Cell Proliferation in the Normal Mouse Mammary Gland and Inhibition by Phenylbutyrate[1]

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Abstract

Ovarian hormones have a pivotal role in the control of proliferation in the mammary gland, and cumulative life-time exposure to ovarian hormones is known to be a determinant of breast cancer risk. We have shown previously that a p.o.-active, long-acting butyrate analogue, sodium phenylbutyrate (PB), reduced proliferation in normal and malignant human breast cells in culture and reduced expression of ovarian hormone receptors, suggesting that PB had cellular effects consistent with decreasing breast cancer risk. The aim of this study was to determine the *in vivo* effects of PB in the normal mammary gland on epithelial cell proliferation, estrogen receptor α (ERα) expression, and cyclin D1 expression. BALB/c mice were treated with PB, delivered by mini-osmotic pumps, for 7 days. Moderate (250 mg/kg/day) and high (500 mg/kg/day) PB treatment resulted in a decrease in proliferation in mammary epithelial cells ($P < 0.001$), determined by bromodeoxyuridine incorporation. Analysis of ERα immunostaining revealed a significant reduction in moderate- and high-treatment groups ($P = 0.01$ and $P = 0.02$), and expression of cyclin D1 was virtually ablated ($P < 0.001$). Histone deacetylase inhibition is a known mechanism of butyrate action, and consistent with this, PB increased levels of acetylated histone H3 in the mammary gland. In summary, PB decreased proliferation in the mammary gland *in vivo* at clinically achievable doses. Decreased proliferation was accompanied by changes in the levels of ERα and cyclin D1. These data show that PB modulates parameters thought to be involved in the carcinogenic process in the normal mammary gland, and compounds in this class may therefore be useful candidates for breast cancer chemoprevention.

Introduction

The cloning of breast cancer predisposition genes, *BRCA1* and *BRCA2* (1–3), has allowed identification of women at increased risk of breast cancer and has focused attention on the need for preventative strategies. The success of the National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention Trial, reporting a significant reduction in breast cancer incidence among the high risk participants on tamoxifen, has shown that chemoprevention in breast cancer is possible (4). However, predominantly ERα[3]-positive tumors were inhibited by tamoxifen in the National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention Trial, and *BRCA1*-associated tumors are predominantly ERα negative (5). Tamoxifen, therefore, may not be effective in the group at highest risk of developing breast cancer (6). There is a need to develop novel strategies to prevent ERα-negative as well as ERα-positive breast cancers.

The ovarian hormones estrogen and progesterone are required for normal mammary development. Female mice lacking ERα have underdeveloped mammary glands, with only rudimentary ducts present at the nipple (7), and progesterone receptor-null mice fail to establish the lobular-alveolar system essential for lactation (8). Ovarian hormones also influence susceptibility to mammary carcinogenesis; women without ovaries have a low risk of breast cancer, and prolonged ovarian activity during life (early menarche or late menopause) is associated with increased risk (9). Sequential rounds of proliferation and cell death in concert with the menstrual cycle (10), during which genetic alterations can accumulate, are thought to underlie this increased risk of breast cancer associated with cumulative hormone exposure. Furthermore, there is increasing evidence that oxidative stress linked to estrogen metabolism and hormonal status can lead to DNA damage and contribute to mammary carcinogenesis (11, 12). Sensitivity of mammary cells to proliferative effects of the ovarian hormones is therefore likely to be a key requirement for their activity as tumor promoters, and agents that decrease ovarian hormone responsiveness could be considered to be potential chemopreventative agents.

A promising preventative agent in human cancer is the short-chain fatty acid, butyrate. Butyrate is a naturally occurring fatty acid derived from a high fiber diet and is produced in the large bowel by bacterial fermentation. Butyrate is also present at low levels in many fruit and vegetables, but its richest source is milk fat (13, 14). Colonic generation of butyrate has been associated with the protective effect of dietary fiber for colon cancer (13, 15). Butyrate has been

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[3] The abbreviations used are: ERα, estrogen receptor α; PB, phenylbutyrate; BrdUrd, bromodeoxyuridine; Ac-H3, acetylated histone H3; OR, odds ratio; CI, confidence interval.

suggested to be a good candidate molecule in protection against breast cancer (16, 17), and one butyrate analogue, tributyrin, was shown to decrease the development of nitrosomethylurea-induced mammary tumors in female rats (18). Butyrate decreases expression of ERα and progesterone receptor (19–21), arrests growth, and induces differentiation in a variety of malignant cell lines in culture (14, 15, 20, 22, 23). Although the molecular mechanisms by which butyrate induces differentiation, cell cycle arrest, and apoptosis are not well understood, it is known that butyrate induces a variety of changes within the nucleus, including histone hyperacetylation (24).

