Nutrition and Cancer

Dietary Exposure to Whey Proteins Alters Rat Mammary Gland Proliferation, Apoptosis, and Gene Expression during Postnatal Development[1]

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ABSTRACT We have found that AIN-93G diets made with whey protein hydrolysate (WPH) reduce 7,12-dimethyl-benz[a]anthracene (DMBA)-induced tumor incidence in Sprague-Dawley (Harlan) rats relative to those fed a diet with casein (CAS). Herein, we replicated these findings in another Sprague-Dawley substrain (Charles River) and examined whether WPH protective effects were associated with altered mammary gland differentiation status and expression of the tumor suppressor phosphatase and tensin homolog deleted in chromosome ten (PTEN). Mammary tumor incidence was lower in DMBA-treated rats fed WPH than in those fed CAS. Mammary glands of WPH- and CAS-fed rats were isolated at weaning [postnatal day (PND) 21–28] and at an early adult stage (PND 50–53) and analyzed for proliferative (proliferating cell nuclear antigen immunoreactivity), apoptotic (terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling), and differentiation (β-casein) indices, as well as for PTEN mRNA and protein levels. PND 50–53 rats fed WPH showed decreased proliferation and increased apoptosis in mammary structures, coincident with increased mammary β-casein gene expression, decreased terminal end-bud numbers, and increased ductal lengths, relative to same-age CAS-fed rats. When challenged with DMBA for 24 h, mammary glands of PND 53 CAS-fed rats had decreased cell survival in both terminal end buds and ductal epithelium, while the mammary glands of WPH-fed rats were not altered from pre-DMBA levels. At 7 d post-DMBA, mammary glands of CAS- and WPH-fed rats exhibited comparable apoptotic indices. Mammary PTEN expression was higher in WPH- than in CAS-fed rats at PND 21–28, but was not different in young adults fed either diet. Results demonstrate that dietary WPH advances mammary gland differentiation during neonatal development and suggest that the transiently increased expression of the pro-apoptotic signal PTEN during a sensitive developmental window may partly underlie the cancer protective effects of WPH. J. Nutr. 134: 3370–3377, 2004.

KEY WORDS: • *mammary gland* • *development* • *cancer* • *PTEN* • *whey proteins*

Breast cancer is a debilitating disease that affects 1 of 9 women in their lifetime and is the leading cause of cancer deaths among women in the Western hemisphere, next to lung cancer (1). Epidemiological studies have suggested that the risk of adult diseases, including breast cancer, has origins during early stages of development that can be influenced by nutrition (2). For example, the linkage of a high intake of soy products by Asian women, especially during adolescence (3,4), to low breast cancer incidence has been widely explored (5), albeit methodological differences and limitations in assessing dietary intake have resulted at times in inconclusive and inconsistent data (6). Our group and others have used the 7,12-dimethyl-benz[a]anthracene (DMBA)[3]-induced rat mammary model to examine the effects of diet on onset of mammary cancer in adult females (7–10). Our studies demonstrated reduced incidence of mammary carcinoma in rats fed soy protein isolate (SPI) or whey protein hydrolysate (WPH) relative to rats fed control diets made with casein (CAS) as the sole protein source (9). Several mechanisms may be working simultaneously to reduce the incidence of chemically induced cancer with dietary intake of SPI or WPH. One mechanism of protection may involve the ability of SPI and WPH to decrease tissue expression of the cytochrome P450 gene family-1 members, which convert the procarcinogen DMBA to the active carcinogen (10). Another mechanism may involve progesterone receptor signaling (11). In this regard, the stage of mammary gland differentiation at the time of carcinogen insult may determine the susceptibility of mammary epithelial cells to abnormal proliferation leading to tumorigenesis (7,8). Hence, it was posited that dietary factors that induce mammary gland differentiation over proliferation are

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[3] Abbreviations used: CAS, casein; DE, ductal epithelium; DMBA, 7,12-dimethyl-benz[a]anthracene; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; PCNA, proliferating cell nuclear antigen; PND, postnatal day; PR, progesterone receptor; PTEN; phosphatase and tensin homolog deleted in chromosome ten; QPCR, quantitative real-time polymerase chain reaction; SPI, soy protein isolate; TEB, terminal end buds; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling; WPH, whey protein hydrolysate.

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likely to be protective (12). Dietary factor–induced enhancement of apoptosis of cells containing genetic lesions and mutations that predispose them to tumorigenesis has been suggested as another viable mechanism for protection (13). Recent studies that focused on genistein, the major phytoestrogen present in soy, documented its ability to decrease the circulating levels of ovarian hormones (14), reduce the percentage of estrogen receptor–positive mammary epithelial cells (15), and upregulate epidermal growth factor receptor expression (16) in rat models. Taken together, these findings demonstrate that dietary factors utilize diverse signaling pathways to confer biological effects in target cells.

PTEN (phosphatase and tensin homolog deleted in chromosome ten), a dual protein and lipid phosphatase that dephosphorylates the 3′ position of the signaling molecule phosphoinositide-3,4,5-triphosphate, plays a critical role in controlling cell survival through its antagonistic effects on the Akt/PK-B pathway (17,18). Recently, PTEN was also demonstrated, via its protein phosphatase activity, to suppress cell growth through its inhibition of the growth factor–activated ras/mitogen activated protein kinase (MAPK) pathways (19). PTEN is mutated in many types of advanced cancers, and loss or diminished expression of PTEN leads to a high incidence of tumors (20,21). A role for PTEN in the etiology of breast cancer is underscored by the findings that decreased PTEN expression is associated with invasive cancer and poor prognosis (22,23). Mice with mammary-specific deletion of the PTEN gene exhibited abnormal proliferation and apoptosis of mammary tissues, leading to neoplasia (24); conversely, PTEN overexpression in the mammary epithelium resulted in severely reduced mammary epithelial cell proliferation and increased apoptosis, pointing to its role in the functional development of the mammary gland (25). Thus, enhancement of PTEN expression leading to inhibition of the Akt/PK-B and/or MAPK mediated cell survival pathways may constitute a contributory factor to the protective mechanisms of dietary components on mammary tumorigenesis.

