Title
IRE1 phosphatase PP2Ce regulates adaptive ER stress response in the postpartum mammary gland.

Permalink
https://escholarship.org/uc/item/4bs6640m

Journal
PloS one, 9(11)

ISSN
1932-6203

Authors
Ren, Shuxun
Lu, Gang
Ota, Asuka
et al.

Publication Date
2014

DOI
10.1371/journal.pone.0111606

Peer reviewed
IRE1 Phosphatase PP2Ce Regulates Adaptive ER Stress Response in the Postpartum Mammary Gland

Shuxun Ren¹, Gang Lu¹, Asuka Ota¹, Z. Hong Zhou⁴, Thomas M. Vondriska¹,², Timothy F. Lane³, Yibin Wang¹,²*

¹ Department of Anesthesiology, Division of Molecular Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States of America, ² Departments of Physiology and Medicine, Cardiovascular Research Laboratories, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States of America, ³ Departments of Obstetrics and Gynecology and Biological Chemistry, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States of America, ⁴ Departments of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States of America

Abstract

We recently reported that the PPM1I gene encodes an endoplasmic reticulum (ER) membrane targeted protein phosphatase (named PP2Ce) with highly specific activity towards inositol-requiring protein-1 (IRE1) and regulates the functional outcome of ER stress. In the present report, we found that the PP2Ce protein is highly expressed in lactating epithelium of the mammary gland. Loss of PP2Ce in vivo impairs physiological unfolded protein response (UPR) and induces stress kinase activation, resulting in loss of milk production and induction of epithelial apoptosis in the lactating mammary gland. This study provides the first in vivo evidence that PP2Ce is an essential regulator of normal lactation, possibly involving IRE1 signaling and ER stress regulation in mammary epithelium.

Introduction

During lactation, mammary gland produces large quantity of protein and lipid in a timely and quantitatively regulated manner. Mammary gland epithelial cells undergo proliferative and hypertrophic response rapidly at the onset of lactation with dramatically induced protein/lipid synthesis and secretion capacities. The endoplasmic reticulum (ER) is the key organelle for protein and lipid synthesis, secretion and post-translational modification [1–3]. Therefore, it is expected that the ER load would be significantly increased in the mammary epithelium during lactation. Overloaded ER would lead to an increase in unfolded or misfolded ER proteins which are able to trigger specific downstream signaling collectively described as the unfolded protein response (UPR). The physiological aspect of UPR is accomplished by compensatory induction of ER chaperones (thus increasing ER capacity) and inhibition of protein translation (thus reducing ER load) in order to restore ER homeostasis [4,5]. The underlying molecular mechanisms of UPR signaling involve several trans-membrane sensing molecules, including inositol-requiring protein-1 (IRE1), PKR-like Endoplasmic Reticulum kinase (PERK) and Activating Transcription Factor 6 (ATF-6) [4,6,7]. However, abnormal UPR as in the case of persistent ER overload, oxidative injury, and other pathological conditions can trigger cellular dysfunction and apoptosis (Ref). Indeed, abnormal UPR has already been implicated in CNS diseases, diabetes, obesity, inflammation and heart diseases [8–13]. It is well recognized that lactation involves abrupt changes in ER load in mammary gland epithelium, and ER stress related genes have been shown to be dynamically expressed during the lactation cycle [14]. However, the importance of ER stress signaling in normal mammary gland physiology has not been demonstrated.

