Progesterone induction of calcitonin expression in the murine mammary gland

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Abstract

Progesterone, via its nuclear receptor, is mandatory not only for the induction and specification of mammary gland ductal side-branching and lobuloalveologenesis but also for carcinogen-induced mammary tumorigenesis. Notwithstanding these recent advances, a more comprehensive molecular explanation of progesterone-induced mammary morphogenesis is contingent upon the identification and characterization of mammary molecular targets that are responsive to the progesterone signal. Toward this goal, we report that calcitonin, a 32 amino acid peptide hormone involved in calcium homeostasis, is exclusively expressed in, and secreted from, luminal epithelial cells within the

mammary gland of the pregnant mouse, and, importantly, its expression is progesterone-dependent. Conversely, the calcitonin receptor is present during all stages of post-natal mammary development examined, is localized to the myoepithelial cell lineage, and is not regulated by progesterone. Because calcitonin induction spatiotemporally correlates with increases in progesterone-induced mammary gland proliferation and structural remodeling, we posit that calcitonin – through its receptor – may be involved in one or both of these progesterone-dependent processes.

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Introduction

Utilizing a progesterone receptor (PR) knockout (PRKO) mouse, in which both isoforms of PR (PR-A and -B) were simultaneously ablated through gene-targeting approaches, we previously demonstrated that the progesterone proliferative signal is essential for pregnancy-associated ductal side-branching and alveologenesis (Lydon *et al.* 1995, Soyak *et al.* 2002). Of importance to breast cancer, removal of the progesterone proliferative signal resulted in a marked reduction in susceptibility of the PRKO mouse to carcinogen-induced mammary tumorigenesis (Lydon *et al.* 1999, Chatterton *et al.* 2002, Medina *et al.* 2003), supporting an essential role for progesterone and its downstream effectors in breast cancer etiology. This latter proposal agrees with recent clinical studies demonstrating that combined estrogen and progesterone (EP) postmenopausal hormone replacement therapies increase breast cancer beyond the risk presented by estrogen treatment alone (Schairer *et al.* 2000). Previous mammary gland transplantation approaches, immunohistochemistry, and more recently the PR-LacZ knockin reporter, have revealed that the progesterone signal emanates exclusively from a subset of luminal epithelial cells in the murine mammary gland (Silberstein *et al.* 1996, Briskin *et al.* 1998, Seagroves *et al.* 2000, Ismail *et al.* 2002). Moreover, the observation that luminal epithelial cells expressing PR

are segregated from, but are in close apposition to, epithelial cells that undergo progesterone-induced proliferation (Seagroves *et al.* 2000, Ismail *et al.* 2002), suggests a paracrine mode of action for progesterone in this tissue. A similar mechanism of action has also been proposed to underlie progesterone-induced proliferation in the rat and human mammary gland (Clarke *et al.* 1997, Russo *et al.* 1999).

Despite recent advances in our understanding of progesterone's role in mammary gland development and tumorigenesis, it remains a challenge to mechanistically define the paracrine pathways through which the PR mediates the progesterone signal. A more comprehensive molecular explanation of progesterone's action in the mammary gland must await the identification and characterization of downstream molecular targets that are directly or indirectly modulated by progesterone.

Previously, calcitonin (CT), a 32 amino acid peptide hormone involved in calcium homeostasis (Sexton *et al.* 1999, Pondel 2000), was identified as a uterine molecular target transiently and specifically induced by progesterone in the early pregnant rat, baboon and human (Ding *et al.* 1994, Kumar *et al.* 1998, 2003). Outside the brain, most of the physiological effects of CT are transduced by the CT receptor (CTR) C1a subtype, a member of a superfamily of heptahelical membrane-spanning G protein-coupled receptors (Brown *et al.* 1996). Interestingly, this receptor

superfamily includes the receptor for the peptide hormone, parathyroid hormone-related protein (PTHrP) (Brown *et al.* 1996); like CT, PTHrP exhibits potent controls on calcium homeostasis (Dunbar *et al.* 1996), but also exerts a pivotal role in mammary gland development and function (Wysolmerski *et al.* 1995). As a corollary to the above, osteoprotegerin ligand (OPGL), which is also involved in bone homeostasis, was recently shown to play a critical role in mammary alveologenesis (Fata *et al.* 2000); interestingly, mammary OPGL expression has been shown to be modulated (in part) by progesterone (Fata *et al.* 2000, Mulac-Jericevic *et al.* 2003).