Whey proteins from milk have been shown to exert multiple health benefits including the enhancement of immune function, modulation of adiposity [see review (26)], and, from our own studies with rat models, protection from DMBA-induced carcinogenesis (9). Because whey has many bioactive components, it is anticipated that these diverse health benefits of whey may be mediated through multiple signaling pathways. In the present study, we tested the hypothesis that WPH reduces chemically induced carcinogenesis by advancing the maturation and differentiation status of mammary cell, in part through regulation of PTEN expression and, hence, cell survival at an early developmental window. Toward this end, we evaluated: *1*) the differentiation, proliferation, and apoptotic status of mammary glands from rats fed CAS or WPH, at weaning [postnatal day (PND) 21–28] and at an early adult (PND 50–53) stage; *2*) PTEN expression in mammary glands from rats fed CAS or WPH diets at these same developmental time points; and *3*) the apoptotic sensitivity and PTEN expression of mammary glands from rats fed CAS or WPH diets when acutely challenged with DMBA.

MATERIALS AND METHODS

Rats, diet, and carcinogen treatment. Time-mated Sprague-Dawley rats were purchased from Charles River Laboratories and housed individually in polycarbonate cages under controlled temperature (24°C), humidity (40%), and light (12-h light/dark cycle). Rats at gestation day 4 were randomly assigned to 1 of 2 semipurified isocaloric diets made according to the AIN-93G diet formula (27) that differed only by protein source: *1*) CAS and *2*) a partial hydrolysate of whey (WPH) (New Zealand Milk Products) as described previously (9). Rats were given ad libitum access to food and water. The litter sizes of CAS and WPH dams did not differ. Ten pups were assigned to each dam for suckling. The offspring were weaned to the same diet as their mothers and were fed the same diets throughout the study. Animal care and handling were in accordance with the Institutional Animal Care & Use Committee guidelines of the University of Arkansas for Medical Sciences.

Experiment 1. At PND 21 and 50, female pups fed CAS- or WPH-based diets ($n = 8$ per diet group) were killed and the inguinal mammary gland (gland 4) pair was removed. The left gland was processed for whole mounts, whereas sections of the right gland were fixed for paraffin embedding, immediately homogenized in TriZol for RNA extraction, or frozen at −80°C for later protein extraction (see below). The rest of the pups at PND 50 ($n = 48$ for CAS, $n = 44$ for WPH) were subjected to DMBA-induced tumorigenesis following previously described protocols (9). Pups were orally gavaged with sesame seed oil (Sigma) containing DMBA (Sigma) at a dose of 0.08 g/kg body wt. Rats were weighed weekly and beginning at 3 wk after DMBA administration were palpated twice weekly for tumors. The initial detection date of tumor for each rat and subsequent appearance of new tumors and their locations were recorded. Rats from both diet groups were killed at 70 d post-DMBA. Tumors were analyzed for pathology, as described in previous studies from this group (9).

Experiment 2. At PND 28 and 53, female pups ($n = 12$) from each diet group were killed, and the inguinal mammary gland (gland 4) pair was removed. The left and right mammary glands were analyzed, as described in Expt. 1 (above). The remainder of the PND 53 pups fed the lifetime CAS- or WPH-based diets ($n = 16$ pups for each diet) were orally gavaged with DMBA (0.08 g/kg rat). At 24 h and 7 d after DMBA administration, female pups ($n = 8$) from each diet group were killed and the mammary gland pair 4 was removed for analyses, as described above (Expt. 1).

Whole-mount, immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling (TUNEL). Mammary gland whole mounts were prepared following previously described protocols from this laboratory (11). For immunohistochemical analysis, tissues were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Five micrometer–thick sections were mounted on poly-lysine–coated microscope slides (Fisher Scientific), deparaffinized, and rehydrated in graded alcohols (11). Endogenous peroxidase activity was quenched in 3% hydrogen peroxide. For antigen retrieval, sections were sequentially microwaved for 105 s at power 10 and then for 10 min at power 1 in Citra Plus (Biogenex). The sections were then placed in a blocking solution (Cas Block; Zymed) for 20 min. Incubations with anti-PTEN antibody [mouse monoclonal IgG raised against the carboxy-terminus of human PTEN (A2B1, Santa Cruz Biotechnology)] and anti-proliferating nuclear antigen (PCNA) antibody [mouse monoclonal IgG raised against rat PCNA (clone PC10, Dako)] diluted at 1:100 and 1:600, respectively, were carried out overnight (PTEN) or for 1.5 h (PCNA) at 4°C in a humidity chamber. Sections were sequentially incubated with biotinylated goat anti-mouse IgG secondary antibody for 30 min and streptavidin-linked horseradish peroxidase (Bio-Rad Laboratories) for 30 min at room temperature. Immunoreactive proteins were detected by incubation with the chromagen 3,3′-diaminobenzidine tetrahydrochloride (Dako), and sections were counterstained with hematoxylin, dehydrated, cleared, and coverslipped for examination under a microscope. Four randomly chosen fields (200X magnification) per slide per rat were counted for the presence of immunostaining. Only cells showing a dark-brown color were scored for positive expression. The proliferation index was calculated as the percentage of PCNA-positive cells relative to the number of cells counted.

