Paracrine-Acting Adiponectin Promotes Mammary Epithelial Differentiation and Synergizes with Genistein to Enhance Transcriptional Response to Estrogen Receptor β Signaling

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Mammary stromal adipocytes constitute an active site for the synthesis of the adipokine, adiponectin (APN) that may influence the mammary epithelial microenvironment. The relationship between “local,” mammary tissue-derived APN and breast cancer risk is poorly understood. Here, we identify a novel mechanism of APN-mediated signaling that influences mammary epithelial cell proliferation, differentiation, and apoptosis to modify breast cancer risk. We demonstrate that early dietary exposure to soy protein isolate induced mammary tissue APN production without corresponding effects on systemic APN levels. In estrogen receptor (ER)-negative MCF-10A cells, recombinant APN promoted lobuloalveolar differentiation by inhibiting oncogenic signal transducer and activator of transcription 3 activity. In ER-positive HC11 cells, recombinant APN increased ERβ expression, inhibited cell proliferation, and induced apoptosis. Using the estrogen-responsive 4X-estrogen response element promoter-reporter construct to assess ER transactivation and small interfering RNA targeting of ERα and ERβ, we show that APN synergized with the soy phytoestrogen genistein to promote ERβ signaling in the presence of estrogen (17β-estradiol) and ERβ-specific agonist 2,3-bis(4-hydroxyphenyl)-propionitrile and to oppose ERα signaling in the presence of the ERα-specific agonist 4,4′,4′-propyl-(1H)-pyrazole-1,3,5-triyl(1H)-trisphenol. The enhancement of ERβ signaling with APN + genistein cotreatments was associated with induction of apoptosis, increased expression of proapoptotic/prodifferentiation genes (Bad, p53, and Pten), and decreased antiapoptotic (Bcl2 and survivin) transcript levels. Our results suggest that mammary-derived APN can influence adjacent epithelial function by ER-dependent and ER-independent mechanisms that are consistent with reduction of breast cancer risk and suggest local APN induction by dietary factors as a targeted approach for promotion of breast health. (Endocrinology 152: 3409–3421, 2011)

Obesity (assessed by body mass index) increases the risk of breast cancer in postmenopausal women by 30–50% (1–3), in part due to aromatization of androstenedione to estrone by adipose tissue and subsequent conversion to the active hormone estradiol. Further, obese women at diagnosis are more likely to have higher grade and poor prognosis regardless of their menopausal status (4–6). The adipose tissue produces and secretes at least 50 different polypeptide hormones termed adipokines that can act in an endocrine, paracrine, or autocrine manner, }

Abbreviations: APN, Adiponectin; APNR, APN receptor; CAS, casein; CSS, charcoal-stripped serum; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E2, 17β-estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; 4xERE-TK, 4xERE-TK-Luciferase; GEN, genistein; MEC, mammary epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PND, postnatal day; PPT, 4,4′,4′-propyl-(1H)-pyrazole-1,3,5-triyl(1H)-trisphenol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; QPCR, quantitative real-time PCR; scRNA, scrambled RNA; siRNA, small interfering RNA; SPI, soy protein isolate; STAT3, signal transducer and activator of transcription 3; TK, thymidine kinase.
thus changing the notion of this tissue from a simple fat depot into a very active endocrine organ (7, 8). Unlike most adipokines, serum adiponectin (APN) level is lower in obese individuals compared with normal weight or lean subjects (9) and is considered to be a link between obesity and breast cancer (10–14).

APN effects are mediated through two types of receptors: APN receptor (APNR)1, which is expressed ubiquitously and has higher affinity for the low molecular weight APN trimer, and APNR2, expressed mainly in the liver and has similar affinity for the low and high molecular weight forms of APN (15). Physiological doses of APN inhibit cell proliferation and/or induce apoptosis of both estrogen receptor (ER)-negative (MDA-MD231) and positive (T47D and MCF-7) breast cancer cell lines in a cell type-specific manner (16–18). A recent study using mouse mammary tumor virus-polyomavirus middle T antigen transgenic mice with decreased APN expression demonstrated that in vivo APN haploinsufficiency facilitates mammary tumorigenesis by down-regulation of tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) activity and activation of phosphatidylinositol 3 kinase/AKT signaling (19).

