by approximately two-thirds (Fig. 2B), and there was no effect on lean body mass (Fig. 2C).

Since estrogens have the capacity to alter energy expenditure and satiety in rodents (22, 55), indirect calorimetry was performed and food intake was also quantified in mice administered vehicle, CE, BZA, or CE + BZA at 10–11 wk of treatment. Compared with vehicle treatment, the three regimens had no discernible effect on O2 consumption, CO2 production, or heat production (Fig. 3, A–C). There were also no observed differences in food intake or activity with CE, BZA or CE + BZA treatment vs. vehicle (Fig. 3, D–G).

Impact on adiposity. To evaluate how the treatments impact different fat depots, inguinal fat pads were weighed at the end of study to assess subcutaneous WAT accumulation, and uterine and mesenteric fat pads were weighed to assess visceral WAT depots (Fig. 4, A–C). E2 or CE caused 71% declines in subcutaneous WAT and 80 and 59–64% decreases in uterine and mesenteric WAT, respectively. Although there were no statistically significant differences, the declines in WAT with BZA treatment alone were qualitatively not as substantial as with E2 or CE alone or CE + BZA. The addition of BZA to CE treatment did not alter the efficacy of CE to lower WAT accumulation. All four treatments lessened BAT abundance by at least 50% (Fig. 4D).

To provide an additional assessment of adiposity, the morphology of adipocytes in the inguinal WAT was evaluated histologically and adipocyte size was quantified (Fig. 5, A–F). E2 decreases not only adipose mass but also adipocyte size, and this is mediated by adipocyte ERα (11, 12). In contrast to the
large adipocytes observed in the WAT of vehicle-treated mice, all treatments caused declines in adipocyte size. As was observed for percent body fat and for the weights of individual WAT depots, the addition of BZA did not alter the ability of CE to cause a decrease in adipocyte size. Collectively, these findings reveal that CE/BZA has equal efficacy as E2 or CE alone to decrease the adiposity caused by the consumption of a Western diet and that both subcutaneous and visceral WAT depots as well as BAT stores are affected.

Impact on hepatic steatosis and hepatic insulin resistance. In addition to their roles in the modulation of adipocyte function, estrogens participate in hepatic lipid homeostasis. The importance of the hormone in this realm is highlighted by the observation that tamoxifen treatment can be complicated by NASH (5, 28, 39, 40). We therefore evaluated the impact of CE/BZA or CE or BZA alone on the hepatic steatosis that accompanies receipt of a Western diet in mice. In the livers of vehicle-treated mice, there was considerable lipid deposition...
noted both histologically (Fig. 6A) and by the quantification of liver triglyceride content (Fig. 6F). E2 or CE caused declines in liver fat accumulation (Fig. 6, B and C), with triglyceride abundance decreased by half (Fig. 6F). Both CE + BZA and BZA alone caused additional marked declines in liver lipid deposition (Fig. 6, D and E), and liver triglyceride content also fell further, to levels that were one-third those obtained with either E2 or CE treatment. Thus, BZA causes dramatic attenuation of hepatic lipid accumulation.

Since hepatosteatosis is a recognized risk factor for the development of hepatic insulin resistance and glucose intolerance (48, 50), PTT were performed to evaluate hepatic glucose production in response to a gluconeogenesis substrate. In contrast to vehicle-treated mice, the increase in serum glucose following pyruvate administration was blunted in mice treated with CE or BZA or CE + BZA (Fig. 6G). Therefore, CE or BZA, alone or in combination, blunt lipid deposition in the liver, and concomitantly they decrease hepatic gluconeogenesis, most likely by improving hepatic insulin resistance.

Impact on glucose tolerance and insulin sensitivity. Global effects of CE + BZA and the agents alone on glucose tolerance and insulin sensitivity were also evaluated. Fasting glucose was lowered by treatment with E2, CE, or CE + BZA (Fig. 7A), and fasting insulin was lowered by all of these regimens and also by treatment with BZA alone (Fig. 7B). GTT were improved in mice given E2, CE, or CE + BZA but not by treatment with BZA alone (Fig. 7, C and D). HOMA-IR were lowered by E2, CE, or CE + BZA but not by BZA treatment alone (Fig. 7E). Thus, the most dramatic effects on global glucose tolerance and insulin sensitivity are with E2, CE alone, or CE + BZA.