TUNEL assay to detect apoptotic cells was performed following the manufacturer's instructions (Oncogene). Paraffin-embedded sections (4 μm) were treated with proteinase K (20 mg/L) for 20 min, rinsed in Tris-buffered saline, and then incubated with terminal deoxynucleotidyl transferase and nucleotide mixture for 1 h at 37°C in a humidified chamber. TUNEL-positive cells were counted from 3 randomly selected fields (200X magnification) per slide, and 3 slides were evaluated for each tissue section, with each section representing

TABLE 1

Primer sequences for quantitative real-time PCR

Gene	Forward primer	Reverse primer
PR	5′-CCAGTCAGTGGCCTTTCCTAAT-3′	5′-GTGTTGTCATGCCCTGCATAAA-3′
PTEN	5′-CAATGTTCAGTGGCGGAACTT-3′	5′-GGCAATGGCTGAGGGAACT-3′
$p21^{WAF}$	5′-CTGGTGATGTCCGACCTGTTC-3′	5′-CTGCTCAGTGGCGAAGTCAAA-3′
β-Casein	5′-TTCATCCTTGCCTGCCTTGT-3′	5′-GCACCTGTCCCATGAGTTTCA-3′
Cyclin D1	5′-TGCCGAGAAGTTGTGCATCT-3′	5′-GAGGGTGGGTTGGAAATGAA-3′
Cyclophilin A	5′-AAGCATACAGGTCCTGGCATCT-3′	5′-TGCCATCCAGCCACTCAGT-3′
IGF-2	5′-GAGCTTGTTGACACGCTTCAGT-3′	5′-TGCGGAAGCAGCACTCTTC-3′
IGFBP2	5′-CACAGCAGGTTGCAGACAGTG-3′	5′-TGACCTTCTCCCGGAACACA-3′
κ-Casein	5′-TCTGTCCTGAACCGCAATCAT-3′	5′-GGCATTGGTTGCCATTTGA-3′

an individual animal. The total number of TUNEL-positive cells was divided by the number of mammary gland structures (terminal end buds, TEB; ductal epithelium, DE) evaluated. For both immunohistochemical and TUNEL assays, tissue sections representing 4 individual rats per diet were analyzed.

RNA isolation and quantitative RT-PCR. Total RNA was extracted from mammary tissues using TriZol reagent (Invitrogen). Integrity of the isolated RNAs was confirmed using the RNA 6000 Nano LabChip kit with the Agilent 2100 Bioanalyzer System (Agilent Biotechnologies). Approximately 1 μg of total RNA was reverse-transcribed in 10 μL of reaction volume using random hexamers and MultiScribe reverse transcriptase in a 2-step RT-PCR reaction (Applied Biosystems). For PCR, primers that span intron/exon junctions were designed using PrimerExpress software (Applied Biosystems) to prevent amplification of residual genomic DNA; these are summarized in **Table 1**. Quantitative PCR (QPCR) was performed with the SYBR Green detection system (Applied Biosystems) using an ABI Prism 7000 sequence detector. Thermal cycling conditions included preincubation at 50°C for 2 min, DNA polymerase activation at 95°C for 1 min, and 40 PCR cycles for 15 s at 95°C and for 1 min at 60°C. The transcript levels for each gene were calculated at cycle threshold values (C_T) at which each fluorescent signal was first detected above background; these were determined using the ABI Prism 7000 SDS software (Version 1.0) (Applied Biosystems). mRNA levels were normalized to that of the housekeeping gene cyclophilin A to control for input RNA and are reported as overall means ± SEM from 8 (Expt. 1) or 12 (Expt. 2) individual rats per dietary group.

Western blotting. Nuclear and cytoplasmic proteins were isolated from frozen mammary glands using an extraction system that utilized low- and high-salt buffers, following the manufacturer's instructions (NE-Per; Pierce Biotechnology). Protein concentrations were determined by Lowry's method, using bovine serum albumin as standard. Proteins (100 μg) were fractionated by electrophoresis in SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Laboratories), and probed using anti-PTEN (1:1000; A2B1, Santa Cruz Biotechnology,) and anti-PCNA (1:1000) antibodies. After incubation with secondary horseradish peroxidase–conjugated antibodies (Santa Cruz Biotechnology), immunoreactive proteins were detected using the Western Lightning chemiluminescence reagents (Pierce Biotechnology). Signals were captured and quantified with the Bio-Rad molecular analyst detection system and the associated Quantity One Software (Bio-Rad). Membranes were stained with Coomassie blue to verify equal protein loading among samples.

Statistical analysis. Results are means ± SEM. Significant differences between two groups were determined by Student's *t* test or Fischer's exact test. Proliferation and apoptotic data were subjected to two-way ANOVA using diet, structure, age, and time of DMBA exposure as the main effects in separate analyses, followed by inspection of all differences between pairs of means by Tukey's test. Differences with P values < 0.05 were considered significant.

RESULTS

Tumor protection by WPH in DMBA-administered rats. Pups fed WPH as the sole protein source showed greater protection from DMBA-induced tumorigenesis when compared to those fed CAS (**Table 2**). At 70 d post-DMBA administration, when 87.5% of CAS-fed rats showed at least 1 tumor, only 56.8% of WPH-fed rats had palpable tumors ($P = 0.001$). Most of these tumors (92.9 and 96%, respectively, for CAS and WPH) showed cancer pathology, as defined in our earlier study (9). Tumor latency differed between diet groups (WPH > CAS; $P < 0.05$), whereas tumor multiplicity did not (Table 2). These results are consistent with our previously published studies (9) and confirm the early protective effects of WPH on tumor initiation.

Mammary cell proliferation and survival. Compared with CAS-fed rats, WPH-fed rats at weaning showed a tendency for decreased PCNA immunostaining in TEB ($P = 0.06$) (**Fig. 1**A). TEB from PND 50 WPH-fed rats displayed less ($P < 0.05$) PCNA immunoreactivity than those of CAS-fed counterparts. Proliferation in lobuloalveolar structures also tended to be diminished by WPH in young adult rats ($P = 0.07$). The levels of PCNA immunoreactivity in DE structures were not affected by diet at either developmental stage (Fig. 1*A* and *B*). Western blot analysis of nuclear extracts prepared from mammary tissues confirmed the significant decrease in levels of PCNA protein with WPH in PND 50 rats, which was not observed in PND 28 rats (Fig. 1*C* and *D*).