Histone deacetylases play important roles in chromatin remodeling and transcriptional regulation. Both histone acetyltransferase and histone deacetylase activities are recruited to target genes in complexes with specific transcription factors, such as nuclear hormone receptors and their cofactors, corepressors such as N-CoR and SMRT, and coactivators (25). Several structurally diverse histone deacetylase inhibitors have been identified, including butyrates, and their activity in inducing differentiation and apoptosis of transformed cells has rendered them potential candidates for cancer therapy (26).

Butyrate causes a decrease in expression of the cell cycle-related protein cyclin D1 at the level of gene transcription (27, 28). Cyclin D1 is involved in normal mammary development (29, 30) and has been implicated in mammary carcinogenesis. Butyrate also increases expression of the cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$, through a process thought to involve histone hyperacetylation. Induction of $p21^{Waf1/Cip1}$ by butyrate is independent of p53, an advantage in potential anticancer and chemopreventive agents because p53 mutations occur frequently in neoplasia (31). Butyrate treatment leads to retinoblastoma protein hypophosphorylation, and this is likely to be involved in the mechanisms underpinning butyrate inhibition of cell proliferation, although it may be distal to down-regulation of cyclin D1 (28, 32).

Despite the demonstrated antiproliferative actions of butyrate, its usefulness as a cancer preventative agent in humans is limited because it is rapidly metabolized and excreted *in vivo*. These limitations have partially been circumvented with the development of stable derivatives or prodrugs of butyrate, such as phenylacetate and PB, which have more favorable pharmacological properties (14). These butyrate prodrugs have been used clinically to treat urea cycle disorders and have been tested for the treatment of several diseases including β-thalassemia (33, 34) and sickle cell anemia (34). PB has also been in trials for use in the treatment of leukemia and has been tested in Phase I trials in patients with a variety of refractory solid tumors (35–37).

We and others have found previously that the stable analogue PB had a similar effect on cell proliferation as butyrate, whereas phenylacetate was less effective at inhibiting proliferation (38, 39). PB inhibited proliferation in both ERα-positive and ERα-negative breast cancer cell lines and a cell line derived from normal mammary tissue (39). PB also induced differentiation, decreased expression of ERα, and decreased expression of cyclin D1, features consistent with a potential breast cancer chemopreventative agent (39). The effect of PB on these end points *in vivo* in the normal mammary gland were unknown.

Although the role of ovarian hormones in mammary development is well described, the mechanisms underlying hormonal control of proliferation are complex. ERα-positive cells are in the minority in the normal breast, and the greater proportion of normal cells proliferating under the influence of ovarian hormone are ERα negative (40, 41). The challenge for primary cancer chemoprevention pertains to protecting nontransformed cells from being initiated, and in the mammary gland similar protection must be extended both to ERα-positive and ERα-negative cells. Therefore, this study has concentrated on the effects of a p.o.-active butyrate analogue *in vivo* on the normal mammary gland as a step in determining whether this class of drugs could be effective in the prevention of breast cancer in women who are at high risk.

Materials and Methods

Animals

Ten-week-old (mature), virgin BALB/c mice were housed in suspended cages with a 12-h light/dark cycle and free access to pelleted food and water. PB (Fyrklovern Scandinavia AB, Kopingsvik, Sweden) was administered via s.c. implantation of Alzet micro/mini osmotic pumps (Alza Corp., Palo Alto, CA). Mice were weighed, and a dose equivalent to 250 or 500 mg/kg/day of PB prepared in saline was administered for 7 days. Control mice received only the vehicle, saline. Estrus stage was determined by vaginal smear and classified according to the stages described by Nelson *et al.* (42). BrdUrd (200 mg/kg; Sigma-Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) was administered by i.p. injection 30 min before sacrifice. All animal experimentation was done in accordance with National Health and Medical Research Council guidelines for animal welfare and with approval from the Westmead Hospital Animal Care and Ethics Committee.

Immunohistochemistry

Cell Proliferation. After sacrifice, the number 4 inguinal mammary gland was removed and fixed in neutral buffered formalin; 4-μm serial sections were cut and stained. BrdUrd staining was essentially the same as described previously (43). Tissue sections were deparaffinized in Histolene (Fronine Pty. Ltd., Riverstone, New South Wales, Australia) and were passed through graded alcohols before being treated with 3% H_2O_2 (v/v) in H_2O. The sections were treated with 1.5 M HCl for 20 min. After denaturation, the slides were quickly washed three times in ice-cold NaCl 0.9% (w/v), Tween 20 1% (v/v; polyoxyethylene sorbitan monolaurate; Sigma Aldrich) and incubated for 1 h with anti-BrdUrd monoclonal antibody (clone BU-1; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) diluted 1:4 in Dulbecco's PBS, 1% (w/v) BSA, and 1% Tween 20 (v/v), pH 7.4 (PBT buffer). The slides were then washed in PBT buffer and incubated with 2.4 μg/ml biotinylated goat antimouse antibody (Biosource International, Camarillo, CA) for 30 min, washed in Dulbecco's PBS, and incubated for 30 min with 0.5 μg/ml streptavidin-peroxidase conjugate (Biosource In-