A role for estrogen in the etiology of breast cancer is supported by increased risk of the disease during conditions of prolonged estrogen exposure, such as early menarche, late menopause, late first full-term pregnancy, and nulliparity (20). Biological effects of estrogen are mediated mainly by two members of the nuclear receptor superfamily, ERα and ERβ. These ER isoforms can form homo- or heterodimers and, via the classical pathway, bind to estrogen response elements (ERE) in target genes or, through nonclassical pathways, interact with other transcription factors (21). Both receptors are coexpressed in approximately 70% of breast tumors, and although ERα is associated with cell proliferation and ERβ with antiproliferative effects, the exact role of ERβ in breast cancer remains controversial (22–24). However, ERα/ERβ ratio is higher in breast tumors compared with normal tissue due to loss of ERβ expression during tumor progression (25–27), suggesting a tumor suppressor role for ERβ (28–33). Genomic and proteomic expression analysis of breast cancer cells indicate that when both ER are coexpressed, ERβ inhibits the overall proliferative/survival actions of ERα (34–43).

The soy isoflavone genistein (GEN) is considered to partly mediate the protective effects of soy-rich diets against breast cancer (44, 45) by its preferential activation of ERβ signaling, as shown by enhanced recruitment of steroid receptor coactivator more strongly to ERβ in the presence of GEN (40, 46). In breast cancer cells expressing both ER, GEN inhibits the proliferative actions of ERα by increasing the expression of a number of ERβ-mediated proteins involved in apoptosis, cell cycle, motility, and lipid metabolism (47). Further, APN induces the expression of both ER in malignant mammary epithelial cells (MEC) (48), suggesting that APN may exert its antitumor effects by regulating the direction of ERα and/or ERβ signaling. However, whether APN similarly functions in normal (nontumorigenic) MEC to influence ERα/ERβ cross talk and, more importantly, whether this function of APN is coregulated by GEN have not been determined.

Here, we evaluated the hypothesis that dietary induction of APN synthesis and/or secretion by mammary stromal adipocytes leads to enhancement of ERβ signaling on neighboring MEC by paracrine-acting APN. We show that lifetime dietary exposure of weaning and young adult female rats to soy protein isolate (SPI) increased APN protein levels in mammary tissue without parallel effects on systemic APN levels. Using nonmalignant human (MCF-10A) and mouse (HC11) MEC, we demonstrate that recombinant APN can enhance differentiation of ER-negative MCF-10A cells by its suppression of basal signal transducer and activator of transcription 3 (STAT3) activity and promote apoptosis and differentiation of ER-positive HC11 cells by its activation of ERβ signaling in cooperation with the ERβ-specific ligand GEN. Further, using specific agonists to ERα [4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol (PPT)] and ERβ [2,3-bis(4-hydroxyphenyl)-propionitrile (DPN)] along with small interfering RNA (siRNA) technologies targeting either receptor isoform, we define novel synergistic roles for APN and GEN in promoting ERα/ERβ cross talk in MEC.

Materials and Methods

Animals and diets

All animal experiments were carried out under protocols approved by The University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Time-mated Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in polycarbonate cages under conditions of 24 C, 40% humidity, and a 12-h light, 12-h dark cycle. At gestation day 4, dams were randomly assigned to one of two semipurified isocaloric diets containing either casein (CAS) (New Zealand Milk Products, Santa Rosa, CA) or SPI (Solae, St. Louis, MO) as sole protein source and formulated following the American Institute of Nutrition-93G guidelines (49), except that corn oil was substituted for soybean oil. SPI contains the isoflavones GEN (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg) as aglycone equivalents. Animals were provided food and water ad libitum. At delivery, all pups from dams of the same diet groups were pooled, and 10 pups (five per sex) were randomly assigned to each dam to nurse. Female pups were weaned at postnatal day (PND)21 to the same diets as their dams and were fed this diet throughout the study. The inguinal mammary glands (number 4)
were collected at PND21 and at PND50 (n = 5 female offspring per PND) and processed for Western blot analyses as described below.

**Cell culture and treatments**

The mouse MEC line HC11 (kindly provided by Jeffrey M. Rosen [Baylor College of Medicine, Houston, TX]) and the human nontumorigenic MEC line MCF-10A (American Type Culture Collection, Manassas, VA) were maintained in growth medium at 37°C in a 5% CO2 incubator as previously described (50, 51). Phenol red-free media supplemented with charcoal-striped serum (CSS) was used for serum starvation (0.5% CSS) and treatments (2.5% CSS). Recombinant mouse or human APN (R&D Systems, Inc., Minneapolis, MN) dissolved in PBS was used at 8 μg/ml and GEN (Sigma Chemical Co., St. Louis, MO) dissolved in dimethylsulfoxide was used at 40 nM. Treatments with PBS or dimethylsulfoxide served as negative controls.

