Published in final edited form as:

J Cell Physiol. 2010 January ; 222(1): 73–81. doi:10.1002/jcp.21922.

Activation of nuclear factor-kappa B in mammary epithelium promotes milk loss during mammary development and infection

Linda Connelly¹, Whitney Barham¹, Rachel Pigg¹, Leshana Saint-Jean¹, Taylor Sherrill², Dong-Sheng Cheng², Lewis A. Chodosh³, Timothy S. Blackwell^{1,2,4}, and Fiona E. Yull^{1,*}

¹ Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee

² Department of Medicine, Vanderbilt University, Nashville, Tennessee

³ Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, Pennsylvania

⁴ Department of Veterans Affairs, Nashville, Tennessee

Abstract

We investigated whether Nuclear Factor-kappa B (NF-κB), which exhibits a regulated pattern of activity during murine mammary gland development, plays an important role during lactation and involution, when milk production ceases and the gland undergoes apoptosis and remodeling. We generated a doxycycline inducible transgenic mouse model to activate NF-κB specifically in the mammary epithelium through expression of a constitutively active form of IKK2, the upstream kinase in the classical NF-κB signaling cascade. We found that activation of NF-κB during involution resulted in a more rapid reduction in milk levels and increased cleavage of caspase-3, an indicator of apoptosis. We also found that activation of NF-κB during lactation with no additional involution signals had a similar effect. The observation that NF-κB is a key regulator of milk production led us to investigate the role of NF-κB during mastitis, an infection of the mammary gland in which milk loss is observed. Mammary gland injection of *E. coli* LPS resulted in activation of NF-κB and milk loss during lactation. This milk loss was decreased by selective inhibition of NF-κB in mammary epithelium. Together, our data reveal that activation of NF-κB leads to milk clearance in the lactating mammary gland. Therefore, targeting of NF-κB signaling may prove therapeutic during mastitis in humans and could be beneficial for the dairy industry, where such infections have a major economic impact.

Keywords

NF-κB; mammary; involution; transgenic; mastitis; milk

Introduction

Nuclear Factor kappa B (NF-κB) activity during mammary gland development

The development of the murine mammary gland is a dynamic process. In virgin mice a simple ductal structure forms throughout the mammary fat pad. During pregnancy there is epithelial cell proliferation and differentiation to secretory alveoli which produce milk during lactation. Upon weaning of pups, the gland undergoes a process termed involution where the gland remodels and there is significant apoptosis of the secretory epithelium

*Correspondence to: Fiona E. Yull, 771 Preston Research Building, 2220 Pierce Avenue, Nashville, TN 37232-6838. Phone: 615-343-2568, Fax: 615-343-2569, fiona.yull@vanderbilt.edu.

(Furth, 1999; Hennighausen and Robinson, 2005; Medina, 1996). The transcription factor nuclear factor kappa B (NF- κ B) has been demonstrated to undergo a regulated pattern of activation during the different stages of mammary gland development. NF- κ B is a dimer formed from a multi-subunit family (Ghosh et al., 1998). The activation of NF- κ B is regulated by the binding of inhibitory proteins termed I κ Bs (Baeuerle and Baltimore, 1988). In the canonical NF- κ B pathway, a dimer composed of the p65 and p50 subunits is held in the cytosol through binding of the inhibitor I κ B α . The phosphorylation of I κ B α by I κ B Kinase 2 (IKK2) acts as a signal for the ubiquitination and degradation of the inhibitor. p50/p65 then translocates to the nucleus and binds to the promoter of target genes (Baldwin, 1996; Ghosh et al., 1998). NF- κ B activity increases during pregnancy, falls during lactation then increases again during involution (Brantley et al., 2000; Clarkson and Watson, 1999). This pattern of activity suggests that NF- κ B plays an important role during pregnancy and involution.

NF- κ B and mammary gland involution

The role of both the classical and alternative NF- κ B signaling pathways during the proliferative phase in pregnancy has been investigated by our laboratory and others using transgenic mouse models (Brantley et al., 2001; Cao et al., 2001; Connelly et al., 2007; Demicco et al., 2005). However fewer studies have focused on the role of NF- κ B during involution. Electrophoretic mobility shift assay using extracts from murine mammary glands demonstrates activation of NF- κ B at 1 hour post-weaning, reaching maximum levels by day 3 of involution (Clarkson et al., 2000). The process of involution involves apoptosis of the secretory alveolar epithelium (Baxter et al., 2007) and NF- κ B has been shown to regulate a number of anti-apoptotic proteins including Bcl-2 and Bcl X_L (Karin and Ben-Neriah, 2000). Thus, it has been suggested that the role of NF- κ B activation during involution may be to act in a protective manner and promote survival of epithelial cells (Clarkson et al., 2000). However, in a recent study where the upstream activating kinase IKK2 was deleted in mammary epithelial cells, a reduction in apoptosis and a delay in involution was observed accompanied by a reduction in the death receptor ligand TWEAK (Baxter et al., 2006). In this study, selective deletion of IKK2 occurred early in life, raising the possibility that IKK2 deletion could impact all stages of development, not just involution, influencing the phenotype observed. To address the question of whether NF- κ B acts in a pro- or anti-apoptotic manner during specific stages of mammary development, we have specifically activated NF- κ B in the mammary epithelium during involution or lactation using a doxycycline inducible system.