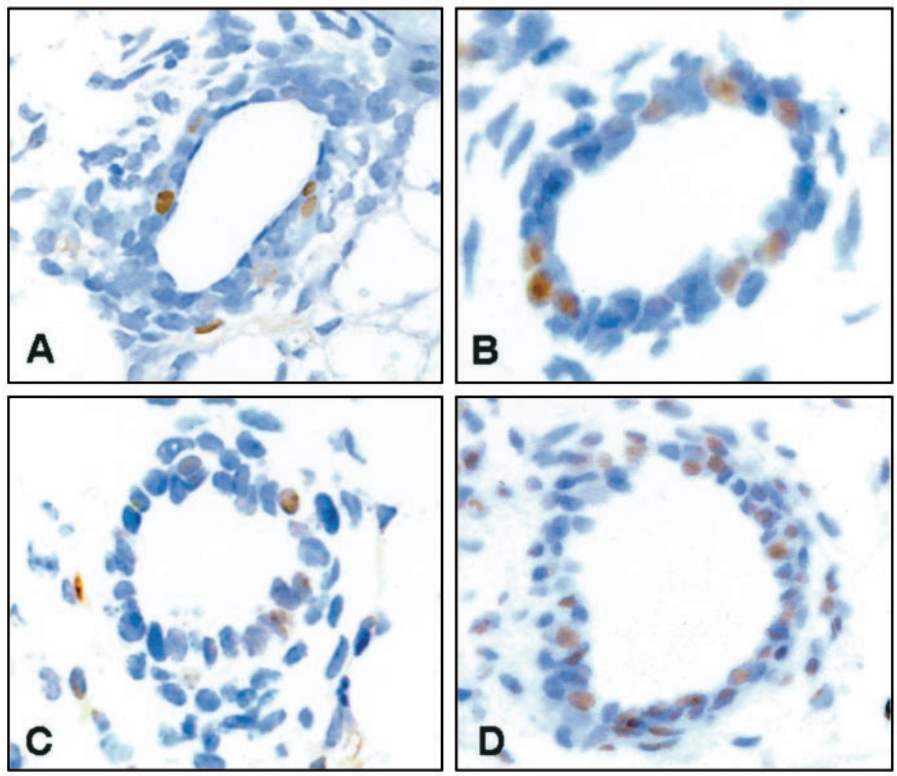

Fig. 1. Immunohistochemical staining. Mouse mammary glands were fixed, sectioned, and stained for incorporation of BrdUrd (S-phase cells; *A*), ERα (*B*), cyclin D1 (*C*), and Ac-H3 (*D*). Antigens were visualized by diaminobenzidine staining (*brown*), and the sections were counterstained with hematoxylin (*blue*). $\times$400.

ternational). BrdUrd incorporation was visualized using a diaminobenzidine substrate kit (Vector Laboratories, Inc., Burlingame, CA) which resulted in a brown stain, and the sections were counterstained with hematoxylin.

Expression of ERα and Cyclin D1. Four-μm serial sections were cut onto Superfrost plus slides (Lomb Scientific, Taren Point, New South Wales, Australia) that had been coated in Mayer's egg albumin (equal volume egg white: glycerol) and left to dry for 72 h at 37°C. Tissue sections were rehydrated, as described above, before being immersed in DAKO antigen retrieval solution (DAKO Australia, Botany, New South Wales, Australia). Slides were microwaved for 2 min 20 s on 100% (850 W) and then for 10 min on 40%, in a NEC microwave oven, model N706E, before being covered with cling wrap and left to stand for 20 min. Slides were then rinsed in water, washed in PBS, and treated with 3% H_2O_2 (v/v) in H_2O. Sections were blocked for 30 min with normal goat serum (Hunter Antisera, Birmingham Gardens, New South Wales, Australia) diluted 1:1 in PBS. All incubations were performed at room temperature in a moist chamber. Sections were incubated with primary antibodies for 1 h. ERα was detected using Novocastra ER antibody clone 6F11 (diluted 1:60; Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom), and cyclin D1 was detected using a primary antibody from Santa Cruz Biotechnology (sc-8396, diluted 1:50; Santa Cruz, CA). The sections were washed in PBS and incubated with 1.8 μg/ml biotinylated goat anti-mouse secondary antibody (Biosource International), and staining was visualized using streptavidin and a detection system as described above for BrdUrd staining.