Of further significance, CT and CTR have been detected in human breast cancer cell lines (Findlay *et al.* 1981, Kurokawa *et al.* 1991) and in primary breast cancers (Gillespie *et al.* 1997), suggesting that aberrant expression of the CT signaling pathway may confer upon breast cancer cells a selective growth advantage and/or an ability to undergo metastasis to other target tissues, such as bone; this latter suggestion is based on CT's previously described bone-resorbing functions (Sexton *et al.* 1999). Recently, the induction of CT and CTR transcripts was detected in the normal mammary gland of the pregnant rat, but not in the virgin gland (Tverberg *et al.* 2000), suggesting that one or more pregnancy hormones may modulate the emergence of this signaling pathway in the rat mammary gland. Based on the above, the mouse – in particular the PRKO mouse – was employed to evaluate whether CT and CTR are expressed in the murine mammary gland, and if so, to determine whether their expression is responsive to the endocrine mammo-genic effects of progesterone.

Materials and Methods

Mice and hormone treatment

Mice, with a C57BL6/129SvJ mixed genetic background, were maintained in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) with a 12 h light:12 h darkness photocycle and fed rodent chow meal (Purina Mills, Inc., St Louis, MO, USA) and fresh water, freely available. The PRKO mouse model has been previously described (Lydon *et al.* 1995).

For total RNA isolation and immunohistochemistry, mammary glands (both thoracics and inguinals) were dissected from mice at various stages of mammary development: virgin (12 weeks old), pregnancy (days 2, 4, 6, 8, 12 and 18), lactation (day 7) and involution (day 7); for timed pregnancies, the morning of observing the vaginal plug was designated as day 0.5 of pregnancy. Tissues were processed for immunohistochemistry or snap frozen on dry ice for RNA isolation.

To elicit ductal side-branching and initial alveologenesis in the intact virgin mouse (12 weeks old), EP (estrogen (1 µg) and progesterone (1 mg) (in sesame oil) in combination) was administered by daily intrascapular s.c.

injections for 7 days, according to procedures described previously (Said *et al.* 1997).

RNA and immunohistochemistry

The RNase protection assay (RPA) was performed using the RPA III kit according to the manufacturer's instructions (Ambion, Inc., Austin, TX, USA); mammary gland RNA isolation was previously described (Lydon *et al.* 1999). The murine CT RPA probe, obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA (ATCC number 1213716), encompassed 350 bp localized within exon 4 of the murine CT gene (Thomas *et al.* 2001). For each developmental time point or hormone treatment group, the CT mean transcript induction ($\pm$ S.E.M.) is presented as a percentage of cyclophilin message from five separate experiments; four mice per time point or hormone-treatment group were used in the RNA and immunohistochemical assays described herein. Radioactive signals from protected fragments were quantified on a Storm 860 Phosphor Imager (Molecular Dynamics, Inc., Sunnyvale, CA, USA), standardized relative to cyclophilin and represented as an average of five experiments $\pm$ S.E.M.