**Serum APN levels**

The concentrations of APN in sera of CAS- and SPI-fed rats collected at PND21 and PND50 were measured using a rat APN ELISA kit (Linco Research, St. Charles, MO). The assay sensitivity was 0.15 ng/ml, and intra- and interassay variations were less than 8.5%.

**Antibodies and Western blot analysis**

Whole-cell extracts were prepared and immunoblotted following previously described protocols (50, 52). Antimouse APN (Abcam, Inc., Cambridge, MA), antiphospho-STAT3 Tyr705 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-STAT3 (Santa Cruz Biotechnology, Inc.) antibodies were each used at 1:1000 dilution. Anti-α-tubulin (Santa Cruz Biotechnology, Inc.) and anti-β-actin (Sigma Chemical Co.) antibodies at 1:2000 dilutions were used as normalizing controls for protein loading. Blots were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL) before reprobing with additional antibodies. Immunoreactive signals were visualized using Amersham ECL Plus (GE Healthcare Life Sciences, Piscataway, NJ) and quantified using the Bio-Rad molecular analyser detection system and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Quantitative real-time PCR (QPCR)**

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). QPCR was carried out using the SYB Green Supermix (Bio-Rad Laboratories) and ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA). Primers (Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org) for PCR were designed to span introns using Primer Express software (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The expression of each target mRNA was calibrated to a standard curve generated using pooled cDNA stocks and normalized to that of TATA-box binding protein (Tbp).

**Cell viability and numbers**

The number of viable cells was evaluated using a cell proliferation assay kit [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); American Type Culture Collection] according to the manufacturer’s instructions. Cells (20,000 cells/well) were seeded in 96-well plates and treated with APN (8 μg/ml) or vehicle (PBS) every 2 d for 6 d. Absorbance values (570 nm) reflect the ability of metabolically active cells to reduce the yellow tetrazolium MTT salts into a purple precipitate. Viable cell numbers were determined under the same treatment conditions with Trypan blue exclusion method (INC Biomedicals, Inc., Aurora, OH). Each experiment was conducted in quadruplicate and repeated twice.

**Acini morphogenesis assay**

MCF-10A cells were seeded on a layer of Matrigel (BD Biosciences, San Jose, CA) in eight-well chamber slides and allowed to form acini as previously described (52). Culture medium containing 2% charcoal-stripped horse serum and 5 ng/ml epidermal growth factor (EGF) without (vehicle alone) or with added APN (8 μg/ml) was refreshed every 4 d. At least 80 acini were counted from five random areas per chamber (×20 objective), with four chambers for each treatment group. Acini number and diameter were assessed at d 12 of culture using a phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany) (×20 objective). Confocal images of 4′,6-diamidino-2-phenylindole-stained acini were collected on a Zeiss LSM510 confocal microscope (×20 objective).

**Fluorescence-activated cell sorting**

Unreated and treated HC11 cells were harvested with trypsin, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol. After propidium iodide (10 μg/ml) staining, at least 10,000 cells were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences). Histograms were generated with the CellQuest software program (BD Biosciences).

**Transient transfection and luciferase assays**

The 4xERE-thymidine kinase (TK)-Luciferase reporter construct was generously provided by Benita S. Katzenellenbogen (University of Illinois, Urbana-Champaign, IL). HC11 cells were cotransfected with the reporter plasmid or empty vector (pGL3B) (each added at 0.5 μg/well) and with Renilla-Luciferase construct (50 ng/well) using Lipofectamine 2000 (Invitrogen), as previously described (52, 53). Incubation with 17β-estradiol (E2) (10 nM), DPN (40 nM), PPT (40 nM), or GEN (40 nM) was carried out without or with APN (8 μg/ml) pretreatment for 24 h. Cells were lysed in buffer (Promega, Madison, WI) and quantitative determination of luciferase activity used a Dual-Luciferase Reporter Assay System (Promega) and a MLX Microplate Luminometer (DynaX Technologies, Chantilly, VA). ERE-Luc activity was normalized to that of Renilla luciferase, which served as an internal control for transfection efficiency. Data are presented as means ± SEM from at least three independent experiments, with each experiment performed in quadruplicates.

**Apoptosis assay**

HC11 cells were seeded in white-walled 96-well plates (15,000 cells/well) and treated with E2 (10 nM) in the presence or absence of GEN (40 nm) and/or APN (8 μg/ml) for 72 h. Cell apoptosis was determined by quantifying caspase-3 and caspase-7 activity using the luminometric Caspase-Glo 3/7 assay.
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kit (Promega) following the manufacturer’s protocol and a MLX Microplate Luminometer (Dynex Technologies).