Immunodetection of Ac-H3 (Lys 9). Sections were cut, dried, rehydrated, retrieved, and quenched as described for ERα and cyclin D1 staining. Sections were blocked for 1 h at room temperature with 5% normal goat serum (Hunter Antisera) diluted in PBS. Sections were then incubated overnight at 4°C with Ac-H3 (Lys 9) antibody (Cell Signaling Technology, Beverly, MA) diluted 1:100 in blocking solution. The sections were washed in PBS and incubated with 1.8 μg/ml biotinylated goat antirabbit secondary antibody (DAKO Australia), and staining was visualized using streptavidin and a detection system as described above for BrdUrd staining.

Data Collection and Analysis

All mice were randomly allocated a unique identifying number, and tissue blocks were independently numbered to ensure that assessment of all immunohistochemical staining was done while blinded to the treatment group. Tissues were stained for BrdUrd, ERα, and cyclin D1 in at least two separate experiments using nonadjacent sections. Greater than 1000 cells were counted per slide, using an Olympus BX-40 microscope at $\times$600. Cells were scored positive or negative for BrdUrd incorporation, and the percentage of positive cells per ductal structure was calculated. Results were analyzed using logistic regression analysis (44). ERα-positive cells and Ac-H3-positive cells were assigned a value of 1, 2, or 3, representing low, medium, or high expression, respectively, in addition to calculating the percentage of positive cells. To combine the percentage of positivity and intensity of

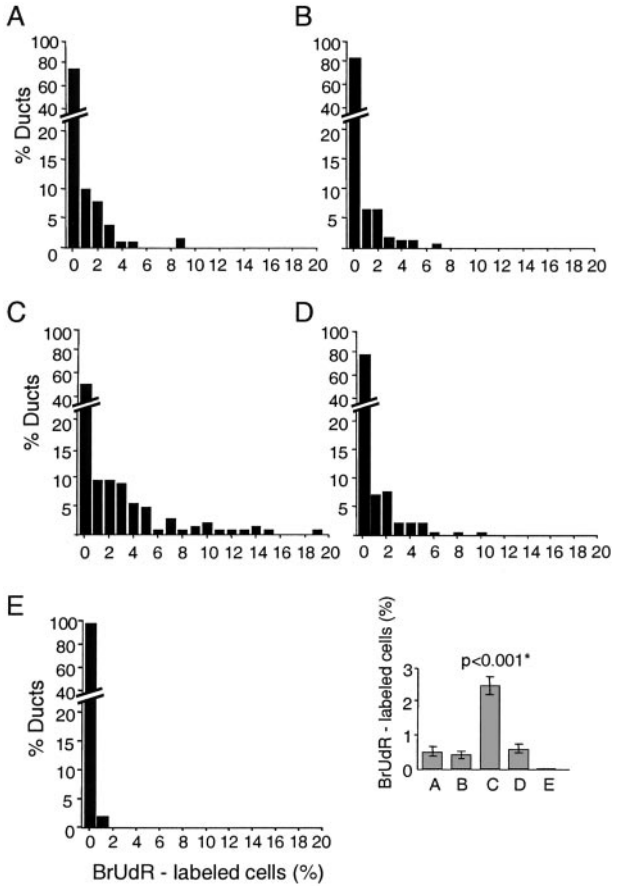

Fig. 2. Proliferation in the normal mouse mammary gland during the estrus cycle. The percentage of S-phase cells (BrdUrd-labeled) was determined immunohistochemically in individual ductal structures in mammary glands from 10-week-old mice in diestrus (*A*), proestrus (*B*), estrus (*C*), metestrus (*D*), and negative control (*E*), ovariectomized mice (cumulative data for ≥6 mice/group). *Inset,* overall proliferation in mammary epithelial cells in diestrus (*A*), proestrus (*B*), estrus (*C*), metestrus (*D*), and ovariectomized mice (*E*). *Bars,* 95% CI. ∗, significance, $P < 0.05$, logistic regression analysis.