Mammary gland infection and NF- κ B signaling

In addition to a role in normal mammary developmental processes such as involution, NF- κ B activation has also been linked with infection in the mammary gland. In bovine studies and a recent murine model, the activation of NF- κ B has been linked with mastitis (Boulanger et al., 2003; Notebaert et al., 2008a). Mastitis is an inflammation of the mammary gland caused by intra-mammary bacterial infection that results in tissue damage and a reduction in milk production (Zhao and Lacasse, 2008). Investigations into the role of NF- κ B in mastitis have focused on its role as an inducer of inflammatory mediators (Boutet et al., 2007; Notebaert et al., 2008b). As both involution and mastitis represent situations where NF- κ B is up-regulated during a time of milk loss and gland re-modeling, it is possible that common signaling pathways are activated during both states.

Generation of murine models to investigate the role of NF- κ B during involution and infection

We generated doxycycline-inducible transgenic mouse models to express an activator or inhibitor of NF- κ B specifically within the mammary epithelium to modulate classical NF- κ B

signaling within defined developmental periods. We found that activation of NF- κ B resulted in a more rapid initial phase of involution, promoting milk clearance and apoptosis. In addition, we found that activation of NF- κ B during lactation with no additional involution signals had a similar effect. We also investigated the effects of NF- κ B signaling using a murine model of mastitis, a pathological event where NF- κ B is activated during lactation. Activation of NF- κ B resulted in increased milk loss and apoptotic signaling that could be decreased by targeted inhibition of NF- κ B signaling. Our findings indicate that NF- κ B is a critical regulator of milk loss during involution and infection, and identify the NF- κ B signaling pathway as a potential target to prevent mastitis-induced milk loss in both humans and the dairy industry.

Materials and Methods

Animal Models

All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Transgenic mice containing the NF- κ B activating (tet-O)₇-FLAG-cIKK2 construct (Cheng et al., 2007) were mated with MMTV-rtTA mice (Gunther et al., 2002; gift from Dr. L. Chodosh, School of Medicine, University of Pennsylvania, Pennsylvania, PA) to obtain transgenic mice carrying both transgenes, which were designated IKMV mice. Mice containing the NF- κ B inhibiting (tet-O)₇-I κ B- α DN-Myc-His construct (Cheng et al., 2007) were mated with MMTV-rtTA mice to obtain transgenic mice carrying both transgenes, which were designated DNMV mice. Littermates lacking one or both transgenes were used as controls. NF- κ B reporter mice termed NF- κ B-GFP-Luciferase (NGL) have been described previously (Everhart et al., 2006). Triple transgenic mice were generated by mating DNMV mice with NGL reporters, these were designated DNMG mice.

IKMV (or littermate controls) mice were maintained on normal water until transgene activation was desired. Mice were mated between 6 and 10 weeks of age. For involution time points, mice were treated with 0.5g/L freshly prepared doxycycline (dox; Sigma-Aldrich) in drinking water at day 8 of lactation. Sucrose (5%) was also added to decrease the bitter taste of dox water. A red bottle was used for dox and 5% sucrose to prevent light-induced dox degradation. On day 9 pups were weaned and glands were collected as 1 day post wean on day 10. For lactation only experiments mice were treated with dox as above from day 8 of lactation and mammary glands were collected on day 10 with no weaning of pups.

NGL mice were mated between 6 and 10 weeks of age. At day 8 of lactation, mice were anesthetized and injected with 5 μ g *E. coli* LPS (serotype O55:B5; Sigma-Aldrich) in PBS through the right number 4 nipple using a 31G needle. As a control, the left number 4 nipple was injected with an equivalent volume of PBS. This gives an internal control for both effects of injection and the brief time taken for imaging and anesthesia. Mice were allowed to recover for 1 hour before being placed back in the cage with their litter. At 6 hours post injection, mice were imaged as described below. Mice were observed after injection and anesthesia to ensure that suckling of pups resumed. At 24 hours post-injection mammary glands were collected and snap frozen.

DNMG females were mated and checked for plugs each morning. After a successful copulation, components of the male ejaculate will coagulate to form a hard plug that occludes the entrance to the vagina. The day that a plug was found was designated day 0. At day 12 of pregnancy mice were treated with dox (0.5g/L). Mammary glands were harvested at day 14 and snap frozen. Luciferase assay was performed on mammary glands as described previously (Connelly et al., 2007).

DNMV (or littermate controls) were maintained on normal water until transgene activation was desired. Mice were mated between 6 and 10 weeks of age. Mice were treated with dox as described above at day 7 of lactation. On day 8, LPS injection was performed as described above. At 24 hours post-injection, mammary glands were collected and snap frozen.