For immunohistochemistry, mammary glands were fixed overnight in Bouin's fixative, followed by thorough washing in 70% ethanol before tissues were processed, embedded in paraffin, and sectioned. CT immunohistochemistry was performed using a rabbit polyclonal antibody to human CT, which cross-reacts with murine CT (NCL-CALp; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Immunohistochemistry for PR expression and 5-bromo-2-deoxyuridine (BrdU) incorporation is described elsewhere (Lydon *et al.* 1999). CTR immunohistochemistry was undertaken using a goat polyclonal antibody raised against the amino terminus of the mouse CTR C1a subtype (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). For dual immunofluorescence, a rabbit anti-mouse cytokeratin 14 (Covance Research Products, Inc., Berkeley, CA, USA) was used in concert with the CTR primary antibody using previous methods (Seagroves *et al.* 2000). To visualize resultant immunocomplexes, donkey anti-goat IgG secondary antibody linked to fluorescein isothiocyanate (green) and donkey anti-rabbit IgG secondary antibody conjugated to Texas Red (red) were used to detect cytokeratin 14 and CTR respectively (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and were in accordance with the procedures detailed in the 'Guide for Care and Use of Laboratory Animals' (NIH publication 85-23).

Results

By the second day of pregnancy, RPA detected CT transcript induction in the murine mammary gland

Figure 1 Parity-induced CT expression in the murine mammary gland. RPA for mammary CT expression is shown in (A) for the 12-week-old virgin mouse (V), during days 2, 4, 6, 8, 12 and 18 of pregnancy, and on day 7 of lactation (L) and involution (IN). CT transcripts are induced in the mammary gland as early as day 4 of pregnancy (black arrow; protected fragment: 350 bp), with maximal transcript levels occurring around day 6 of pregnancy. Mammary CT expression was not detected toward the end of pregnancy (day 18), or during the stages of lactation and involution. Cyclophilin was included as a loading control (gray arrow; protected fragment: 100 bp). Indicated on the right-side of the gel are probe controls in the presence of yeast RNA consisting of CT or cyclophilin (CY) antisense probes in the absence (–) or presence (+) of RNase. Both marker lanes are denoted by M. The graph in (B) quantitatively displays the induction of CT expression (shown in (A)) as a percentage of cyclophilin mRNA. CT immunohistochemical analysis of the mammary gland of the 12-week-old virgin, pregnant mouse (day 6), late pregnant mouse (day 18) and of the lactating mouse (day 7) is shown in that order in (C–F). Note the induction of CT expression is restricted (arrow) to the mammary epithelial compartment of the parous mouse (D). Scale bar in (C) measures 100 μ m and also applies to (D–F). The RPA and immunohistochemical data are representative of five separate experiments in which four mice per time point were used for each experiment (20 mice per developmental time point in total).

Figure 2 CT is exclusively expressed in and secreted from the luminal epithelial cell lineage of the mammary gland of the parous mouse. (A) High-magnification of Fig. 1D reveals that mammary luminal epithelial cells express and secrete CT (black arrow). Note the mitotic figure (white arrow) in the luminal epithelial compartment, indicative of an actively proliferating tissue. (B) Higher magnification of a longitudinal section of a duct in (A) reveals the presence of CT in the luminal (brown arrow) and in the luminal–myoepithelial region (black arrow). (C) reveals significant BrdU incorporation (black arrow) in the mammary gland of the 6-day pregnant mouse. Inset shows numerous luminal epithelial cells that express PR (black arrow) at this time. (D) Is a higher magnification of a longitudinal section shown in (C) clearly showing luminal (black arrow) and myoepithelial (gray arrow) proliferation. Scale bar in (A) equals 50 μ m and also applies to panel (C); scale bar in inset denotes 50 μ m, whereas scale bar in (B) indicates 20 μ m and also applies to (D).

(Fig. 1A and B). The highest levels of CT expression were reached by days 4–6 of pregnancy, only to decline by mid-pregnancy (day 12) to levels observed on day 2 of pregnancy. Mammary transcripts for CT were detected neither during the later time points of pregnancy (day 18) nor during the stages of lactation (day 7) and involution (day 7). As in the rat (Tverberg *et al.* 2000), CT expression was not detected in the mammary gland of the 12-week-old virgin mouse; however, unlike the rat, CT expression was restricted to the first half of pregnancy in the mouse. Immunohistochemistry confirmed that the virgin mammary gland did not express CT protein (Fig. 1C); however, significant CT expression was detected in the mammary gland of the 6-day pregnant mouse (Fig. 1D (arrow)). As expected, CT protein was not detected in the mammary gland of the late-pregnant mouse (day 18) or of the lactating mouse (Fig. 1E and F respectively); CT

immunoreactivity was not observed in the involuting mammary gland (data not shown).