Mammary tissues from PND 28 and 50 rats fed the control diet (CAS) had comparable numbers of TUNEL-positive cells in TEB (**Fig. 2**A and *B*). By contrast, the number of staining (apoptotic) cells was lower in DE of casein-fed PND 50 rats (0.30 ± 0.08 cells/structure; $n = 4$ rats) compared to younger rats (0.82 ± 0.06 cells/structure; $n = 4$ rats; $P = 0.002$) (Fig. 2*A* and *B*). PND 28 rats fed CAS- and WPH-based diets did

TABLE 2

Mammary tumor protection in rats fed a WPH-based diet[1]

Diet	*n*	Tumor multiplicity[2]	Tumor latency[3]	Tumor incidence[4]	Tumor pathology[5]
		n/rat	*d*	%	
CAS	48	4.5 ± 0.50	54.3 ± 1.6	87.5	92.9
WPH	44	3.8 ± 0.65	61.7 ± 1.8*	56.8#	96.6

[1] Values are means ± SEM or %. *Different from CAS, $P < 0.05$ (*t*-test). #Different from CAS, $P = 0.001$ (Fisher's exact test).

[2] Mean number of tumors/rat.

[3] Lag time between DMBA administration and tumor development per rat.

[4] Percentage of tumor-positive rats at 70 d post-DMBA.

[5] Percentage of rats with tumors showing cancer pathology at 70 d post-DMBA.

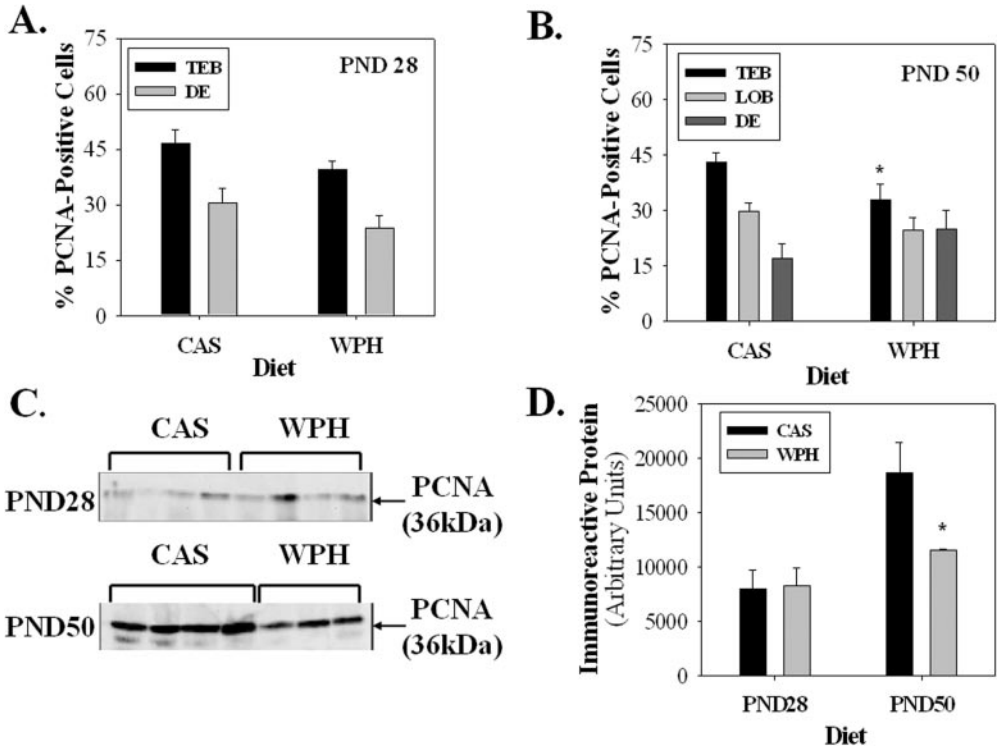

FIGURE 1 Cell proliferation status of mammary glands of CAS- and WPH-fed rats at PND 28 and 50, assayed by PCNA immunohistochemistry and Western blot. PCNA immunostaining (*A*, *B*) is expressed as a percentage of the total number of TEB and DE cells for a given field. Data are means ± SEM, $n = 4$, with 2 sections evaluated from each of 4 rats per diet. (*C*) Western blot analysis of nuclear extracts from mammary tissue of PND 28 and 50 rats fed CAS- or WPH-based diets using anti-PCNA antibody. Each lane contains 100 μg total protein and represents an individual rat. (*D*) Graphical representation of immunoreactive PCNA (mol wt 36 kDa) in *C* above (means ± SEM). Diet affected the proliferative response at PND 50 (*$P < 0.05$). Diet-by-structure ($P = 0.02$) (*B*) and diet-by-age ($P < 0.05$) (*D*) interactions were observed by two-way ANOVA.

not differ in the numbers of TUNEL-positive cells in TEB and DE (Fig. 2*A*). However, both types of mammary structures in PND 50 WPH-fed rats had more TUNEL-positive cells than CAS-fed rats (Fig. 2*B*).

The incidence of mammary carcinomas has been shown to be positively correlated with the number of TEB in the mammary glands of young adult virgin rats at the time of carcinogen exposure (28). To investigate the effect of WPH on TEB numbers in mammary glands of rats immediately prior to DMBA administration, we examined whole mount preparations from mammary tissues of young adult (PND 53) rats fed CAS or WPH. Relative to CAS-fed rats, those fed a WPH-based diet had fewer ($P < 0.05$) mammary TEB and showed a tendency ($P = 0.07$) for increased ductal extension into the mammary fat pad (**Fig. 3**). For a given area (27.4 mm^2), the mean numbers of TEB for WPH- and CAS-fed rats were 15 and 20, respectively ($n = 4$ rats/diet). Further, for WPH-fed rats, the mean distance of the TEB from the main ductal structure was 22% greater than for CAS-fed rats ($n = 4$ rats/diet, $P = 0.07$).