Data analysis

Computer-assisted statistical analyses were performed using the StatView 5.0 program for Windows. Data were analyzed by Student’s t test, one-way ANOVA, or two-way ANOVA. Differences between means in two-way ANOVA were further analyzed by Tukey’s test. A value of $P < 0.05$ was considered significant.

Results

Dietary SPI increases mammary APN expression

To date, most studies linking APN to obesity-related breast cancer are based on systemic APN levels; hence, the relationship between mammary APN expression and breast cancer occurrence has not been fully determined. Here, we examined whether early exposure to dietary SPI that promoted mammary epithelial differentiation in vivo (54) and conferred protection from chemical-induced mammary tumor formation in female rat offspring (55, 56) is associated with higher APN production in mammary adipocytes. Mammary tissues from female rats exposed to dietary SPI or the control diet CAS beginning at gestation d 4 until tissue collection at PND21 and at PND50 were assessed for APN expression by Western blotting. Levels of APN protein (molecular mass 30 kDa) were higher in mammary tissues of weanling and young adult rats exposed to SPI than to CAS diets (Fig. 1, A and B). Serum APN levels increased with age (PND50 > PND21) (Fig. 1C) and were within the reported physiological range for humans and mice (57). Despite the lower body weights of PND21 and PND50 rats exposed to dietary SPI when compared with the control group (54), there was no comparable increase in systemic APN levels with diet, as was shown for mammary tissue (Fig. 1, A–C). These data provide the first in vivo evidence for dietary regulation of “local” mammary APN expression and suggest that mammary adipose-derived APN may be a mediator of the mammary tumor protective effects of dietary SPI.

APN inhibits mammary epithelial proliferation and promotes cellular apoptosis

The tumor protective actions of APN on breast epithelium are likely mediated at multiple levels, including proliferation, apoptosis, insulin sensitivity, growth factor sequestration, recruitment of proinflammatory cytokines, and angiogenesis (58). To mechanistically dissect the functional outcomes of greater mammary adipocyte APN production/secretion with SPI dietary exposure (Fig. 1, A and B) on neighboring epithelial cells, mouse nontumorigenic MEC HC11 were treated with recombinant mouse APN (8 μg/ml) and assessed for cell proliferation/viability and apoptosis status relative to control (vehicle only treated) cells. Cells administered growth media without added EGF for the same period as APN (no EGF, Fig. 2A) served as positive control in these experiments. APN treatment for 24 or 48 h decreased cell viability, as measured by the MTT assay, relative to control cells (Fig. 2A). Consistent with the MTT assay, APN treatment decreased cell numbers (0.64 ± 0.03 × 10^6) relative to control group (0.83 ± 0.02 × 10^6; $P < 0.05$) (Fig. 2B). Fluorescence-activated cell sorting analysis of cells treated with APN for 12 h showed an increase in the percentage of cells in the sub-G_0 (apoptotic) phase, with no changes noted at other cell cycle stages (Fig. 2, C and D). The higher apoptotic status with APN treatment was correlated with an early, although transient, decrease (compare 6 and 24 h) in transcript levels for survivin (Fig. 2E), the antiapoptotic protein normally up-regulated in human breast cancer (59), relative to untreated cells. APN did not alter the expression
of its receptors APNR1 and APNR2 (Fig. 2F), which have been shown to mediate its pleiotropic actions in target cells (15).

**APN enhances mammary epithelial differentiation**

To further address APN regulation of mammary epithelial function as an underlying mechanism for mammary tumor protection, APN effects on differentiation were assessed in two MEC lines using distinct outcomes. Expression of β-casein mRNA levels, a marker of mammary epithelial differentiation, was evaluated in the ER-positive HC11 cells treated with recombinant mouse APN or vehicle, in the presence of ovine prolactin (5 μg/ml; ovine prolactin-21, AFP-10692C) for 48 h. As shown in Fig. 3A, APN up-regulated β-casein mRNA levels by 6.2-fold (P = 0.006) relative to control (vehicle) cells. In the ER-negative, nontumorigenic human MEC MCF-10A plated on Matrigel-coated chamber slides, APN promoted the formation of acini structures with hollow lumen (Fig. 3, B and C), resembling the morphogenesis of the mammary gland during pregnancy (60). The shift toward the formation of larger acini [40–90 (×10²) μm² range] from the smaller-sized structures [<10 (×10²) μm² range] was observed as early as d 6 (data not shown) and persisted through d 12 of APN treatment (Fig. 3, B–D), indicating induction by APN of early lobuloalveolar differentiation. These results suggest that APN promotes the differentiation of nontumor MEC, irrespective of ER status.