Close scrutiny of the mammary gland of the 6-day pregnant mouse revealed that the luminal epithelial cell was the cell of origin for CT expression and secretion (Fig. 2A; black arrow). In concordance with this observation, pro-CT protein was recently detected in the ductal lumen of the human mammary gland (Struck *et al.* 2002), further supporting mammary CT as a product of the luminal epithelial cell lineage. Although CT was derived from luminal epithelial cells, the secreted protein was detected both in the ductal lumen and in the luminal–myoepithelial junctional region (Fig. 2B; brown and black arrows respectively); however, CT was not detected in periductal fibroblasts or in the outer stromal compartment. The striking increase in CT expression in the mammary luminal epithelial cell of a 6-day pregnant mouse closely

Figure 3 (A) RPA demonstrates that CT is induced within 7 days of EP treatment in the WT virgin (V) but not in the similarly treated PRKO mouse. The graph in (B) shows the quantification of CT induction (shown in (A)) as a percentage of the loading control, cyclophilin; error bar denotes S.E.M. The RPA data are representative of five experiments in which four mice per hormone treatment and genotype were examined per experiment (20 mice per hormone treatment and genotype in total).

paralleled the noticeable increase in pregnancy-induced proliferation of the luminal and myoepithelial cell at this time (Fig. 2C and D). The increase in mammary epithelial CT expression and proliferation correlates with robust PR expression (inset in Fig. 2C) at this stage of mammary development, an observation consistent with recent PR-LacZ knockin studies (Ismail *et al.* 2002). Future studies, using alternative approaches (such as CT *in situ* hybridization analysis coupled with PR immunohistochemistry), will aim to determine whether the subgroup of luminal epithelial cells that express the PR are the cells of origin for CT expression and secretion.

Because mammary gland CT expression was restricted to the first half of murine pregnancy, we evaluated whether progesterone, through the PR, specifically induced CT in the mammary gland. In response to a 7-day daily treatment of estrogen and progesterone, mammary CT transcripts were induced in the adult wild type (WT) virgin but not in the similarly treated PRKO (Fig. 3A and B), demonstrating that progesterone, not estrogen, is responsible for the induction of CT expression, and that this induction is mediated by the PR; similar findings have been reported for the uterus (Ding *et al.* 1994, Kumar *et al.* 1998, 2003).

As observed during early pregnancy, CT protein induction correlated with an increase in luminal epithelial cell proliferation in the EP-treated WT mouse (Fig. 4; compare A and B with C and D). Interestingly, despite the

presence of PR in the virgin gland (Fig. 4E), mammary CT is not expressed at this developmental stage (Fig. 4A), supporting an obligate role for ligand-dependent activity for PR in the induction of CT expression (Fig. 4B). Unlike progesterone-treatment alone (Ismail *et al.* 2002), a 7-day EP treatment did not suppress PR expression in the mammary gland (Fig. 4F); estrogen is included to maintain PR levels.

To support the concept that an intact CT signaling pathway may be operative in the mammary gland, CTR expression was evaluated during the salient stages of mammary gland development. Unlike CT, CTR is expressed in the virgin mammary gland (Fig. 5A) and is restricted to the myoepithelial cell lineage (black arrow); CTR is not expressed in the luminal cell (red arrow). CTR is present during all stages of mammary development including the later stages of pregnancy (day 18) (Fig. 5B); Fig. 5C and D are higher magnifications of A and B respectively. To further confirm that CTR expression is limited to the myoepithelial cell lineage (and not to periductal fibroblasts), dual immunofluorescence for cytokeratin 14 (Fig. 5E) and CTR (Fig. 5F) was performed; cytokeratin 14 is a cellular marker for the myoepithelial cell (Smith *et al.* 1990). In the case of a longitudinal section of a main epithelial duct, Fig. 5G demonstrates that CTR coexpresses with cytokeratin 14 in the myoepithelial cell type (white arrow).