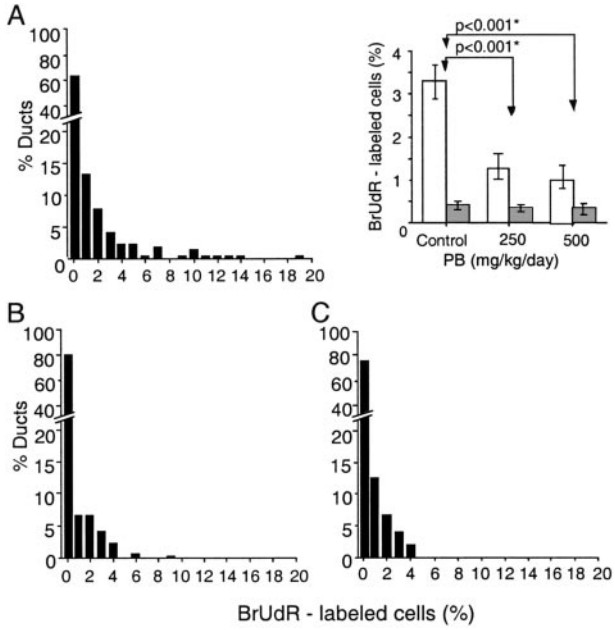

Fig. 3. Effect of PB on proliferation in the mammary gland. The percentage of S-phase cells (BrdUrd-labeled) was determined immunohistochemically in individual ductal structures in mammary glands from 10-week-old mice treated with vehicle control for 7 days (*A*), treated for 7 days with 250 mg of PB/kg/day (*B*), and treated with 500 mg PB/kg/day for 7 days (*C*; cumulative data for ≥6 mice/treatment group). *Inset,* proliferation in mammary epithelial cells between treatment groups. *Bars,* 95% CI. ∗, significance, $P < 0.05$, logistic regression analysis. Estrus stage was determined in each mouse using vaginal smears. □, estrus phase; ▨, diestrus, proestrus, and metestrus, combined for illustrative purposes.

expression, a histoscore value was calculated: [(number of high cells × 3) + (number of medium cells × 2) + (number of low cells)/(actual number of positive cells)]/[(actual number of positive cells)/(total number of cells)].

ERα histoscore values were analyzed using repeated measures ANOVA (44). Ac-H3 staining was analyzed using one-way ANOVA, with Bonferroni's correction for multiple comparisons. Cyclin D1 expression was determined by scoring cells as positive or negative as described above for BrdUrd expression. The percentage of positive cells was calculated, and results were analyzed using logistic regression analysis (44). The statistical software package SPSS for Windows, Version 10.0, was used for all analyses.

Results

Proliferation in the Normal Adult Mouse Mammary Gland. BrdUrd staining detects proliferating cells in S-phase, and as expected, staining was nuclear and positive cells were predominantly luminal cells found within ducts and in terminal ductal structures (Fig. 1*A*). A large range was found in the percentage of proliferating cells in each ductal structure from 0 to 19%. The overall mean of positive cells was low (0.92%; $n = 25$ mice). Proliferation is known to vary through the menstrual cycle in women and the estrus cycle in rodents (10). To determine the intrinsic difference in proliferation, BrdUrd incorporation was measured in the different stages of the estrus cycle, diestrus, proestrus, estrus, and metestrus. In control mice, proliferating cells were observed in all stages of the cycle (Fig. 2, *A–D*). Significantly higher levels of proliferation were observed during estrus, $P < 0.001$ (OR, 5.1; 95% CI, 4.2–6.1), coinciding with high circulating levels of ovarian hormones (Ref. 45; Fig. 2, *inset*). After ovariectomy, BrdUrd staining was virtually ablated (Fig. 2*E*), as anticipated, given the known involvement of ovarian hormones in mammary epithelial cell proliferation.

Effect of PB on Proliferation in the Normal Adult Mouse Mammary Gland. BrdUrd incorporation was measured in the normal mammary gland of mice continuously infused with PB or vehicle control for 7 days. Two doses of PB, 250 and 500 mg/kg/day, were used. These doses were equivalent to those used clinically, with few side effects, and in humans are regarded as a moderate and high dose (46). In control mice, the maximal proportion of BrdUrd-labeled cells/duct was 19% (Fig. 3*A*), whereas after treatment with 250 mg PB/kg/day, maximal labeling/duct was reduced to

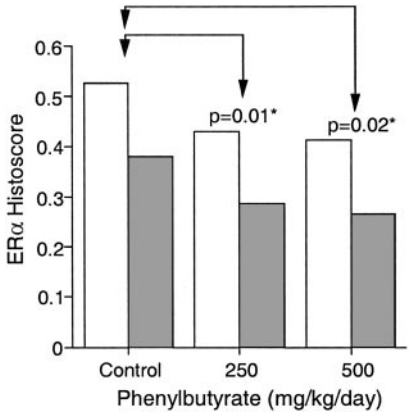

Fig. 4. Effect of PB on ERα expression in the mammary gland. ERα levels in mammary epithelial cells were evaluated immunohistochemically and expressed as a histoscore, combining the percentage of positively stained cells and intensity (assigned a value of 1–3). Mice were treated with vehicle-control or 250 or 500 mg PB/kg/day for 7 days (≥6 mice/treatment group). Estrus stage was determined in each mouse using vaginal smears. □, estrus phase; ▨, diestrus, proestrus, and metestrus, combined for illustrative purposes. ∗, significance, $P < 0.05$ compared with control, repeated measures ANOVA.