All experiments were carried out with litters of between 6 to 8 pups. All mice were on an FVB strain background.

Histology

Number 4 inguinal mammary glands were fixed in 10% formalin overnight at 4 °C. The glands were then dehydrated in a graded ethanol series followed by xylenes and embedded in paraffin. 5µm sections were prepared and stained with hematoxylin and eosin (Vanderbilt University Medical Center Immunohistochemistry Core Laboratory).

Electrophoretic Mobility Shift Assay

Mammary gland nuclear extracts were prepared and EMSA performed as previously described (Connelly et al., 2003; Connelly et al., 2007).

Western Blot analyses

Whole cell mammary gland extracts were prepared and Western blot performed as described (Connelly et al., 2007) however blocking and antibody detection was performed using the Odyssey Infrared Imaging System (Licor) as follows. Nitrocellulose membranes were blocked for one hour in a 1:1 mix of Odyssey Blocking Buffer (Licor) and PBS. Primary antibodies: Cleaved Caspase-3, Bid and Bcl-2 (Cell Signaling Technology); β -Casein (Santa Cruz Biotechnology); Anti-C-myc (Sigma-Aldrich); rabbit polyclonal IgG Actin and mouse monoclonal IgG Actin (Santa Cruz Biotechnology) and Anti-Flag M2 (Sigma-Aldrich), were diluted in a 1:1 mix of Odyssey Blocking Buffer and PBS with 0.1% Tween-20. Membranes were incubated with primary antibody overnight at 4° C and then washed with a 1:1 mix of Odyssey Blocking Buffer and PBS. Secondary antibodies (Licor Biosciences Odyssey Donkey anti-Rabbit IRDye 680, Licor Biosciences Odyssey Donkey anti-Goat IRDye 800 CW, or Licor Biosciences Odyssey Donkey anti-Mouse IRDye 800 CW) were diluted in a 1:1 mix of Odyssey Blocking Buffer and PBS with 0.1% Tween-20. Membranes were incubated with secondary antibody for 30 minutes, washed with 1:1 Odyssey Blocking Buffer and PBS, and visualized using the Licor Odyssey Infrared Imaging System.

Quantitative RT-PCR

Mammary gland total RNA was extracted using Trizol (Invitrogen) and the RNeasy Mini Kit (Qiagen). RNA was subsequently treated with the DNase-free kit (Ambion). Equal concentrations of RNA were subject to reverse transcription using Superscript II Reverse Transcriptase (Invitrogen). PCR was performed using the Applied Biosystems Stepone Plus Real Time PCR system using SYBR Green PCR Master Mix (Applied Biosystems) and the following primers: TNF- α and TWEAK (Baxter et al., 2006); Bcl-2 Forward 5'-GCTGCTAGAAGCTTTGAAGAAGGC-3' and Bcl-2 Reverse 5'-TGTGGATGACTGAGTACCTGAACC-3'; Bid Forward 5'-CCATGTAGGTGGGCTTCTGTCTAA-3' and Reverse 5'-CATGGTTTGAGATCAGCCATTCGG-3'; GAPDH Forward 5'-TGAGGACCAGGTTGTCTCCT - 3' and Reverse 5'-CCCTGTTGCTGTAGCCGTAT - 3'; Beta-casein Forward 5'-GCTCAGGCTCAAACCATCTC - 3' and Reverse 5'-TGTGGAAGGAAGGGTGCTAC - 3'. Each primer pair was tested and melt curves analyzed to ensure that only a single amplicon was generated. Each experimental or control

sample was assayed in triplicate for target gene or GAPDH (reference gene) and the average of these values was used as C_T . To perform statistical analyses and for graphical representation, target gene C_T values (A) and GAPDH C_T values (B) were both expressed as exponents of 2, and data represented as the ratio of $2^A/2^B$, or $2^{(A-B)}$. To obtain fold-change values, the average target gene C_T value for experimental samples (X) was subtracted from the average GAPDH C_T value (G) for experimental samples, and expressed as an exponent of 2, yielding the value $2^{(G-X)}$. The average target gene C_T value for control samples (X') was subtracted from the average GAPDH C_T value (G') for control samples, and expressed as an exponent of 2, yielding the value $2^{(G'-X')}$. Fold change was then determined as the inverse ratio of normalized experimental to normalized control sample values, being expressed as $1/(2^{(G-X)}/2^{(G'-X')})$, or $1/(2^{(G-X) - (G'-X')})$.

Bioluminescence imaging and photon count quantification

Mice were anesthetized and luciferin (1 mg/mouse in 100 μ l isotonic saline) was administered by i.v. injection. Mice were imaged with an intensified charge-coupled device (ICCD) camera (IVIS 200; Xenogen). For the duration of photon counting, mice were placed inside a light tight box that housed the camera. Light emission from the mouse was detected as photon counts by the ICCD camera and customized with image processing hardware and software (Living Image software; Xenogen). The imaging duration (30 s) was selected to avoid saturation of the camera during image acquisition. Quantitative analysis was performed by defining a standard area over the number 4 mammary gland and determining the total integrated photon intensity over the area of interest. The photon intensity of LPS-injected glands was calculated as a percentage of the photon intensity for the control PBS-injected glands. For presentation, a digital false-color photon emission image was generated for each captured image. Removal of females for imaging totaled no more than 5 minutes with recovery from anesthesia observed 1–2 minutes after return to the cage. Pup suckling was observed to resume immediately after the females were returned to the cage.