Discussion

During the onset of pregnancy, the pre-implantation uterus and mammary gland undergo striking changes in proliferation and structural remodeling, both of which are critically dependent on PR function (Soyal *et al.* 2002). In the case of the PRKO mammary gland, removal of the progesterone proliferative signal abrogates both tertiary ductal side-branching and lobuloalveologenesis (Lydon *et al.* 1995). Because this specific stage of progesterone-induced mammary morphogenesis occurs during pregnancy and because an early or late first pregnancy differentially impacts the life-long risk for breast cancer (Henderson *et al.* 1993), delineating the downstream signaling pathways through which progesterone induces these morphological changes is of paramount importance. Toward this goal, RPA and immunohistochemical approaches in combination with the PRKO mouse unequivocally demonstrated that progesterone, through its receptor, can induce CT expression in the luminal epithelial cell of the murine mammary gland. In contrast, immunohistochemistry revealed that CTR is localized to the myoepithelial cell lineage and its expression is not affected by progesterone. The difference in the temporal expression profiles for CT and CTR between the mouse and rat reflects a fundamental difference in mammary physiology in these two rodent species.

Figure 4 The PR requires progesterone to induce CT expression and luminal epithelial proliferation in the murine mammary gland. (A, C, and E) Correspond to the 12-week-old virgin mouse, whereas (B, D and F) represent the 7-day EP-treated age-matched virgin. Panels (A and B), (C and D) and (E and F) show CT, BrdU, and PR immunoreactivity respectively. Note the increase in CT protein expression in response to EP treatment (compare (A) with (B) (arrow)) which correlates with an increase in the proliferative status of the gland, as judged by an increase in cellular BrdU incorporation (compare (C) with (D) (arrow)). In (E), note that PR expression alone (arrow) is not sufficient to induce CT expression (compare (A and E) with (B and F)). Scale bar in (A) denotes 100 μ m and applies to all panels.

The restricted expression of CT and CTR to the luminal and myoepithelial cell lineages respectively raises a number of important points.

First, like the uterus (the archetypal progesterone target tissue), an intact CT signaling pathway may be intrinsic

to the murine mammary epithelium; however, this assumption has yet to be functionally validated.

Secondly, if future studies demonstrate a functional mammary CT signaling pathway, the segregation of CT and CTR expression at the cellular level would suggest

Figure 5 Immunohistochemistry reveals CTR expression is limited to the myoepithelial cell lineage (black arrow) of the mammary gland of the 12-week-old virgin (A) and mid-pregnant mouse (B); unlike CT, CTR is not expressed in the luminal epithelial compartment (red arrow). Higher magnifications of (A) and (B) are shown in (C) and (D) respectively. Double immunofluorescence for the cytokeratin 14 (E) and CTR (F) confirms CTR expression is confined to the myoepithelial cell lineage: in the longitudinal section of a typical medial duct, (G) represents the overlay of (E) and (F) and clearly shows cellular co-expression for both proteins in the myoepithelial cellular compartment (white arrow). Scale bar in (A) and (C) denotes 50 μ m and applies to (B) and (D) also. Scale bar in (E) represents 20 μ m and also applies to (F) and (G).

that progesterone could instigate a molecular dialog between the myo- and luminal epithelial cell lineages. That CT could act as a paracrine mediator in such a dialog is supported by the fact that: (i) uterine CT was shown to exert its effects through a paracrine circuit during murine embryo implantation, with maternally derived CT and embryo-derived CTR making up this circuit (Wang *et al.*

1998); (ii) in the anterior pituitary gland, CT acts as a paracrine signaling pathway between the gonadotrope and lactotrope cell types to suppress prolactin secretion (Ren *et al.* 2001); (iii) the closely related PTHrP also makes up a paracrine signaling pathway that is indispensable for early mammary development (Wysolmerski *et al.* 1995); and (iv) progesterone – an upstream affecter of CT expression

in the mammary gland and uterus – is known to exert its functional effects through paracrine mechanisms in both target tissues (Briskin *et al.* 1998, Kurita *et al.* 1998).