Impact on uterus. The impact of the interventions on the female reproductive tract was determined by assessments of uterotrophic response. Predictably, both E2 and CE alone caused increases in uterine wet weight (Fig. 8). In contrast, neither BZA treatment nor the administration of both CE and BZA caused a uterotrophic response, with the addition of BZA blunting the uterotrophic response to CE. Thus, in contrast to the changes observed in body weight, adiposity, hepatic steatosis, and glucose homeostasis, CE + BZA has negligible if any impact on the female reproductive tract.

DISCUSSION

Typical hormone replacement therapy that can favorably impact the metabolic consequences and other complications of menopause consists of a combination of an estrogen with a progestin, such as CE + MPA. However, concerns have been raised about potential negative effects of progestin, and therapy with an estrogen alone is associated with undesired cancer-promoting and uterotrophic effects (7, 24, 26). Seeking a new means to leverage the beneficial actions of estrogens on body composition and glucose homeostasis (38) without estrogen-related effects on reproductive tissues or hormone-responsive cancers, we have determined how CE alone, BZA alone, and the combination of CE + BZA impact metabolic parameters in Western diet-fed female mice. The studies of CE are the first in an animal model to quantify the metabolic actions of the most common form of estrogen used clinically in women. Mirroring the effects of E2, we found that body weight and adiposity were protected not only by CE but also by the novel SERM BZA and by CE + BZA. Glucose intolerance and insulin resistance were improved with CE + BZA, and the impact of CE + BZA was comparable to that with E2 or CE alone. The effect of the TSEC (CE + BZA) on glucose homeostasis in the Western diet-fed mice was more dramatic than the lack of impact observed in a previous trial in postmenopausal women, most likely because the women were not obese (33). Whereas E2 or CE alone invoked a uterotrophic response, CE + BZA or BZA alone had minimal effect on the uterus. Thus, we have revealed that combination treatment with CE + BZA has a number of
favorable metabolic actions comparable to E2 or CE alone without impacting the female reproductive tract.

Evaluations of energy utilization and food intake showed no effects of E2, CE, BZA, or CE+BZA. However, it should be noted that the majority of the changes in weight that occurred with treatment took place during the first 6 wk of the regimens, and the energy- and activity-related measurements were done during the second half of the treatment period at 10–11 wk. It is possible that metabolic or behavioral alterations triggered by the earlier body composition changes hindered the capacity to detect the initiating processes through the measurements done at the later time point. Another possibility is that the effects of the treatments were due to small differences in energy intake or expenditure that were not readily detected but accumulated over time to have substantial impact on body composition and weight (52).

The effects of CE, BZA, or CE + BZA on body weight were related to decreases in fat mass. The form of ER by which BZA or CE + BZA impacts adiposity is likely ERα, which is the receptor subtype that primarily influences metabolic processes. ERα−/− mice are obese, whereas ERβ−/− mice display no change in adiposity (23, 27, 44), and potential roles for ERβ in metabolism are principally apparent in the absence of ERα or they oppose the demonstrated metabolic functions of ERα (12, 20). The specific populations of ERs by which CE + BZA attenuate adiposity likely include ERα in adipocytes, since adipocyte-specific deletion of ERα prevents E2 effects on fat depot size (12). E2, CE, CE + BZA, and BZA alone all caused a decrease in adipocyte size, which is a known impact of E2 (11).

The loss of visceral WAT with CE + BZA may underlie the observed improvements in glucose tolerance and insulin sensitivity, because surgical removal of visceral fat but not subcutaneous fat in rodents improves glucose homeostasis (21). A possible mechanistic link between CE + BZA action on adipose tissue and the normalization of glucose homeostasis is also supported by the prior finding that adipose ERα deletion in mice yields a loss of the favorable effects of E2 on GTT (12). In contrast to CE or CE + BZA, BZA alone had no effect on GTT or HOMA-IR, and this may have been related to the tendency for BZA alone to have less effect on VAT depot size than when CE was administered. We also observed a decrease in BAT abundance with CE + BZA as well as with the other treatments. It is difficult to propose which ER is operative in these responses, because although ERα may be the prevalent