In UPR signaling, IRE1 activity has the unique ability to stimulate multiple pathways, one with adaptive, the other with detrimental, effects in cells. Oligomerization of IRE1 activates RNase activity and downstream splicing of X-box binding protein-1 (XBP1) mRNA as well as degradation of 28S rRNA. XBP1 acts as a transcription factor to induce chaperone protein expression; loss of 28S rRNA due to IRE1-dependent RNase activity leads to inhibition of protein synthesis [15–19] [20]. On the other hand, IRE1 activation can also cause cell death via TRAF2-ASK1-JNK pathway [21–24]. A key step in IRE1 activation is the oligomerization and subsequent trans-autophosphorylation. Recently, we discovered that an ER localized protein phosphatase, PP2Ce, dephosphorylates IRE1 with high selectively, resulting in a potent IRE1 inhibitory effect [25]. The PP2Ce coding gene, ppm1H, is significantly associated with obesity and metabolic disorder based on genome wide association studies [26] [25], providing genetic evidence for a link between IRE1 regulation and metabolic diseases. However, specific contribution of PP2Ce in mammary gland function and ER stress regulation has not been investigated.

In the present study, we found that PP2Ce was significantly expressed in mammary gland epithelium and dynamically regulated at the onset of lactation, correlating with similar expression profile of IRE1ζ. Genetic inactivation of PP2Ce in...
female mice caused lactation defects associated with abnormal UPR and enhanced stress signaling. In addition, abnormal ER ultra-structure and a significant induction of apoptosis was detected in the lactating mammary epithelium of PP2Ce deficient mice comparing the wildtype controls. Therefore, this study shows for the first time a critical function for PP2Ce in UPR and lactation in mammary gland.

Materials and Methods

Animals

The ppm1k knockout and wildtype mice were established in C57BL6 background as described [25]. Specifically, a IRES-neo-lacZ cassette was inserted at mouse ppm1l exon 4 region, replacing 183 bp of the coding sequence while directing the lacZ expression by the ppm1l locus. The knockout allele was identified by genomic DNA PCR for genotyping using a pair of oligoes, A1 for mouse genomic sequence and A2 for Neo cassette sequence (see below for sequences). A1: GCCTCTGTAAAAGGACTGCAGGACG. A2: GGGTGGGATTAGATAAATGCCTGCTCT. Homozygous KO mice were generated by breeding two heterozygous KO mice and maintained by breeding homozygous KO males with heterozygous KO females. All animals were maintained and bred at certified animal facility of University of California, at Los Angeles. All animal work were conducted following protocols approved by UCLA Office of Animal Research Oversight.

Immunoblotting

Polyclonal antibody against β-Actin, p-JNK, JNK, p-p38, p38, caspase-12 and activated caspase-12 were purchased from Cell Signaling and used following manufacturer’s recommendations.

Realtime RT-PCR analysis

Total RNA was extracted from tissues using Trizol Reagent (Invitrogen) according to manufacturer’s instructions. 5 μg total RNA was reverse transcribed into the first-strand cDNA using Superscript First-strand synthesis Kit (Invitrogen) with oligo-dT primers. Then, cDNA transcripts were quantified by iCycler iQ Real-Time PCR Detection System (Bio-RAD) using iQ SYBR Green Supermix (Bio-RAD). Each reaction was performed in duplicate and values were averaged to calculate the relative expression level. The RT-PCR primers used are listed below: PP2Ce: 5’- TTGTCTCAGATCACAAGCC; 3’- AGATGTCGCTGTCGCTCTCT. IRE1α: 5’- AGGTTGGAGATGCTGGTCTGGGATG; 3’- TGGGGATCCATAGCAATCAT. EDEM: 5’- TCTGTGGACAAACGTCTTCG; 3’- GGCGCATGTAGATGCTCTTT. Bip: 5’- GAGGCTGTAGCCTATGGTGC; 3’- TTTGGTTAGGGGTCGTTCACC. CHOP: 5’- TATCTCATCCCAGGAAACG; 3’- GGGCACTGACCACTCTGTTT. GAPDH: 5’- TCCTGCACCCACCACTGCTTTAG; 3’- GATGACTTGCCACAGGCGTTG.
Whole-mount carmine and β-galactosidase staining of mouse mammary glands