Data analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using Odyssey V3.0 software (Licor). All data are plotted graphically as mean vertical bars representing S.E. A Student's *t* test was used to assess differences between experimental conditions. A probability (*p*) value of < 0.05 was taken as an appropriate level of significance.

Results

Characterization of a novel transgenic model to activate NF- κ B in mammary epithelial cells

In order to generate a mouse model to specifically activate classical NF- κ B signaling in the mammary gland we utilized the tet-on system. This involved the crossing of two transgenic mouse models (Figure 1). The first transgenic contains the mouse mammary tumor virus-reverse tetracycline transactivator (MMTV-rtTA) which leads to the expression of the rtTA protein in mammary epithelial cells as previously described (Gunther et al., 2002). The second transgenic contains the tet operon promoter element upstream of a FLAG-tagged constitutively activated human IKK2 (cIKK2) gene and has also been previously described (Cheng et al., 2007). Treatment of double transgenic mice with doxycycline (dox) in drinking water leads to mammary epithelial cell expression of the cIKK2. These mice are termed IKMV.

In order to confirm that doxycycline treatment induced expression of the FLAG-tagged cIKK2, IKMV mice and littermate controls lacking one or both transgenes were treated with

dox (0.5g/L) in drinking water. Mammary glands were harvested after 48hrs of dox treatment, whole cell extracts were made, and western blot was performed to detect the FLAG-tag on the cIKK2 protein. Expression of the cIKK2 was observed in IKMV mice, but not in controls, indicating that expression was dox-inducible (Figure 2A). To determine specificity of transgene expression, whole cell extracts were made from mammary glands, kidney, liver, salivary glands and hearts of IKMV mice treated with dox as above and western blot performed (Figure 2B). Transgene expression was observed in mammary gland tissue but not in kidney, liver or heart. A lower level of transgene expression was observed in the salivary gland as has been previously reported for the MMTV-rtTA mouse model (Gunther et al., 2002).

Having confirmed mammary transgene expression, nuclear extracts were prepared from IKMV and control mice treated with 0.5g/L dox for 48hrs. EMSA was performed to determine effects on NF- κ B activity (Figure 2C). A large increase in shift of radio-labeled consensus NF- κ B oligo was observed in IKMV nuclear extracts compared to control, indicating that expression of cIKK2 led to activation of NF- κ B signaling. Specificity of binding was confirmed by the addition of excess cold probe.

Activation of NF- κ B signaling during early involution accelerates milk clearance and apoptosis

Next, we investigated the impact of up-regulation of NF- κ B activity on early stages of mammary gland involution. At day 8 of lactation, IKMV or control females were started on dox treatment (0.5g/L). At day 9, pups were weaned to initiate involution and mammary glands were collected on day 10. Hematoxylin and Eosin (H and E) staining of mammary glands from IKMV females showed a loss of milk and a collapse of secretory structures as compared with control (Figure 3A). Western blot revealed a dramatic reduction in the milk protein beta-casein in mammary gland extracts from IKMV mice as compared with control in agreement with the lack of milk on the H and E section (Figure 3B). This was accompanied by an increase in the presence of cleaved Caspase-3 in IKMV mammary glands, as compared to controls, suggesting that increased apoptosis was occurring in the IKMV mammary glands. In order to investigate whether activation of NF- κ B signaling affects beta-casein mRNA expression, real time RT-PCR was performed. A 10-fold reduction in beta-casein mRNA levels was observed in IKMV mammary gland RNA samples as compared to control (Figure 3C). The expression of apoptotic proteins was further investigated by real time RT-PCR (Figure 4A). A 48-fold increase was observed in the expression of TNF- α in IKMV mammary gland RNA as compared with control. No significant change was observed in the expression of TWEAK. There was a 7-fold increase in Bcl-2 and a 10-fold increase in expression of Bid in IKMV mammary gland RNA as compared with control. The increase in Bcl-2 and Bid was confirmed at the protein level by western blot (Figure 4B).