Expression of growth and differentiation genes in the developing mammary gland. The basal expression levels of genes with known functions in cellular proliferation and differentiation (25,29–31) and representing signaling pathways (e.g., PR, PTEN, IGFs) operative in mammary gland development (32,33) were measured in weaning (PND 21–28) and young adult (PND 50–53) CAS-fed rats (**Fig. 4**). When normalized to that of the constitutively expressed gene cyclophilin A, the expression of these genes fell into three categories: *1*) increased (PR, β- and κ-caseins, PTEN); *2*) decreased (cyclin D1, IGF-2); and *3*) did not differ (IGFBP2) with age.

WPH effects on mammary gland gene expression. PTEN transcript levels were greater ($P < 0.05$) in mammary glands of WPH-fed rats at weaning relative to that of CAS-fed rats of the same age (**Fig. 5**). However, mammary PTEN levels did not differ for young adult (PND 50) rats fed either diet. At PND 21–28, there was no association between WPH-en-

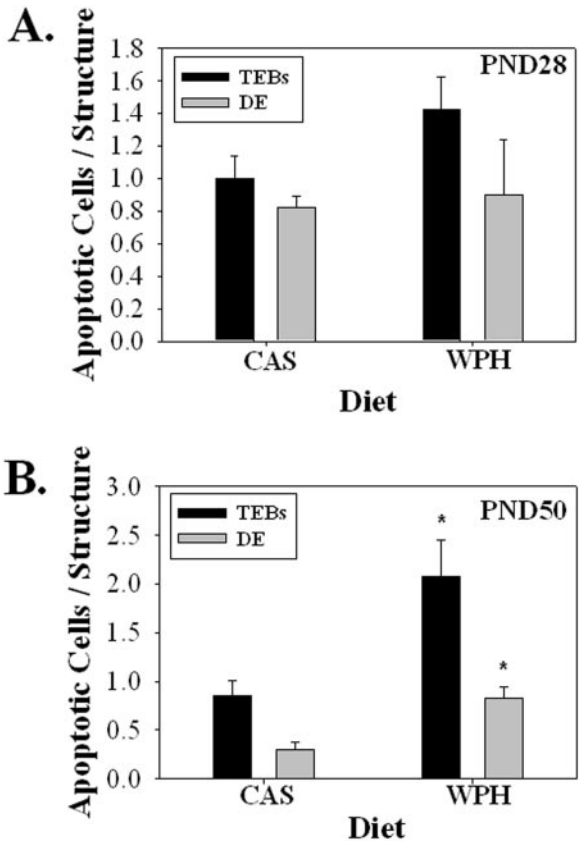

FIGURE 2 Apoptosis in mammary glands of CAS- and WPH-fed rats at PND 28 (*A*) and 50 (*B*). The numbers of stained nuclei in TEB and DE cells from 3 randomly selected fields per slide, with 3 slides evaluated from each of 4 rats ($n = 4$) per diet are presented as means ± SEM. Diet affected the apoptotic response at PND 50 (*$P < 0.05$).

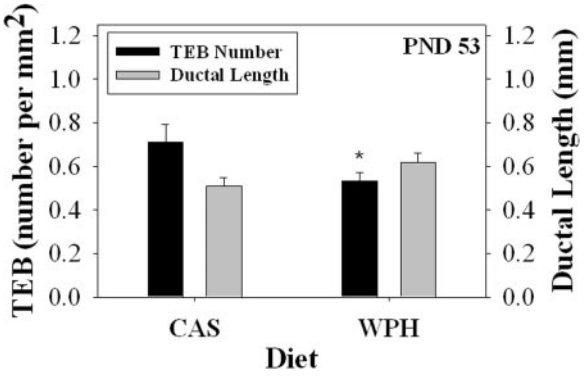

FIGURE 3 Whole-mount analysis of mammary glands of CAS- or WPH-fed rats at PND 53. Quantitative data of TEB number and of ductal length (in millimeters) are means ± SEM. For TEB, 3 (27 mm^2) areas were analyzed per slide per rat, with $n = 4$ rats evaluated per diet. For ductal length, 25 ducts were analyzed per slide per rat, with $n = 4$ rats evaluated per diet. Diet affected TEB numbers (*$P < 0.05$).

hanced expression of the PTEN gene and the relative expression levels of the genes encoding the mammary gland differentiation marker β-casein, the cell cycle components cyclin D1 and $p21^{WAF/CIP1}$, and IGF-2, a positive regulator of PTEN gene expression (34), all of which did not differ with diet (Fig. 5). On the other hand, there was an inverse association between PTEN and the receptor for progesterone PR, with the latter decreasing ($P < 0.05$) with dietary WPH. At PND 50, the expression of these genes was not altered by WPH relative to CAS, except for β-casein, which was greater ($P < 0.05$) in mammary glands of WPH-fed rats.

PTEN protein increased ($P < 0.05$) in cytosolic but not in nuclear extracts from mammary glands of PND 21–28 WPH-fed rats relative to that of CAS-fed rats of the same age (**Fig. 6***A* and *B*). TEB, but not DE, of rats fed WPH showed increased ($P < 0.05$) immunoreactive PTEN expression (Fig. 6C). Consistent with RNA data, immunoreactive PTEN protein did not differ in cytosolic and nuclear fractions of mammary glands from PND 53

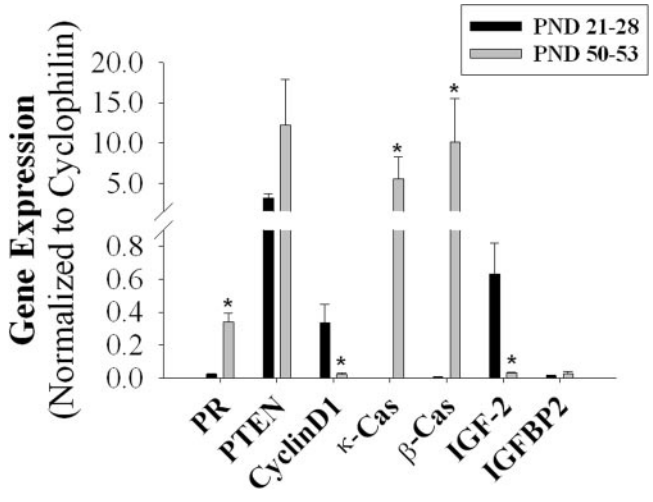

FIGURE 4 Expression of growth- and differentiation-associated genes in mammary glands of rats fed AIN-93G diet with CAS at PND 21–28 (weaning) and PND 50–53 (early adult stage). Levels of RNA transcripts (means ± SEM; $n = 12$ rats) were measured by QPCR and normalized to cyclophilin A. *Difference at $P < 0.05$ between developmental stage for each gene was analyzed by Student's *t* test.