Female mice were killed around 10 wk, freshly dissected mammary gland tissue were flatly placed on glass slides and fixed in Carnoy’s solution (70% ethanol, 20% chloroform, and 10% glacial acetic acid) for 1 hr at room temperature. The fixed glands were washed in 70% ethanol for 15 min and then rinsed in water for 5 min. The mammary glands were stained overnight at 4°C in carmine aluminum solution (1 g carmine, sigma C1022 and 2.5 g aluminum potassium sulfate (Sigma A7167) in 500 ml water). The glands were then dehydrated progressively in 70%–95%–100% ethanol, cleared in xylene for 30 min, and mounted on glass slides with Permount (Fisher Scientific, Suwanee, GA). For β-gal staining, same tissues were 0.2% paraformaldehyde in PBS (0.1 M, PH = 6.9) with 2 mM MgCl₂ and 5 mM EGTA overnight at 4°C. After washing three times with PBS, the tissue samples were incubated for 2 h at 30°C in X-gal reaction buffer containing 5 mM K-Ferricyanide, 5 mM K-Ferrocyanide, 0.02% NaDecoxycholate, 0.01% NP-40, 2 mM MgCl₂ and 1 mg/ml X-gal for 24 to 48 h at 30°C. The tissues were then washed with PBS. The stained tissues were viewed under Nikon SM21500 dissection microscope and images were recorded digitally.

Histological analysis

Mammary gland samples were dissected from 10 month old virgins or female animals at postpartum day 0 and 2. The tissues were fixed overnight in 10% formalin (SIGMA HT501128-4 L) and processed as following: 40°C in automatic tissue processors through 50, 80, 95, 100% ethanol and xylene, then embedded in paraffin molds at 60°C. Blocks were sectioned in a microtome at 4 μm followed by deparaffinization and rehydration through 3 cycles of xylene and graded ethyl alcohols to PBS. The sections were blocked 30 min at room temperature in TNB blocking buffer (PerkinElmer, NEL 701) and then incubated with WAP primary antibody (1: 100; Santa Cruz, sc-43302) for overnight. Washed twice with PBS and stained with fluorochrome-coupled anti-goat secondary antibodies (Molecular Probes, A-11055), samples were mounted for microscopic inspection in mounting medium (DAPI, Vector Lab. Inc.). Images were viewed from a Nikon Ccnfocol fluorescent microscope according to manufacturer’s instruction. For transmission electron microscopy, the tissues were fixed, sectioned, mounted as described [27] and imaged at UCLA Electron Imaging Center for Nanomachines.
Statistical Analysis

All statistical analysis was performed using student t-test for comparison between two groups and ANOVA test for multi-group comparisons. \( p < 0.05 \) was accepted as significant.

Results

PP2Ce expression in lactating mammary gland

Using a mouse line with lacZ knocked in at the \( ppm1l \) locus [25], we first determined the expression of PP2Ce in intact mammary gland using whole-mount X-gal staining. As shown in Figure 1, a high level of X-gal positive staining reflecting the PP2Ce expression pattern was detected in the epithelium of mammary gland. Therefore, \( ppm1l \)-driven expression of X-gal in the knock-out reveals \( ppm1l \) expression in the mammary gland, which is confirmed by analysis via qRT-PCR. Using qRT-PCR, we found PP2Ce mRNA levels to be significantly induced in the mammary gland of the postpartum animal as compared with that in virgin females (Figure 2A). In parallel with PP2Ce expression, IRE1\( \alpha \) mRNA was also significantly induced in the mammary glands of lactating mice versus virgin females (Figure 2B). Therefore, PP2Ce and IRE1\( \alpha \) are both induced at the onset of lactation in postpartum mammary glands.