In a previous study using genomewide profiling of mammary tissue of weanling rats exposed to CAS or SPI via maternal diet (54), we found that expression levels of the transcription factor STAT3 were attenuated with dietary exposure to SPI, relative to CAS. Given the reported inhibition by APN of STAT3 signaling (61), the constitutive activation (measured as tyrosine phosphorylation) of STAT3 in breast cancer (62), and findings that promotion of tumor cell survival is partly mediated by activated STAT3 through up-regulation of the antiapoptotic protein survivin (59, 62), we evaluated whether enhanced differentiation of MEC HC11 and MCF-10A induced by APN is mediated by APN inhibition of STAT3 signaling. Western blot analyses showed comparable levels of total STAT3 protein (using anti-STAT3 antibodies) in control and APN-treated HC11 and MCF-10A cells (Fig. 3, E and F). However, whereas HC11 cells demonstrated undetectable/low activated STAT3 levels (anti-pSTAT3 immunoreactivity) with or without APN treatment (Fig. 3E), MCF-10A cells showed robust STAT3 activity that was significantly attenuated by APN (Fig. 3F). These results suggest that context-dependent signaling mechanisms may underlie the biological response of MEC to the protective effects (e.g. increased differentiation) of APN.

**FIG. 2.** APN inhibits proliferation and promotes apoptosis of ER-positive HC11 cells. A, HC11 cells treated with APN (8 μg/ml) for 24 or 48 h had decreased cell proliferation compared with control cells (vehicle treated) as measured by the MTT assay. Cells incubated in medium without added EGF for the same duration served as a positive control for decreased cell growth. B, Cells were seeded in 100-mm dishes and treated in same manner as for MTT assay. Viable cells were quantified by the trypan blue exclusion method after 12 h of treatment using a hemocytometer. C and D, Control and APN-treated cells were analyzed by fluorescence-activated cell sorting. E and F, Transcript levels of the antiapoptotic protein survivin and of APNR1 and APNR2 were quantified by QPCR and normalized to Tbp. Results are mean ± SEM from two independent experiments performed in triplicate; *, P < 0.05 relative to control.
APN promotes ERβ transcriptional activity

In light of earlier findings suggesting an inverse estrogen (E2)/APN connection in human breast cancer cells (63), we addressed the participation of ER on APN signaling in the nonmalignant, ER-positive HC11 cells. We first evaluated whether APN altered the expression levels of ERα and ERβ isoforms in these cells at the mRNA and protein levels, by QPCR and Western immunoblottings, respectively. Cells treated with APN for 6 h showed lower ERα (2-fold) and higher ERβ (2-fold) transcript levels, relative to control (vehicle treated) cells (Fig. 4A). A more robust effect (~10-fold) of APN on ERβ protein expression was observed when compared with vehicle-treated cells, in contrast to the lack of effect noted on ERα protein levels (Fig. 4, B and C). On a per protein basis, however, expression levels of ERα were greater than those of ERβ in these cells. Thus, APN may promote ERβ signaling in HC11 cells by increasing ERβ relative to ERα gene and protein expression.