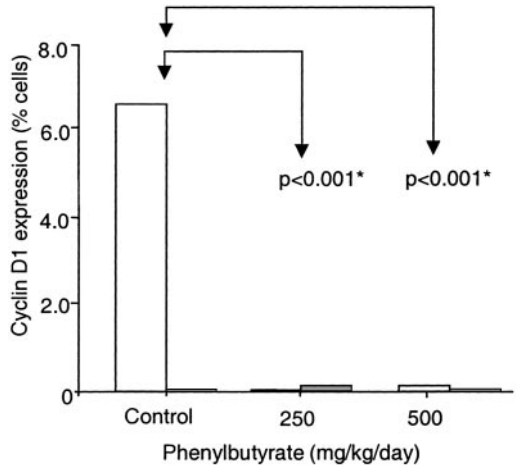

Fig. 5. Effect of PB on cyclin D1 expression in the mammary gland. The proportion of mammary epithelial cells expressing cyclin D1 was determined immunohistochemically. Ten-week-old mice were treated with vehicle-control or 250 or 500 mg PB/kg/day for 7 days (≥6 mice/treatment group). Estrus stage was determined in each mouse using vaginal smears. □, estrus phase; ▨, diestrus, proestrus, and metestrus, combined for illustrative purposes. ∗, significance, $P < 0.05$, logistic regression analysis.

9% (Fig. 3*B*) and further reduced to 4% after treatment with 500 mg PB/kg/day (Fig. 3*C*). There was a concomitant increase in the proportion of ductal structures containing no proliferating cells.

Given the marked difference in proliferation observed in different phases of the estrus cycle, estrus stage was determined on day 6 for each mouse treated with either vehicle control or PB and at the time of sacrifice on day 7. The effect of PB on proliferation was adjusted for the intrinsic effects of estrus cycle using logistic regression analysis. Treatment with PB resulted in a significant decrease in proliferation of normal mammary epithelial cells for 250 mg/kg/day ($P <$ 0.001; OR, 0.49; 95% CI, 0.39–0.61) and 500 mg/kg/day ($P < 0.001$; OR, 0.36; 95% CI, 0.28–0.47; Fig. 3, *inset*). The effects of a moderate dose of PB was not significantly different from that at the high dose. The greatest effect of PB on BrdUrd labeling was observed in mice in estrus, when proliferation in the mammary gland was highest (Fig. 3, *inset*). The results for diestrus, proestrus, and metestrus, although statistically analyzed as separate stages of the cycle, have been combined for illustrative purposes in Fig. 3, because proliferation (compare Fig. 2) and effects of PB were similar in these stages.

Effect of PB on ERα Expression. The ovarian hormone estrogen, acting via its nuclear receptors, has a fundamental role in the control of mammary epithelial cell proliferation. We have shown previously that PB down-regulated the expression of ERα in human breast cancer cells in culture (39); however, the effect of PB on ERα expression in mammary cells *in vivo* was not known. Expression of ERα was measured immunohistochemically in the mammary glands of mice after 7 days continuous infusion with PB or vehicle control. ERα staining was nuclear and predominantly confined to luminal epithelial cells (Fig. 1*B*). The overall percentage of ERα-positive cells in control mice ($n = 21$) was 31.1% (±0.5% SE); however, individual ducts and terminal ductal structures varied considerably in the proportion of ERα-positive cells observed (range, 0–81.3%). Heterogeneity in staining intensity was observed between positively stained cells. ERα expression was therefore analyzed using a modified histoscore, taking into account both intensity and percentage of positive cells.

ERα expression was higher in the mammary glands of mice in estrus ($P < 0.001$, repeated measures ANOVA), where higher proliferation was also observed, compared with those in metestrus, diestrus, and proestrus (Fig. 4). Treatment with a moderate dose of PB (250 mg/kg/day) resulted in a significant decrease in ERα histoscore ($P = 0.01$), adjusted for estrus cycle effects on ERα expression. ERα histoscore was similarly reduced after infusion with a high dose of PB (500 mg/kg/day; $P = 0.02$). Reduced ERα expression after PB treatment was observed in both the high and low proliferative phases of the estrus cycle.

PB Decreases Expression of Cyclin D1 in the Normal Mammary Gland. Expression of cyclin D1 in the normal mouse mammary gland was nuclear and predominantly found in luminal epithelial cells (Fig. 1*C*). Cyclin D1 is expressed in proliferating cells in the G_1 phase of the cell cycle. The number of cyclin D1-expressing cells in the normal mammary gland was low, consistent with its cell cycle-restricted expression. In control mice, the overall mean of cyclin D1-positive cells was 2.3%; however, individual ducts and terminal ductal structure varied considerably in the proportion of cyclin D1-positive cells observed (range, 0–14.7%). Expression of cyclin D1 was significantly greater in mice in estrus compared with non-estrus phases (Fig. 5, logistic regression analysis; $P < 0.001$; OR, 43.9; 95% CI, 19.5–99.0). The expression profile of cyclin D1 was therefore similar to that found for BrdUrd incorporation (Fig. 2), reflecting the expected pattern of epithelial cell proliferation in the