Figure 6. Stress signaling activation in postpartum PP2Ce deficient mammary gland. A. Immunoblot of phospho-JNK, phospho-p38 and Caspase 12 activation (cleaved) levels in PP2Ce +/- and PP2Ce -/- mammary tissue at day 0 (L0) or day 2 post parturition (L2). B. Quantification of signaling activation from at least 3 samples from each group. #, \( p < 0.05 \) WT vs. KO.

doi:10.1371/journal.pone.0111606.g006

Figure 7. Effect of PP2Ce deficiency on ER stress response. mRNA levels of adaptive ER stress response markers BIP and EMDM, and maladaptive ER stress response marker CHOP in wildtype (WT) and \( pp2ce/- \) (KO) mammary tissue at day 0 (L0) and day 2 (L2) postpartum. * \( p < 0.05 \), ** \( p < 0.01 \) by ANOVA test.

doi:10.1371/journal.pone.0111606.g007

Figure 7. Effect of PP2Ce deficiency on ER stress response. mRNA levels of adaptive ER stress response markers BIP and EMDM, and maladaptive ER stress response marker CHOP in wildtype (WT) and \( pp2ce/- \) (KO) mammary tissue at day 0 (L0) and day 2 (L2) postpartum. * \( p < 0.05 \), ** \( p < 0.01 \) by ANOVA test.
Mammary gland defects in PP2Ce deficient females

PP2Ce is essential for normal lactation

To determine the functional role of PP2Ce in mammary gland function, a genetic knockout mouse line was created as described [25,26]. Inactivation of PP2Ce expression in mammary gland was validated at mRNA level by qRT-PCR (Figure 2A). In virgin females, histological analysis of mammary gland showed similar gross morphology and branching profile between the wildtype and PP2Ce knockout (KO) mice (Figure 3). The litter sizes produced by the PP2Ce KO females are similar at birth comparing with those produced by the PP2Ce heterozygous females, however, all offspring from the PP2Ce KO females died within two days postnatally regardless of their own genotypes (Table 1). Upon closer inspection, we found that the neonates from the PP2Ce KO mothers died from a lack of milk consumption. Consequently, by switching to foster mothers of wildtype (WT) background, the neonates from the PP2Ce KO females were successfully weaned. In contrast, those produced by the PP2Ce heterozygous females, however, all offspring from the PP2Ce KO mice (Figure 3). In WT KO mammary tissues, we indeed observed a marked induction of apoptosis (Figure 8B). Therefore, PP2Ce is essential for adaptive ER stress signaling, milk production and cellular survival in lactating mammary cells.

PP2Ce KO induces apoptotic cell death in postpartum mammary epithelium

It is well established that constitutive activation of IRE1 signaling can trigger apoptosis via caspase-12 activation. In the PP2Ce KO mammary gland, we indeed observed a marked induction of apoptosis based on TUNEL analysis in postpartum tissue (Figure 8A and C), which was associated with caspase-12 activation (Figure 6). Ultra-structure analysis also detected signs of enlarged ER lumen and mitochondrial swelling, supporting the status of pathological ER stress response and potential involvement of apoptosis (Figure 8B). Therefore, PP2Ce is essential for adaptive ER stress signaling, milk production and cellular survival in lactating mammary cells.

Discussion

ER stress signaling is important in both physiological UPR as well as ER stress-induced programmed cell death. In this study, we found that an IRE1 specific phosphatase, PP2Ce, is essential for normal lactation function in the postpartum mammary gland. A sharp increase in protein and lipid synthesis, as is observed during normal lactation, increases ER load dramatically; therefore, it would be expected that an adaptive ER stress response would be induced in the lactating mammary gland. It is surprising, however, that a detailed characterization and demonstration of the importance of ER stress signaling in the lactating mammary gland has not been reported to date. A recent study in lactating bovine confirmed a coordinated change in ER stress genes during the lactation cycle [14]. In agreement with this observation, we found significant induction of IRE1α and the downstream adaptive genes BiP and EDEM in the postpartum mouse mammary gland. However, these adaptive changes were attenuated in the PP2Ce deficient mice. In contrast, pathological ER stress markers, including CHOP expression, JNK activation and caspase-12 activation were observed in the postpartum PP2Ce deficient mammary gland. To our knowledge, this in vivo observation represents the first evidence to implicate the importance of PP2Ce in mammary gland physiology. The correlated

PP2Ce Regulates ER Stress in Mammary Gland
changes in ER stress signaling in the PP2Ce deficient mammary gland supports the potential role of PP2Ce as an IRE1 regulator in this process as demonstrated in vitro [25], although other uncharacterized downstream targets of PP2Ce can also contribute to the phenotype observed.