Activation of NF- κ B signaling during lactation promotes milk clearance and apoptosis

As activation of NF- κ B during involution results in dramatic effects, we were interested in whether NF- κ B activity alone was sufficient to lead to milk loss and apoptosis in the absence of other involution signals. At day 8 of lactation, IKMV or control females were started on dox treatment (0.5g/L) and mammary glands were collected at day 10 of lactation. As observed in the previous studies, activation of NF- κ B during lactation led to a dramatic reduction in milk loss and structural collapse visualized in H and E sections from mammary glands from IKMV females compared to controls (Figure 5A). These structural alterations were accompanied by a large reduction in the levels of the milk protein beta-casein in IKMV mammary gland extracts compared to controls (Figure 5B) and cleaved Caspase-3. A 50-fold reduction in beta-casein mRNA levels was observed in IKMV RNA samples as

compared to control (Figure 5C). Real time RT-PCR analysis of apoptotic proteins revealed a marked induction of TNF- α expression in IKMV mammary glands as compared with controls, while no significant differences were observed in TWEAK or Bcl-2 (Figure 6A). A 23-fold increase in Bid was observed in IKMV mammary gland RNA as compared to controls (Figure 6A). The lack of significant change in Bcl-2 and increase in Bid in IKMV mammary glands as compared to controls was confirmed at the level of protein expression by western blot (Figure 6B).

NF- κ B activation and milk protein loss are observed in a murine model of mastitis

The observation that NF- κ B activation during lactation leads to milk loss in the mammary gland led us to wonder whether NF- κ B is a critical regulator of milk loss associated with mastitis. It has recently been demonstrated that NF- κ B is activated in a murine model of mastitis (Notebaert et al., 2008a); however, the effects of NF- κ B activation on milk levels are not known. Since mastitis and the associated loss of milk can be distressing in humans and economically devastating in the dairy industry, we decided to investigate whether NF- κ B was linked to this effect. We utilized our NF- κ B reporter mouse model (termed NGL) in which NF- κ B signaling leads to the expression of a GFP/luciferase reporter that can be monitored by *in vivo* imaging giving a direct read out of NF- κ B activation (Everhart et al., 2006; Connelly et al., 2007). This model allows us to show that NF- κ B is activated and is driving the expression of downstream target genes. In order to determine the penetrance of injection through the nipple, we injected a lactating female with 50 μ l of blue ink and removed the mammary gland (Figure 7A). This demonstrated that injection via the nipple leads to distribution throughout the ductal tree. NGL mice were injected with *E.coli* LPS (5 μ g) directly into the right number 4 mammary gland while the left number 4 mammary gland was injected with an equivalent volume of PBS as a vehicle control. At 6 hours post-injection, mice were injected intravenously with luciferin and imaged using an intensified charge-coupled device (ICCD) camera. A representative image is shown (Figure 7B) demonstrating the activation of NF- κ B in the mammary gland injected with LPS but not in the control gland injected with PBS. Photon counts from replicate mammary gland injections of LPS were calculated as a percentage of the basal levels observed in PBS controls (Figure 7B). The glands injected with LPS had a significant increase in luciferase activity (246.5 ± 52.27 %; n=5) as compared with PBS controls, indicating that intraductal LPS activates NF- κ B. To determine the effect on mammary gland milk protein levels, mammary glands were collected at 24 hours after injection and western blot performed to detect beta-casein levels (Figure 7C). Bands were quantified by densitometry, normalized to Actin loading control and LPS beta-casein levels were represented as a percentage of the control PBS beta-casein levels in the same mouse. A significant decrease in beta-casein levels was observed in the LPS treated glands (75.64 ± 4.911 %; n=7) compared with the PBS controls. There was no cleavage of Caspase 3 observed in either PBS or LPS treated mammary glands (data not shown).

Inhibition of NF- κ B decreases milk loss in LPS-treated mammary glands

In order to determine whether the reduction in milk levels was directly mediated via NF- κ B signaling, we generated a transgenic model in which NF- κ B could be inhibited specifically within the mammary epithelial cells. We crossed the MMTV-rtTA transgenic with a transgenic line in which expression of a dominant negative form of the I κ B α (which is resistant to degradation) is driven by the tet operon (Cheng et al., 2007). In this model termed DNMV, treatment with dox leads to an inhibition of classical NF- κ B signaling in mammary epithelial cells. We confirmed expression of the myc-tagged transgene by performing western blots using extracts from mammary glands of DNMV and control mice that had been treated with doxycycline for 48 hours (Figure 8A). To determine the effectiveness of NF- κ B inhibition in the DNMV model, these mice were crossed with NGL

reporter mice to create a triple transgenic termed DNMG and mice were studied after treatment with dox (0.5g/L) from day 12 to day 14 of pregnancy. We chose to study mice during pregnancy because we and others have shown that NF- κ B activity is elevated during pregnancy (Brantley et al., 2000; Clarkson and Watson, 1999) and a large number of epithelial cells, where transgene expression occurs, are present at this time. Mammary glands were collected and luciferase assay performed. A significant reduction in luciferase activity was observed in DNMG mice as compared with NGL controls indicating the successful inhibition of NF- κ B by the DN-I κ B α transgene (Figure 8B).