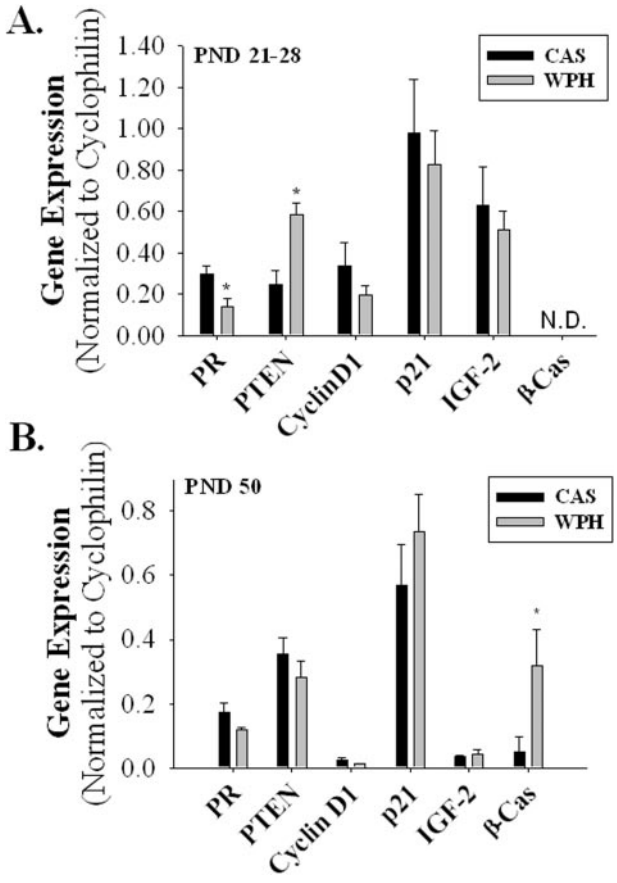

FIGURE 5 Expression of selected genes in mammary glands of rats fed AIN-93G diets with CAS or WPH as sole protein source. (*A*) PND 21/28 (weaning). (*B*) PND 50 (early adult stage). Levels of RNAs (means ± SEM; $n = 8–12$) were measured by QPCR and normalized to cyclophilin A. *Difference at $P < 0.05$ between CAS- and WPH-fed groups for each gene was analyzed by Student's *t* test. N.D. = not detectable.

rats fed CAS- and WPH-based diets as well as in corresponding mammary TEB and DE (data not shown).

Apoptotic status and PTEN expression in DMBA-exposed mammary glands. To further examine whether mammary gland status at the time of carcinogen administration (PND 53) differed for CAS- and WPH-fed rats, mammary tissues from rats fed the 2 diets were evaluated for TUNEL-positive cells and PTEN expression at 24 h and 7 d after DMBA administration (time of DMBA at +1 and +7, respectively; **Fig. 7**). At 24 h post-DMBA, mammary gland structures (TEB and DE) from CAS-fed rats exhibited increased apoptosis relative to that of rats fed the same diet prior to DMBA administration ($P < 0.05$ for both; Fig. 7A). By contrast, there was no change in the apoptotic index of mammary gland structures from WPH-fed rats pre- and 24 h post-DMBA (TEB, $P = 0.18$, DE, $P = 0.79$). At 7 d post-DMBA, the number of TUNEL-positive cells in mammary structures of CAS-fed rats decreased ($P < 0.05$) to pre-DMBA levels. There was a diet-by-duration of DMBA exposure effect in the apoptotic indices for TEB ($P = 0.07$) and DE ($P = 0.04$).

The lower apoptotic index in DE of WPH-fed rats, relative to CAS-fed rats, at 24 h post-DMBA paralleled the reduced mammary PTEN levels in DE (Fig. 7B). No difference was noted in the levels of immunoreactive PTEN in TEBs of CAS- and WPH-fed rat mammary glands 24 h post-DMBA, consis-