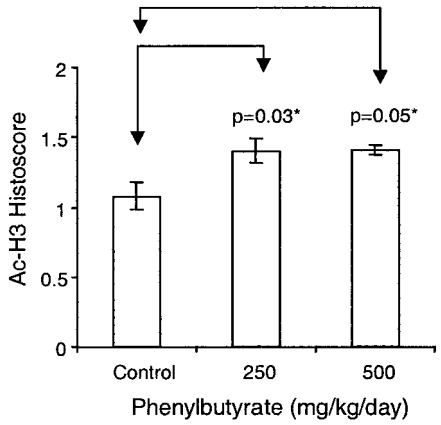

Fig. 6. Effect of PB on levels of acetylated histone in the mammary gland. Ac-H3 levels in mammary epithelial cells were evaluated immunohistochemically and expressed as a histoscore, combining the percentage of positively stained cells and intensity (assigned a value of 1–3). Mice were treated with vehicle-control or 250 or 500 mg PB/kg/day for 7 days (≥6 mice/treatment group). ∗, significance, $P < 0.05$, compared with control; one-way ANOVA, with Bonferroni's correction for multiple comparisons. *Bars,* 95% CI.

mammary gland. Treatment with a moderate dose of PB and for 7 days resulted in a significant decrease in cyclin D1 expression, adjusted for estrus cycle effects (Fig. 5; $P < 0.001$; OR, 0.08; 95% CI, 0.05–0.13), and a similar decrease was observed after treatment with high-dose PB (Fig. 5; $P < 0.001$; OR, 0.07; 95% CI, 0.04–0.13). The reduction in cyclin D1 expression resulting from exposure to a moderate dose of PB was not significantly different from that at the high dose. Cyclin D1 expression was barely detectable in either control or treated mice in metestrus, diestrus, and proestrus.

PB Increases Acetylation of Histone H3 in the Normal Mammary Gland. Butyrate is known to inhibit histone deacetylase, and PB treatment has been shown to increase acetylation of histone H3 in mononuclear cells *in vivo* (47). Expression of Ac-H3 at lysine 9 was measured immunohistochemically in the mammary glands of mice after 7 days of continuous infusion with PB or vehicle control. Ac-H3 staining was nuclear, and the majority of epithelial cells were positive (Fig. 1*D*). A high degree of positivity was also observed in stromal cells. Heterogeneity in staining intensity was observed in adjacent cells; therefore, Ac-H3 staining was analyzed using a modified histoscore, taking into account both intensity and percentage of positive cells. There was no difference in levels of Ac-H3 staining through the estrus cycle; however, treatment with both moderate (250 mg/kg/day) and high (500 mg/kg/day) doses of PB resulted in a significant increase in Ac-H3 levels ($P = 0.03$ and 0.05, respectively; Fig. 6). The increase in Ac-H3 expression resulting from exposure to a moderate dose of PB was not significantly different from that at the high dose ($P = 1.0$).

Discussion

We have shown that a p.o.-active butyrate analogue, PB, has activity in the normal mouse mammary gland at clinically relevant and achievable doses. Butyrate is known to induce differentiation, G_1 arrest, and cell death by apoptosis in a variety of cell types in culture. However, the effects of butyrates on normal tissues *in vivo* are largely unknown. The efficacy of new candidate preventative agents, such as the butyrates, in the normal breast is largely unexplored, and the effectiveness of physiologically and therapeutically relevant formulations of such agents was not known in the normal breast.

Cumulative lifetime exposure to ovarian hormones increases breast cancer risk, and the majority of human breast cancers are ERα positive (9). However, ERα-positive cells are in the minority in the normal breast, and the greater proportion of proliferating cells lack expression of ERα (40, 41). These findings indicate that ERα-negative mammary epithelial cells proliferate under the influence of ovarian hormones, probably via hormone induction of paracrine growth factors, and may give rise to ERα-negative breast tumors. Because proliferation in ERα-negative cells is still under hormonal control, albeit indirectly, this idea remains consistent with the hormonal etiology of human breast cancer (48). ERα-negative breast tumors tend to be more aggressive, with a worse clinical outcome, compared with ERα-positive breast tumors (49), and the majority of *BRCA1*-associated breast cancers are ERα negative (5). A preventative strategy aiming for a reduction in proliferation of normal mammary cells and preneoplastic cells would therefore need to encompass inhibition of both ERα-positive and ERα-negative cells.

Our previous data in cultured cell lines showed that PB inhibited proliferation of both ERα-positive and ERα-negative breast cancer cells (39). The ability to equally inhibit proliferation of ERα-positive and -negative cancer cells is an attractive attribute for breast cancer prevention, as mentioned above. However, these data suggested that inhibition of growth in cell culture was not directly mediated by down-regulation of ERα, because proliferation was inhibited in cells lacking the receptor. Nevertheless, PB is likely to dampen the proliferative effects of ovarian hormones in the mammary gland *in vivo* by decreasing ERα expression and inhibiting paracrine growth factor signaling.