To investigate whether NF- κ B inhibition in mammary epithelium could block the reduction in beta-casein expression found in the LPS-induced mastitis model, DNMG mice or littermate controls were treated with dox (0.5g/L) starting at day 7 of lactation. On day 8, LPS (5 μ g) was injected into the right number 4 mammary gland and an equivalent volume of PBS was injected into the left number 4 mammary gland in the same mouse. Twenty-four hours post-injection, mammary glands were harvested and western blot performed on whole cell extracts to determine the effects of NF- κ B inhibition on beta-casein protein levels (Figure 8C). Bands were quantified by densitometry, normalized to Actin loading control, and LPS-treated beta-casein levels were represented as a percentage of the PBS-treated beta-casein levels in the same mouse. A similar reduction in beta-casein protein expression was observed in the LPS-treated glands of DNMG littermate controls (lacking one or both components of the transgene system) as in LPS-treated glands of NGL mice (77.10 ± 6.353 % of PBS control; $p < 0.05$, $n = 4$). As shown, DNMG mammary glands had no reduction in beta-casein levels after LPS treatment relative to PBS injected control glands. PBS-treated DNMG glands had similar levels of beta-casein to PBS-treated NGL or DNMG littermate controls suggesting that the expression of the inhibitor did not significantly change the baseline level of beta-casein expression (data not shown). These findings indicate that NF- κ B is required for the reduction of beta casein expression after LPS treatment and suggest that NF- κ B directly regulates milk production by the mammary epithelium.

Discussion

In the current study, we have generated a novel transgenic model to activate the classical NF- κ B signaling pathway in the mammary epithelium. We have employed the doxycycline tet-on system which allows for both spatial and temporal regulation of transgene expression. We show that dox treatment of IKMV females leads to the expression of a constitutively active form of IKK2 in the mammary gland resulting in an increase in NF- κ B signaling. We initially used this model to investigate the effects of NF- κ B activation during the early stages of involution. We found that NF- κ B activation resulted in a more rapid loss of milk and secretory structures in the gland as compared to controls. The reduction in milk was confirmed by a loss of the mRNA and protein expression of the milk protein beta-casein. The rapid milk loss observed when NF- κ B was activated during involution led us to investigate whether NF- κ B activation alone, in the absence of normal signals to stimulate involution, could have the same effect. Treatment of IKMV mice with dox during lactation did indeed result in milk loss and increased apoptosis indicating that NF- κ B signaling alone was sufficient to initiate this effect.

The loss of milk upon NF- κ B activation during involution and lactation was accompanied by the detection of cleaved Caspase 3, a marker of apoptosis. Thus it appears that the apoptosis associated with involution is enhanced in the IKMV females and that activation of NF- κ B during lactation can initiate apoptotic signaling. A number of studies provide evidence of the anti-apoptotic role of NF- κ B; however, recent studies also suggest a pro-apoptotic role for NF- κ B in some circumstances (Karin and Ben-Neriah, 2000; Kucharczak et al., 2003; Radhakrishnan and Kamalakaran, 2006). Our data suggests that NF- κ B signaling during

early stages of involution exerts an overall pro-apoptotic effect. This may be ultimately due to the balance of anti- and pro-apoptotic signals initiated by NF- κ B. In the only other *in vivo* model where the role of NF- κ B has been investigated during involution it was shown that deletion of IKK2 during involution was associated with a reduction in expression of the TNF superfamily members TNF- α and TWEAK (Baxter et al., 2006). Interestingly, upon activating IKK2, we observe a large increase in TNF- α expression but no changes in levels of TWEAK. The interactions of the various family members involved in NF- κ B signaling are complex thus, the differences between the observations of Baxter et al. 2006 and those in the current study may reflect the difference in direct activation of NF- κ B by constitutive activation of IKK2 rather than the absence of IKK2. The increased levels of TNF- α would be expected to result in activation of death receptor signaling. The expression of TNF- α has previously shown to be elevated at early stages of involution where it has been suggested that the extrinsic apoptotic pathway is the key mediator with intrinsic apoptotic signaling becoming relevant at later stages (Clarkson et al., 2004). Our current data are consistent with this hypothesis.

We also investigated levels of the anti-apoptotic protein Bcl-2, which can block apoptosis via the mitochondrial signaling pathway (Danial, 2007). An increase in Bcl-2 was observed when NF- κ B was activated during involution. However, the extent of activation of the extrinsic apoptotic signaling pathway at this stage of early involution may override the effects of smaller increases in Bcl-2. In addition, levels of Bid were increased in IKMV transgenics treated in both the lactation and involution models. Bid is pro-apoptotic and links peripheral death pathways with the mitochondrial apoptotic pathway through interactions with Bax and Bak (Yin, 2006). An increase in full length Bid was observed although the truncated form of the protein was not detected. This may be due to the short half life of truncated Bid (< 1.5 hours; Breitschopf et al. 2000). It has also been reported that full length Bid demonstrates pro-apoptotic activity (Sarig et al., 2003; Valentijn et al., 2004). Therefore, the expression of Bid may negate the anti-apoptotic effects of Bcl-2. When NF- κ B was activated during lactation, there was no change observed in Bcl-2 levels suggesting that co-operative signals induced by involution are required to lead to transcription of this protein. Although we focused on NF- κ B activity at the initiation of involution, NF- κ B continues to be increased during later phases of involution (Brantley et al., 2000; Clarkson and Watson, 1999); therefore, further investigations into the role of NF- κ B at later time points are required. We speculate that these studies may reveal a larger role for the intrinsic mitochondrial apoptosis pathway, particularly Bcl-2, and regulation of these mediators by NF- κ B.