To evaluate the functional consequence of the preferential induction by APN of ERβ relative to ERα expression in mammary epithelium, the transcriptional response of HC11 cells to ERβ agonists DPN and GEN (20-fold higher affinity for ERβ than for ERα) (40) were compared with those of E2 (binds ERα and ERβ with similar affinities) and ERα-specific agonist PPT, in control and APN-treated cells. Cells were transfected with the estrogen-responsive 4xERE-TK-Luciferase (4xERE-TK-Luc) construct as a reporter for ER transactivation. In cells transfected with the 4xERE-TK-Luc plasmid, treatment with E2 and with PPT resulted in similar (2-fold) increases in Luc promoter activity, relative to untreated cells (Fig. 4D). GEN elicited a modest, although significant, increase in Luc reporter activity relative to nontreated cells; the magnitude of the increase was lower than that obtained with E2 and PPT treatments (Fig. 4D). DPN-treated cells displayed Luc reporter activity that, although not statistically significant, was numerically higher to those of untreated cells. Because both ligand-activated ER isoforms are known to bind the ER recognition sequence in the 4xERE-TK-Luc construct with comparable affinities, results are consistent with the predominant expression of ERα relative to ERβ in non-APN-treated HC11 cells (Fig. 4, B and C). Interestingly, APN treatment had no effect on the magnitude of ER-mediated transcriptional responses individually elicited by E2, DPN, or GEN (which all bind ERβ) (Fig. 4D), despite APN induction of ERβ expression levels in these cells (Fig. 4, A–C). These collective data suggest that APN actions may be manifest only under specific cellular contexts, possibly when both ER are functionally active (i.e., ligand activated) or under conditions...
FIG. 4. APN induces ERβ expression and synergizes with GEN to promote ERβ transcriptional activity. A, Transcript levels of ERα and ERβ were quantified by QPCR in HC11 cells treated with APN (8 μg/ml) or vehicle (control) for 6 h. B, Western blot analysis of ERα and ERβ in lysates from cells treated as in A. Each lane contains 20 μg of total protein. C, Protein levels in B were quantified by densitometry and normalized to β-actin; *, P < 0.05 relative to control. D–H, HC11 cells were transfected with 0.5 μg of 4xERE-TK-Luc promoter/reporter construct in the presence or absence of control siRNA (scRNA) or siRNA targeting ERα or ERβ and treated with APN and specific ER ligands, alone and in combination. D, Cells were treated with vehicle (control), E2, DPN, PPT, or GEN in the presence or absence of APN (8 μg/ml) for 24 h, and lysates were subsequently analyzed for luciferase activity. Effects of GEN and APN on the transcriptional response of E2 (E), DPN (F), and PPT (G) were analyzed. H, Effects of knockdown of ERα and ERβ by respective siRNA on Luciferase reporter activity in cells treated with E2 + GEN + APN were evaluated, relative to nontargeting (scRNA) siRNA. Values are means ± SEM from at least three independent experiments performed in triplicate. Means with different letters differed from control (D–G) or nontargeting scRNA (H) at P < 0.05. RLU, Relative luminescence unit.
require the availability of ER-specific ligands (e.g., GEN for ERβ) that can recruit specific coactivators and/or repressors (46).

**GEN synergizes with APN to influence ligand-activated ERβ transcriptional activity**

We have previously shown that GEN at physiologically relevant doses (40 nM, 2 μM) elicited increased differentiation, decreased proliferation, and promoted apoptosis of MEC in vitro, consistent with it being a major bioactive component of soy foods with breast cancer protective effects (50–52, 64). Our findings, thus far, suggest that in vivo, mammary stromal adipocytes and neighboring epithelial cells constitute coordinate targets of GEN action. To examine whether stromal adipocyte-derived APN promotes GEN action in MEC by enhancing GEN activation of ERβ signaling, we evaluated whether GEN influences the transcriptional activity of GEN in the presence of the physiologically relevant ligand E2 and compared these effects with those elicited with specific ERβ (DPN) and ERα (PPT) agonists. HC11 cells were treated with E2 (10 nM), DPN (40 nM), or PPT (40 nM) in the presence or absence of GEN (4 μg/ml) and/or GEN (40 nM), and 24 h later, 4xERE-Luc activity was analyzed. GEN in the absence of GEN had no effect on basal ERE-TK-Luc activity of E2 (Fig. 4E). Similar for E2, GEN had no significant effect on DPN-activated promoter activity in the absence of GEN (Fig. 4F). By contrast, GEN increased PPT-activated transcriptional responses in non-GEN-treated cells (Fig. 4G). Interestingly, APN cotreatment with GEN significantly influenced these cells’ transcriptional responses to all ligands (Fig. 4, E–G). In particular, APN enhanced Luc promoter activity in GEN + E2-treated (Fig. 4E) and GEN + DPN-treated (Fig. 4F) cells while decreasing this activity in PPT + GEN-treated cells to PPT-alone levels (Fig. 4G).

Given that GEN preferentially binds ERβ, the above findings are consistent with GEN + APN promoting ERβ signaling by increasing ERβ homodimer (with DPN) or ERα/ERβ heterodimer (with E2) transcriptional activities at the expense of ERα homodimer-mediated (with PPT) transactivity. To address this, ERα or ERβ expression was knocked down in APN + GEN + E2-treated cells transfected with 4xERE-TK-Luc plasmid, using a pool of siRNA targeting each ER isoform. A decrease by approximately 25% in 4xERE-TK-Luc promoter activity was observed with ERα siRNA or ERβ siRNA added at equivalent concentrations (50 nM), relative to cells treated with nontargeting siRNA [scrambled RNA (scRNA), 50 nM] (Fig. 4H). We determined 50 nM as an optimal dose with effective knockdown (~70%) at the mRNA level (data not shown). Interestingly, the magnitude of the decrease in promoter activity achieved by targeting either ERα or ERβ with respective siRNA was comparable with the extent of promoter activity induction in APN + GEN + E2-treated cells, when compared with those treated with E2 + GEN in the absence of GEN (Fig. 4E).