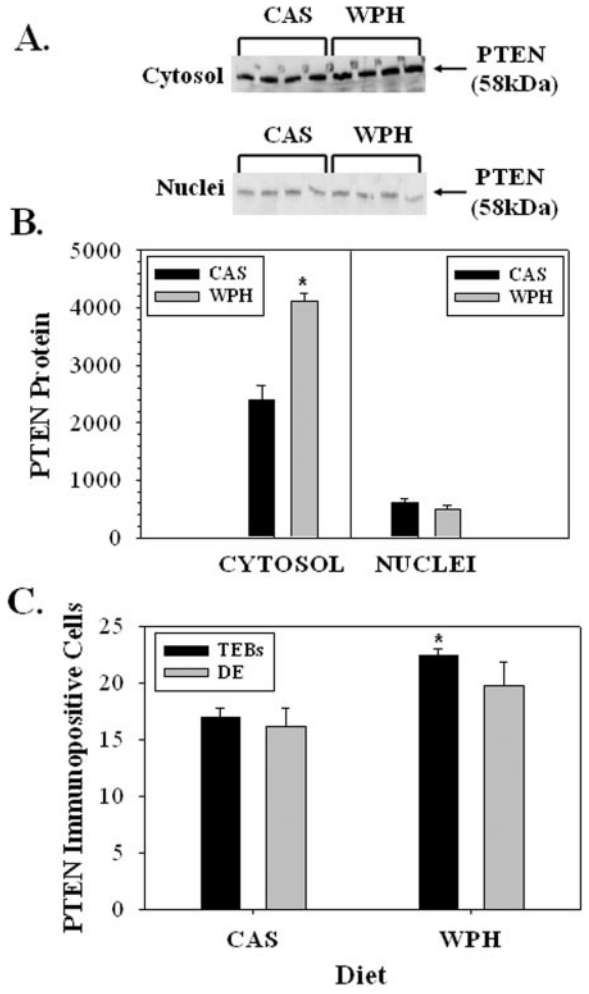

FIGURE 6 Expression at PND 21–28.of PTEN in mammary glands of CAS- and WPH-fed rats. (*A*) Western blot of mammary cytosolic and nuclear extracts from rats fed CAS-or WPH-based diets (n = 4 rats/diet). Each lane contains 100 μg total protein and represents an individual rat. (*B*) Graphical representation of immunoreactive PTEN (mol wt 58 kDa) in *A* above (means ± SEM). (*C*) Immunostaining for PTEN in TEB or DE structures. The numbers of PTEN-positive cells were counted from 4 randomly chosen fields (200× magnification) per slide per rat, and data are means ± SEM from 4 rats per diet. *Difference at $P < 0.05$ between CAS and WPH was analyzed by two-way ANOVA. There was a diet-by-structure interaction ($P = 0.004$).

tent with comparable numbers of TUNEL positive cells. Mammary PTEN expression in TEBs and DE of CAS- or WPH-fed rats did not differ at 7 d post-DMBA, as observed for their respective apoptotic indices.

DISCUSSION

The present study examined whether the protective effects conferred by dietary exposure to WPH against DMBA-induced mammary tumorigenesis were associated with the altered expression of the tumor suppressor PTEN (20,21,24). Results showed that: *1*) PTEN expression at the level of mRNA and protein is elevated in mammary tissue of weanling rats fed WPH relative to those fed CAS as the only protein source; *2*) increased mammary PTEN expression at weaning temporally preceded increased differentiation of mammary tissues in young adults; and *3*) mammary tissue from rats administered dietary WPH responded differently to the procarcinogen DMBA from those fed CAS, as measured by apoptotic status. The mechanism(s) by which dietary WPH increases mammary PTEN gene expression at weaning and alters the molecular and cellular properties of the mammary gland, leading to increased differentiation and distinct responses to DMBA in young adults, is not known. However, based on the documented functions of PTEN in regulating proliferation, apoptosis, and differentiation of mammary cells (24,25,30), coupled with the enhanced expression of PTEN by WPH in TEB, which constitute the most proliferative compartment (28) and are also sites of substantial apoptosis (35) within the developing mammary gland, we suggest that activation of PTEN signaling pathways at an early developmental window, partly at the level of TEB, constitutes a plausible mechanism by which WPH protects against chemically induced tumorigenesis.

We show here that expression of specific genes in mammary glands of rats exposed to dietary WPH varied with developmental stage. While mammary PR and PTEN gene expression at weaning was altered with diet, this difference was not sustained in young adults. On the other hand, there was an increase in β-casein transcript levels with WPH in mammary tissues of young adults, but not in weanling pups, consistent with greater mammary gland differentiation. The modest degree of changes induced by WPH in a limited number of genes evaluated here is in accord with the absence of drastic modifications in the differentiation status of mammary glands from WPH-fed rats relative to those fed CAS, as measured by changes in TEB numbers and extent of ductal elongation. Nevertheless, it was of interest to note that albeit modest, the altered variables translated to a distinct response of the mammary glands to the procarcinogen DMBA. In particular, whereas mammary tissues from CAS-fed rats showed a significant increase in number of apoptotic cells in TEB and DE structures 24 h post-DMBA administration, those from WPH-fed rats were more resistant to apoptosis and did not exhibit corresponding changes in number of TUNEL-positive cells. Thus, seemingly minor advances in the developmental status of mammary tissues resulting from early life exposure to WPH may confer partial resistance to environmental insults later in life.

In these studies, we examined the tumor suppressor PTEN as a possible mediator of WPH action in developing mammary glands. Although basal PTEN expression in these tissues showed only a tendency ($P = 0.08$) to increase between weaning and the early adult stage, WPH significantly increased mammary PTEN expression (RNA and protein) at weaning, although this effect of WPH did not persist in young adults. The time lag between the observed increase in PTEN expression at weaning and the changes in proliferation and apoptotic status, as well as in the expression of the differentiation marker β-casein only in young adults, suggests a lack of a direct effect of PTEN on the proliferative and apoptotic status of mammary cells. We posit that the delayed consequence of enhanced PTEN expression may more reflect the limited number of mammary cells targeted by WPH, whose negative regulation by PTEN results in apoptosis and their subsequent removal from developing mammary tissues. It has been suggested that cancer arises from a series of sequential mutations occurring as a result of genetic instability and environmental insults (36,37); hence, tumors could initiate from only a few hundred breast cancer cells (38). Moreover, some tumors express early developmental markers, coincident with loss of PTEN expression, suggesting a functional correlation among differentiation status, PTEN, and tumorigenesis (39). Thus it is possible that the enhanced expression of the pro-apoptotic PTEN in cells targeted by WPH could favor the removal of a population of undifferentiated cells with high