Since we had demonstrated that activation of NF- κ B during lactation could lead to a premature involution of the gland, we were interested in investigating the role of NF- κ B during mastitis, where milk loss is observed. We found that LPS treatment of the mammary gland leads to the activation of NF- κ B and reduction in milk protein levels. Subsequently, we demonstrated that mammary epithelial NF- κ B was responsible for this effect. It has previously been shown that the activation of NF- κ B in mouse mammary epithelial cells *in vitro* leads to a reduction in beta-casein expression (Geymayer and Doppler, 2000; Safieh-Garabedian et al., 2004). Our study links activation of NF- κ B to milk loss using an *in vivo* model of mastitis and suggests that targeting NF- κ B may prevent the milk loss associated with mastitis. It is interesting to note that the activation of NF- κ B by injection of LPS, unlike results in IKMV transgenic mice, did not lead to initiation of apoptosis, as determined by cleavage of Caspase-3. In addition, the reduction in beta-casein levels was relatively modest in the LPS-induced mastitis model compared to the impact of cIKK2 expression in IKMV mice. Although the differential phenotypes may be explained by differences in the magnitude of NF- κ B activation in each system, we believe that the major factor is that the transient effect of a single injection of LPS does not fully recapitulate the effects of a

persistent infection. The observation that a transient mimic of infection results in milk loss but not immediate initiation of apoptosis suggests that restoration of milk production and the continuation of lactation could be achieved through NF- κ B inhibition as a therapeutic strategy. However, therapeutic interventions to block NF- κ B in the setting of mastitis should proceed with caution because of the potential for negative effects of NF- κ B inhibition on host defense.

In summary, we have generated a novel inducible mouse model to specifically modulate NF- κ B in the mammary epithelium during involution and lactation. We have used this to demonstrate that activation of NF- κ B during lactation and at early stages of involution promotes apoptosis and milk loss. Furthermore, we demonstrate the relevance of these findings in a mastitis model by showing that NF- κ B activation by LPS during lactation also results in the loss of milk from the mammary gland and that this effect can be inhibited by blockade of NF- κ B signaling. These findings demonstrate that during normal development and infection, NF- κ B is a critical regulator of lactation.

Acknowledgments

FEY is supported by grants: CA113734.

Contract Grant Sponsor: NIH; Contract Grant Numbers: CA113734

References

- Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science*. 1988; 242(4878):540–546. [PubMed: 3140380]
- Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*. 1996; 14:649–683. [PubMed: 8717528]
- Baxter FO, Came PJ, Abell K, Kedjouar B, Huth M, Rajewsky K, Pasparakis M, Watson CJ. IKKbeta/2 induces TWEAK and apoptosis in mammary epithelial cells. *Development*. 2006; 133(17):3485–3494. [PubMed: 16887827]
- Baxter FO, Neoh K, Tevendale MC. The beginning of the end: death signaling in early involution. *J Mammary Gland Biol Neoplasia*. 2007; 12(1):3–13. [PubMed: 17340185]
- Boulanger D, Bureau F, Melotte D, Mainil J, Lekeux P. Increased nuclear factor kappaB activity in milk cells of mastitis-affected cows. *J Dairy Sci*. 2003; 86(4):1259–1267. [PubMed: 12741551]
- Boutet P, Sulon J, Closset R, Detilleux J, Beckers JF, Bureau F, Lekeux P. Prolactin-induced activation of nuclear factor kappaB in bovine mammary epithelial cells: role in chronic mastitis. *J Dairy Sci*. 2007; 90(1):155–164. [PubMed: 17183084]
- Brantley DM, Chen CL, Muraoka RS, Bushdid PB, Bradberry JL, Kittrell F, Medina D, Matrisian LM, Kerr LD, Yull FE. Nuclear factor-kappaB (NF-kappaB) regulates proliferation and branching in mouse mammary epithelium. *Mol Biol Cell*. 2001; 12(5):1445–1455. [PubMed: 11359934]
- Brantley DM, Yull FE, Muraoka RS, Hicks DJ, Cook CM, Kerr LD. Dynamic expression and activity of NF-kappaB during post-natal mammary gland morphogenesis. *Mech Dev*. 2000; 97(1–2):149–155. [PubMed: 11025216]
- Breitschopf K, Zeiher AM, Dimmeler S. Ubiquitin-mediated degradation of the proapoptotic active form of bid. A functional consequence on apoptosis induction. *J Biol Chem*. 2000; 275(28):21648–21652. [PubMed: 10801801]
- Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, Karin M. IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell*. 2001; 107(6):763–775. [PubMed: 11747812]
- Cheng DS, Han W, Chen SM, Sherrill TP, Chont M, Park GY, Sheller JR, Polosukhin VV, Christman JW, Yull FE, Blackwell TS. Airway epithelium controls lung inflammation and injury through the NF-kappa B pathway. *J Immunol*. 2007; 178(10):6504–6513. [PubMed: 17475880]