**APN/GEN promotion of ERβ signaling is associated with up-regulation of proapoptotic and prodifferentiation gene expression**

To determine whether APN promotion of GEN-mediated ERβ signaling may underlie the observed in vivo enhancement of MEC differentiation associated with exposure to SPI or GEN-supplemented diets, leading to mammary tumor protection (50–52, 55, 56, 64), we determined the expression of select proapoptotic (Bad, p53), antiapoptotic (Bcl2, survivin), and prodifferentiation/proapoptotic (Pten) genes in APN + GEN-treated cells cotreated with either E2, DPN, or PPT. Because APN induced ERβ signaling in the absence (Fig. 4, A–C) or presence of E2 (Fig. 4E), and in view of previous findings showing enhancement of cell proliferation by E2 upon blockage of ERβ in HC11 cells (65), the activity of proapoptotic proteins caspase-3 and caspase-7 was initially evaluated (Fig. 5A). Further, cells cotreated with APN + GEN + E2 showed increased Pten and decreased survivin expression, with no comparable changes in Bad, Bcl2, and p53 transcript levels, when compared with non-APN-treated controls (Fig. 5B). Cells cotreated with GEN in the presence of GEN + APN elicited changes in gene expression levels consistent with GEN + GEN inhibition of ERα signaling and promotion of ERβ-mediated transcriptional responses (Fig. 5C). In particular, transcript levels for Bad, p53, and Pten were increased, whereas those for Bcl2 and survivin were attenuated with GEN alone, consistent with the extent of promoter activity induction in APN + GEN + E2-treated cells, when compared with those treated with E2 + GEN in the absence of APN (Fig. 4E).

**Discussion**

In this study, we present a novel mechanism for dietary regulation of ERβ signaling in MEC involving the adipo-
to achieve enhanced differentiation and integrate the local stromal adipocyte environment in orchestrating ER-dependent and ER-independent mechanisms to promote neighboring epithelial resistance to tumorigenic agents (66).

A major novel finding of the present study is the robust local induction of APN protein in mammary tissue by dietary intake of soy protein at peripuberty, a critical stage of mammary gland development. Although decreased serum APN levels are associated with increased risk and aggressiveness of breast cancer (12–14), a detailed role for local mammary tissue-derived APN in the promotion of breast cancer has not been fully established. Our current and previous (54) analyses of mammary glands of peripubertal rats from dams consuming SPI diets at amounts modeling the regular intake of soy-rich foods by the Asian population demonstrate the impact of early maternal diet on the adiposity of the developing mammary gland. Further, our identification of APN as a diet-regulated mammary adipocyte-secreted protein that mediates differentiation events in normal (nontumorigenic) ER-positive (HC11) and ER-negative (MCF-10A) MEC has important implications for the suggested intrauterine origins of breast cancer risk. In support of the latter, recent findings implicate a role for maternal obesity in the programming of APN signaling in offspring (67) and for systemic APN in children as a presumptive determinant of obesity-related diseases in later adult life (68–70).

Another novel finding from this study is the demonstration of APN/ER cross talk, leading to the promotion of ERβ transcriptional activation in normal MEC. Although a connection between APN and ER signaling in breast cancer cells has been previously raised (63), the current study takes a further step by addressing the influence of the dietary bioactive factor GEN in APN/ER cross talk in normal (nonbreast cancer) epithelial cells. We showed that APN in the presence of GEN modified the direction of ERβ isoform expression in HC11 cells, resulting in a higher ratio of ERβ to ERα at the levels of transcript and protein. Second, APN in concert with GEN altered the transcriptional responses of HC11 cells

![Graph](https://academic.oup.com/endo/article-abstract/152/9/3409/2457215)