Thirdly, the detection of CTR expression in neoplastic mammary luminal epithelial cells in culture (Kurokawa *et al.* 1991) and in primary mammary tumors (Gillespie *et al.* 1997) implies that CTR induction within the luminal epithelial lineage (instead of the myoepithelial cell) may be linked to neoplastic transformation of this cell type – the most prevalent form of breast cancer. Extending this notion further, abnormal CTR expression in the luminal cell lineage could conceivably set in train an autocrine circuit for CT signaling, clearly at odds with the proposed paracrine pathway for CT signaling in the normal mammary gland. Indeed, previous PR immunolocalization studies on estrogen receptor/PR-positive human mammary tumors suggest that progesterone-induced luminal epithelial proliferation may switch from a paracrine signaling pathway in the normal mammary gland to an autocrine loop in neoplastic tissue (Clarke *et al.* 1997).

Collectively, the data suggest that the functional influence of the progesterone signal may extend (via the CT signaling pathway) to the myoepithelial cell lineage, at a time when the mammary gland is undergoing progesterone-induced mammary proliferation and tissue remodeling. Although speculative, it is not inconceivable that for normal progesterone-induced luminal epithelial cell proliferation to occur in the mammary gland, the myoepithelial compartment may need to receive a progesterone signal (via CT) to proliferate and reorganize to accommodate the increase in the number of luminal epithelial cells arising from the progesterone proliferative signal. To achieve this myoepithelial reorganization, CT may act by altering local cellular calcium homeostasis. Changes in calcium levels are known to impact cell–cell adhesion (Sjaastad & Nelson 1997), thus the hypocalcemic effects of CT may control the disassembly of cellular junctional complexes within the myoepithelial compartment to facilitate the extensive structural remodeling that occurs in the mammary gland during pregnancy or in response to EP treatment. Moreover, a recent report documenting CT modulation of uterine E-cadherin (Li *et al.* 2002) could represent an additional mechanism by which CT disassembles mammary epithelial junctional complexes.

The foregoing speculations must be tempered, however, by the recent observation that the CT knockout (CTKO) mouse (Hoff *et al.* 2002) does not exhibit overt defects in fertility or mammary development; a knockout for CTR has yet to be reported. Although the absence of a mammary phenotype in the CTKO mouse argues that CT is not required *per se* for normal mammary development, the data do not exclude the possibility that parallel progesterone-dependent signaling pathways compensate for CT's absence (considering progesterone's pivotal role

in mammary morphogenesis, built-in degeneracy downstream of the PR would not be surprising). Moreover, with the emergence of receptor-activity modifying proteins (McLatchie *et al.* 1998, Christopoulos *et al.* 1999, Sexton *et al.* 2001), the possibility that mammary CTR may project all or part of its downstream effects independently of CT ligand must be considered, especially in light of the marked difference in spatiotemporal expression profiles for mammary CT and CTR.

Irrespective of the possible mechanisms of CT action in the mammary gland, we report the expression and cellular localization of CT and its receptor in the murine mammary gland. Importantly, as previously documented for the uterus, progesterone (via PR) specifically induces mammary gland CT expression, which is the first reported target gene regulated by progesterone in both the uterus and mammary gland. Apart from attempting to address an acknowledged insufficiency in our mechanistic understanding of progesterone's role in mammary gland morphogenesis and function, this study underscores the importance of further delineating the molecular nature of the cross-talk that exists between progesterone and CT signaling pathways *in vivo*, particularly in light of the continued use of these hormones in postmenopausal treatments.

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