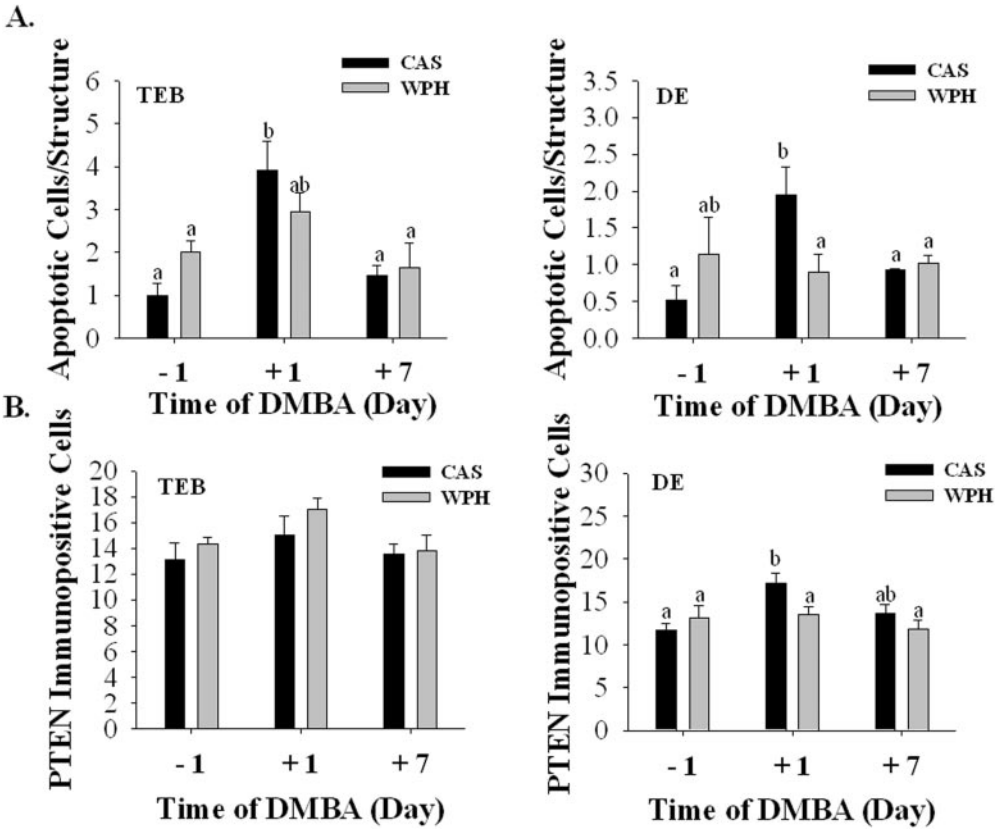

FIGURE 7 Apoptotic status and PTEN expression prior to and after DMBA administration in mammary glands of PND 53 rats fed CAS- or WPH-based diets. Time of DMBA administration (Day, d) is designated as: −1 (24 h before), +1 (24 h after), and +7 (7 d after) DMBA. (*A*) Numbers of apoptotic cells in TEB and DE as determined by TUNEL were measured in $n = 4$ rats per diet group. (*B*) PTEN-positive cells in TEB and DE detected by immunohistochemistry using anti-PTEN antibody were measured in $n = 4$ rats per diet group. Means without a common letter differ ($P < 0.05$). There was an interaction for apoptotic cell numbers between diet and time-of-DMBA exposure for TEB ($P = 0.07$) and DE ($P = 0.04$) and for PTEN immunoreactivity for DE ($P = 0.07$) but not TEB, as determined by two-way ANOVA.

tumorigenic potential early in development, leaving a population of mammary cells with phenotypes that are less susceptible to genetic mutations at a later life stage. The latter would likely manifest as cells with less proliferative, and hence enhanced, differentiation status. Indeed, our data provide support to this possibility because mammary glands from WPH-fed rats at PND 50–53 exhibit: *1*) decreased PCNA immunoreactivity; *2*) increased numbers of apoptotic cells; *3*) increased β-casein gene expression; *4*) decreased numbers of TEB; *5*) increased ductal extension into the fat pad; and *6*) less responsiveness to chemical carcinogen (DMBA) challenge, subsequent to enhanced PTEN expression at weaning. Although we did not directly test the above possibility, subsequent studies of mammary glands from CAS- and WPH-fed rats at or prior to weaning for expression of epithelial progenitor cell markers such as keratin 6 and Sca-1 (39,40), whose levels we anticipate to decrease with WPH as a function of neonatal development, should support this hypothesis.

Two signaling pathways involving PTEN could potentially mediate the protective effects of WPH on chemically induced tumorigenesis. Our results showing the coincident decrease in PR transcript levels with increased PTEN expression at weaning suggest a role for progesterone-mediated PR signaling. Ligand-bound PR has been shown to inhibit PTEN activity in the rat uterus (41), and endometrial adenocarcinoma expressing high levels of PR has low or no PTEN expression (42). Because mice null for the PR gene are less susceptible to DMBA-induced tumorigenesis, suggesting a critical role for PR in tumor development (29), it is possible that decreased PR activity at an early developmental window results in increased PTEN signaling, leading to inhibition of protein kinase (e.g., Akt/PK-B and MAPK) activation that promotes cell survival and proliferation. In this regard, constitutive activation of Akt/PK-B is a characteristic of breast cancer cell lines and primary invasive breast cancers (43). The signaling pathway mediated by the epidermal growth factor receptor family member Her-2/neu may also be involved in the tumor-protective effects of WPH, because overexpression of Her-2/neu occurs in ~30% of breast cancers (44) and is associated with constitutive activation of PI3-and Akt/PK-B kinases (45), both of whose actions are inhibited by PTEN (46). Moreover, PTEN and EGFR signaling pathways also interact, albeit in an opposing manner, via PTEN inhibition of the EGFR-mediated activation of the Ras/Raf/ERK pathway of MAP kinase signaling (47). The relevance of PTEN/EGFR "cross-talk" in the genesis of breast cancer is underscored by the findings that sensitivity to the EGFR-selective tyrosine kinase inhibitor "Iressa" is lost in PTEN-mutant cells (48,49). Further analysis of the functional interactions between PTEN and components of PR and of EGF signaling pathways, respectively, will likely provide insights into the mechanisms of cellular transformation and cell cycle regulation affected by dietary factors.

In conclusion, dietary WPH can lead to transient induction in PTEN expression at a sensitive developmental window (weaning), possibly resulting in long-term consequences on mammary gland differentiation. The latter is manifested by higher basal apoptotic index in TEB and resistance to DMBA-induced cell damage in TEB and DE. Studies using rats exposed to WPH and purified fractions thereof, at specific windows of early development, should help delineate the signaling pathways mediating the protective effects of early diet intervention on adult onset of breast cancer.

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