ER stress regulation is important for many physiological functions, including inflammation and metabolism [28–30]. Dysfunction of ER stress signaling has been implicated in many human diseases, such as cancer, obesity, diabetes, neurodegenerative diseases and atherosclerosis [31]. In fact, the gene coding for PP2Ce, *ppm1l*, was first identified as candidate disease-causing gene for complex traits associated with metabolic syndrome [26] [25]. Our study adds normal mammary gland function as one more important organ where PP2Ce plays a critical role. This observation fits with the physiological demands on the mammary gland: few if any other tissues endure such a sudden increase (in both magnitude and speed) of protein and lipid synthesis and secretion. As an adaptive process, ER stress signaling functions to meet protein/lipid demand with synthesis capacity, clearly an important requirement for the mammary gland supports the potential role of PP2Ce as an IRE1 regulator in in vivo experiments: SR GL AO HZ. Analyzed the data: SR TV HZ TL YW. Performed the experiments: SR YW TL. Conceived and designed the experiments: SR YW TL. The authors wish to acknowledge the excellent technical support from Ms. Haiying Pu in this study.

**Author Contributions**
1. Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, et al. (2006) Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. J Cell Biol 172: 383–393.
2. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2003) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107: 891–899.
3. Yawaki T, Hosoda A, Okada T, Kamigishi Y, Nomura-Furusawa et al. (2001) Translational control by the ER transmembrane kinase ribonuclease IRE1 under ER stress. Nat Cell Biol 3: 138–144.
4. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, et al. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptotic and cytotoxicity by amyloid-beta. Nature 403: 98–103.
5. Nishitoh H, Matsuzawa A, Tohjima K, Saegusa K, Takeda K, et al. (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 16: 1345–1355.
6. Zhang K, Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. Nature 454: 455–462.
7. Schroeder M, Kaufman RJ (2005) The mammalian unfolded protein response. Annu Rev Biochem 74: 739–789.
8. Wistow J, Kaufman RJ (2006) ER stress and the unfolded protein response. Curr Mol Med 6: 1–12.
9. Thomas SE, Dalton LE, Daly ML, Malzer E, Marciniak SJ (2010) Diabetes as a disease of endoplasmic reticulum stress. Diabetes Metab Res Rev 26: 611–621.
10. Urano F, Wang X, Berrhetti A, Zhang Y, Chung P, et al. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 267: 664–666.
11. Vidal R, Caballero B, Couve A, Hetz C (2011) Converging pathways in the occurrence of endoplasmic reticulum (ER) stress in Huntington's disease. Curr Mol Med 11: 1–12.
12. Berrhetti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2006) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2: 326–332.
13. Wu J, Kaufman RJ (2006) From acute ER stress to physiological roles of the unfolded Protein Response. Cell Death Differ 13: 374–384.
14. Petrich BG, Gong X, Lerner DL, Wang X, Brown JH, et al. (2002) c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. Circ Res 90: 640–647.
15. Tama I (2010) The role of endoplasmic reticulum stress in the progression of atherosclerosis. Circ Res 107: 859–866.
16. Lu G, Ota A, Ror S, Frankin S, Rau CD, et al. (2013) PPM11 encodes an inositol requiring-protein 1 (IRE1) specific phosphatase that regulates the functional outcome of the ER stress response. Molecular Metabolism.
17. Chen Y, Zhu J, Penn Y, Xing P, Pinto S, et al. (2008) Variations in DNA elucidate molecular networks that cause disease. Nature 452: 429–433.
18. Potrich BG, Gong X, Lerner DL, Wang X, Brown JH, et al. (2002) c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. Circ Res 90: 640–647.