**FIG. 5.** Synergistic actions of APN and GEN enhance apoptosis in MEC. A, HC11 cells were treated with E2 (10 nM) in the presence or absence of GEN (40 nM) and/or APN (8 μg/ml) for 72 h and subsequently assessed for caspase-3/7 activity, as described under Materials and Methods. Means with different letters differed from control at P < 0.05. Representative graph from two independent experiments, each performed in quadruplicate is shown. B–D, Gene expression of proapoptotic, differentiation-related (Bad, p53, and PTEN), and antiapoptotic (Bcl2, survivin) proteins was quantified by QPCR in cells treated with E2 + GEN (B), DPN + GEN (C), and PPT + GEN (D) in the presence (+APN) or absence (−APN) of APN for 24 h. Tbp was used as a normalizing control; *, P < 0.05 relative to (−) APN group. Bad, Bcl2-associated agonist of cell death; p53, tumor suppressor p53; Bcl2, B-cell leukemia/lymphoma 2; RLU, relative luminescence unit.
to E2 and pure ERα and ERβ agonists. With E2, which normally induces formation of ERα homodimers and ERα/β heterodimers, and with PPT, which induces solely ERα homodimers, the presence of APN + GEN promoted the formation of ERα/β heterodimers and/or ERβ homodimers at the expense of ERα homodimers, thus enhancing GEN-mediated ERβ activation. Although we did not quantify the magnitude of the shift from ERα to ERβ signaling with APN + GEN cotreatments, the functional outcome of increased ERβ signaling was manifested as higher expression of proapoptotic and prodifferentiation genes, both of which are hallmarks of decreased breast cancer risk. The demonstration that Pten expression was similarly induced by APN + GEN in concert with E2 or DPN but not PPT suggests Pten as a gene target of ERβ transactivation in MEC. Further, the induction of Bad, Bcl2, and p53 expression by APN + GEN + DPN and the inhibition of their respective expression by APN + GEN + PPT reflect the opposing effects of ERβ on ERα transcriptional activity. These findings are consistent with ERβ attenuation of ERα-mediated transcriptional activation (34–42, 43) and the negative consequence of ERβ signaling on ERα-enhanced epithelial proliferation (65). Importantly, these results support the notion that mammary adiposity (using APN as a measure) regulates the balance of ERα vs. ERβ signaling in the presence of selective estrogen modulators, to alter MEC phenotype. Although it is also possible that APN action may occur via non-classical (ligand independent) mechanisms involving MAPK-induced ER phosphorylation (71), we believe that this is unlikely, because APN effects on induction of ERβ transcriptional activity occurred only in synergy with the selective ERβ ligand GEN. Our findings that APN elicited a decrease in ERα transcript but not protein levels raise the possibility of distinct transcriptional and posttranslational regulation by APN of ER proteins; however, to our knowledge, this has not been previously reported.

An important feature of APN that was additionally demonstrated in the present study is the ability of this adipokine to induce mammary epithelial differentiation by a mechanism independent of ER signaling. Specifically, in ER-negative MCF-10A cells, APN promotion of epithelial differentiation was associated with its inhibition of activated STAT3 signaling as measured by decreased levels of phosphorylated STAT3. These results are consistent with previous studies documenting multiple proapoptosis signaling cascades blocked by APN (58) and underscore the broad implications of dysfunctional adipose tissue on numerous metabolic, inflammatory, and chronic diseases. Further, given that the lack of ER signaling simulates that of prepuberty and postmenopausal status in women, these findings provide a mechanism by which adiposity can influence breast cancer risk.

In summary, we identified dietary regulation of mammary-specific APN production to occur at an early developmental window, and which may guide the direction of ligand-activated ER signaling in neighboring epithelial cells (Fig. 6). This model predicts that maintenance of dysfunctional adipose tissue elicited by an obesogenic state will have significant deleterious consequences to mammary breast health beginning at puberty. Our results also raise the intriguing (although yet untested) possibility that increased exposure to environmental agents with distinct selective ER modulator activities in the face of the obesity pandemic among children and young adults (72) may underlie, in part, the rising incidence of breast cancer worldwide.
Acknowledgments

We thank Dr. Frank A. Simmen and Dr. Stewart MacLeod (University of Arkansas for Medical Sciences) for insightful discussions during the course of this study.

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This work was supported by grants from the United States Department of Agriculture (CRIS 6251-510002-06S, Arkansas Children’s Nutrition Center), the University of Arkansas Children’s University Medical Group, and the Fashion Footwear of New York (funded through University of Arkansas for Medical Sciences) (R.C.M.S.). O.M.R. is supported by the Department of Defense Breast Cancer Research Program Predoctoral Fellowship W81XWH-10-1-0047.

Disclosure Summary: The authors have nothing to disclose.

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