The expression of cyclin D1 was also decreased in mammary cells *in vivo* by PB in this study. Cyclin D1 has been implicated in mammary carcinogenesis. Overexpression of cyclin D1 in transgenic mice led to the development of mammary adenocarcinomas (50), cyclin D1 is overexpressed in almost 50% of human breast cancers (51), and cyclin D1-deficient mice are resistant to mammary cancers induced by the *neu* and *ras* oncogenes (52). In addition, cyclin D1 is overexpressed in the majority of tumors arising in *BRCA1* knockout mice (53) and in half of the *BRCA1*-associated human breast cancers (54). Cyclin D1 is therefore a rational target for mammary chemoprevention.

The relationship between cyclin D1 and ERα down-regulation is intriguing. Functional interactions have been described previously between ERα and cyclin D1. Estrogens stimulate G_1-S-phase transition associated with increased cyclin D1 expression and activation of cyclin-dependent kinases (55, 56), and estrogen can transcriptionally activate cyclin D1 expression, depending on the cellular context (57). This could suggest that the PB-associated decrease in cyclin

D1 is secondary to decreased expression of ERα. However, our previous data in cell lines showed that cyclin D1 expression was decreased in both ERα-positive and -negative cell lines, suggesting that the PB-mediated reduction in expression of cyclin D1 is independent of reduction of ERα expression (39). Further evidence that reduction in cyclin D1 expression is a key feature of butyrate-induced inhibition of proliferation is provided by investigation of butyrate-resistant clones. Although butyrate down-regulated cyclin D1 in parental HeLa cells, as has been shown in number of cell lines, it failed to regulate expression of cyclin D1 in a butyrate-resistant HeLa clone (58).

The mechanisms of butyrate action are not fully understood. The effects of butyrate on gene expression have been attributed to histone hyperacetylation (24), and an increase in acetylated histone immunostaining was observed in the mammary epithelial cells of PB-treated mice. Other histone deacetylase inhibitors also decrease expression of cyclin D1 (59), suggesting that this butyrate effect may be mediated by histone deacetylase inhibition. The effects of butyrate on the expression of cyclin D1 are mediated via defined sequences in the cyclin D1 promoter (27). A fragment of a novel butyrate-induced transcript (B-ind1) has been shown to block Rac1-mediated nuclear factor-κB activity (60). It has been shown previously that activation of Rac1 enhances expression of cyclin D1 through a nuclear factor-κB site in the proximal promoter (61). Therefore, butyrate may decrease cyclin D1 expression by interrupting Rac1 signaling.

Butyrate, and other histone deacetylase inhibitors, have been shown to suppress nuclear factor-κB activity in colon cells (62–64). In breast cancer cells, blocking nuclear factor-κB activation not only inhibits cell proliferation but also antagonizes the antiapoptotic role of this transcription factor, particularly in ERα-negative breast cancer cells (65). *In vivo* and *in vitro* experiments have suggested the involvement of nuclear factor-κB in ER-negative mammary epithelial cell-mediated tumorigenesis (65). Butyrate inhibition of nuclear factor-κB activity is therefore one potential mechanism of chemoprevention.

Butyrates are considered to be safe, having a low incidence of side effects, and are used in children for the treatment of urea cycle disorders and hematological diseases such as β-thalassemia (33, 34). Although the currently available oral formulations of PB still require frequent dosing with large numbers of capsules or tablets to sustain effective plasma drug concentrations, alternative derivatives and delivery strategies are being developed that may overcome these limitations (36, 66). In addition, other potent histone deacetylase inhibitors, such as the well-tolerated antiepileptic drug valproic acid, are also being investigated for anticancer activity (67). In this study, little difference in effect was observed between what is considered in humans to be a moderate dose and a high dose. Therefore, potentially, the dose may be able to be further reduced without a decrease in effectiveness. Similarly, only one, relatively short, time point was investigated. Lower doses over a longer time course may have similar effects in the mammary gland from those observed here.

Agents that decrease hormone responsiveness and down-regulate cyclin D1 are likely to decrease breast cancer risk. We have shown that PB has these effects in normal mammary cells *in vivo* and as such may be considered a candidate primary chemopreventative agent. Other histone deacetylase inhibitors (tributyrin and suberanilohydroxamic acid) have been shown to inhibit carcinogen-induced rat mammary carcinogenesis (18, 68). The effectiveness of butyrate analogues in the rodent mammary gland *in vivo* and in normal and malignant human breast cells in culture adds further evidence that this class of compounds warrants consideration as preventative agents in women at high risk of developing breast cancer.

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