Fig. 5. CE + BZA attenuates diet-induced increases in adipocyte size. Vehicle (A), E2 (B, 1.6 µg/day), CE (C, 5 mg·kg−1·day−1), BZA (D, 3 mg·kg−1·day−1), or CE + BZA (E) were administered to ovariectomized female mice receiving a Western diet. At 12 wk of study, the inguinal fat pad was harvested, sectioned, and stained with hematoxylin & eosin. Representative images from each treatment group are shown (×10 magnification). F: adipocyte size was quantified on n = 3–4 mice/group. Values are means ± SE. *P < 0.05 vs. vehicle-treated (ANOVA and Tukey’s post hoc testing, F = 4.9).
ER in BAT (53), ERα−/− mice show no change in BAT abundance (8, 27). In addition to the improvements in diet-induced adiposity and glucose intolerance and insulin resistance observed with CE/BZA treatment, we determined that the combination attenuates hepatic steatosis. Equal blunting of steatosis was found with CE/BZA and BZA alone, and either strategy yielded considerably greater attenuation of liver fat accumulation than E2 or CE alone. PTTs indicative of relative hepatic insulin sensitivity were also normalized. Whether the improvements in liver lipid accumulation may have caused the favorable effects on hepatic insulin sensitivity is unclear, because there is ongoing debate whether or not there is mechanistic linkage between hepatic steatosis and hepatic insulin resistance (17). Whether it is ERα or ERβ likely mediating the actions of BZA or the TSEC (CE/BZA) on fatty liver is also difficult to conjecture, because in some reports elevations in liver triglyceride content with high-fat diet are not improved by an ERα-specific agonist but instead by an ERβ-specific agonist (34, 57), yet the hepatic steatosis found in male aromatase-null mice is effectively attenuated by an ERα-specific agonist (5). Interestingly, ERα in hepatocytes per se may have negligible influence on either hepatic fat or hepatic insulin sensitivity because, although global ERα−/− mice have defective insulin suppression of endogenous glucose production, which is primarily derived from the liver (3), hepatocyte-specific ERα deletion does not alter liver lipid content or glucose or...
insulin sensitivity (36). The possible roles of ERα in cell types other than the hepatocyte, or the participation of ERβ in the actions of CE + BZA on hepatic lipid and glucose regulation, deserve inquiry in future studies. In the meantime, BZA or CE + BZA warrants consideration as a novel endocrine-based means to combat fatty liver disorders without estrogen-related impact on the female reproductive tract or hormone-sensitive cancers. The need for new treatment strategies is great, because nonalcoholic fatty liver disease (NAFLD), which ranges from benign hepatic steatosis to the more severe nonalcoholic steatohepatitis (NASH) and cirrhosis, afflicts 10–25% of individuals in Western countries and up to 30% of Americans, having become a major health concern due to its association with the metabolic syndrome (1, 15, 31, 45).

The metabolic effects of CE + BZA are best put into perspective by comparing the current findings with the metabolic actions of the commonly clinically employed SERMS raloxifene and tamoxifen. Studies in postmenopausal women indicate that, in contrast to estrogen-based interventions, raloxifene does not improve type 2 diabetes management (2, 13). As mentioned previously, whereas estrogens blunt fatty liver (28), tamoxifen causes an increase in the risk of hepatic steatosis (9, 25, 41). In contrast, the present preclinical experiments have revealed that the TSEC comprising CE + BZA has beneficial effects on both type 2 diabetes and fatty liver without invoking a uterotrophic response. Considering the prior preclinical observations regarding BZA attenuation of estrogen-dependent breast cancer growth (49, 56), CE + BZA may offer an

![Fig. 7. CE + BZA causes improvements in glucose tolerance and insulin sensitivity. Following ovariectomy, female mice were placed on Western diet and treated with vehicle, E2 (1.6 μg/day), CE (5 mg·kg⁻¹·day⁻¹), BZA (3 mg·kg⁻¹·day⁻¹), or CE + BZA. At 12 wk of study, fasting glucose (A) and insulin (B) were measured, glucose tolerance tests (GTT) were performed (C and D, with D showing GTT area under the curve, or AUC), and homeostasis model assessment of insulin resistance (HOMA-IR) was calculated (E). Values are means ± SE.

*P < 0.05 vs. vehicle-treated (ANOVA and Tukey’s post hoc testing, F = 14.3, 3.3, 24.7, and 8.2 for A, B, D, and E, respectively).

![Fig. 8. CE + BZA does not invoke a uterotrophic response. Uterine wet weight was measured following 12 wk of Western diet feeding and treatment with vehicle, E2 (1.6 μg/day), CE (5 mg·kg⁻¹·day⁻¹), BZA (3 mg·kg⁻¹·day⁻¹), or CE + BZA. Values are means ± SE. *P < 0.05 vs. vehicle-treated, †P < 0.05 vs. CE alone (ANOVA and Tukey’s post hoc testing, F = 50).

![Graph](http://ajpendo.physiology.org/) by 10.220.32.246 on June 23, 2017 http://ajpendo.physiology.org/ Downloaded from...
advantageous means to leverage the metabolic actions of estrogens for therapeutic advantage without adverse impact on the female reproductive tract or breast.

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