- Clarkson RW, Heeley JL, Chapman R, Aillet F, Hay RT, Wyllie A, Watson CJ. NF-kappaB inhibits apoptosis in murine mammary epithelia. *J Biol Chem*. 2000; 275(17):12737–12742. [PubMed: 10777569]
- Clarkson RW, Watson CJ. NF-kappaB and apoptosis in mammary epithelial cells. *J Mammary Gland Biol Neoplasia*. 1999; 4(2):165–175. [PubMed: 10426395]
- Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res*. 2004; 6(2):R92–109. [PubMed: 14979921]
- Connelly L, Jacobs AT, Palacios-Callender M, Moncada S, Hobbs AJ. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J Biol Chem*. 2003; 278(29):26480–26487. [PubMed: 12740377]
- Connelly L, Robinson-Benion C, Chont M, Saint-Jean L, Li H, Polosukhin VV, Blackwell TS, Yull FE. A transgenic model reveals important roles for the NF-kappa B alternative pathway (p100/p52) in mammary development and links to tumorigenesis. *J Biol Chem*. 2007; 282(13):10028–10035. [PubMed: 17261585]
- Daniel NN. BCL-2 family proteins: critical checkpoints of apoptotic cell death. *Clin Cancer Res*. 2007; 13(24):7254–7263. [PubMed: 18094405]
- Demicco EG, Kavanagh KT, Romieu-Mourez R, Wang X, Shin SR, Landesman-Bollag E, Seldin DC, Sonenshein GE. RelB/p52 NF-kappaB complexes rescue an early delay in mammary gland development in transgenic mice with targeted superrepressor IkappaB-alpha expression and promote carcinogenesis of the mammary gland. *Mol Cell Biol*. 2005; 25(22):10136–10147. [PubMed: 16260626]
- Everhart MB, Han W, Sherrill TP, Arutiunov M, Polosukhin VV, Burke JR, Sadikot RT, Christman JW, Yull FE, Blackwell TS. Duration and intensity of NF-kappaB activity determine the severity of endotoxin-induced acute lung injury. *J Immunol*. 2006; 176(8):4995–5005. [PubMed: 16585596]
- Furth PA. Introduction: mammary gland involution and apoptosis of mammary epithelial cells. *J Mammary Gland Biol Neoplasia*. 1999; 4(2):123–127. [PubMed: 10426390]
- Geymayer S, Doppler W. Activation of NF-kappaB p50/p65 is regulated in the developing mammary gland and inhibits STAT5-mediated beta-casein gene expression. *FASEB J*. 2000; 14(9):1159–1170. [PubMed: 10834938]
- Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 1998; 16:225–260. [PubMed: 9597130]
- Gunther EJ, Belka GK, Wertheim GB, Wang J, Hartman JL, Boxer RB, Chodosh LA. A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. *FASEB J*. 2002; 16(3):283–292. [PubMed: 11874978]
- Hennighausen L, Robinson GW. Information networks in the mammary gland. *Nat Rev Mol Cell Biol*. 2005; 6(9):715–725. [PubMed: 16231422]
- Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*. 2000; 18:621–663. [PubMed: 10837071]
- Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene*. 2003; 22(56):8961–8982. [PubMed: 14663476]
- Medina D. The mammary gland: a unique organ for the study of development and tumorigenesis. *J Mammary Gland Biol Neoplasia*. 1996; 1(1):5–19. [PubMed: 10887477]
- Notebaert S, Carlsen H, Janssen D, Vandenabeele P, Blomhoff R, Meyer E. In vivo imaging of NF-kappaB activity during *Escherichia coli*-induced mammary gland infection. *Cell Microbiol*. 2008a; 10(6):1249–1258. [PubMed: 18241210]
- Notebaert S, Demon D, Vanden Berghe T, Vandenabeele P, Meyer E. Inflammatory mediators in *Escherichia coli*-induced mastitis in mice. *Comp Immunol Microbiol Infect Dis*. 2008b; 31(6):551–565. [PubMed: 18243314]
- Radhakrishnan SK, Kamalakaran S. Pro-apoptotic role of NF-kappaB: implications for cancer therapy. *Biochim Biophys Acta*. 2006; 1766(1):53–62. [PubMed: 16563635]

- Safieh-Garabedian B, Mouneimne GM, El-Jouni W, Khattar M, Talhouk R. The effect of endotoxin on functional parameters of mammary CID-9 cells. *Reproduction*. 2004; 127(3):397–406. [PubMed: 15016959]
- Sarig R, Zaltsman Y, Marcellus RC, Flavell R, Mak TW, Gross A. BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. *J Biol Chem*. 2003; 278(12):10707–10715. [PubMed: 12519725]
- Valentijn AJ, Gilmore AP. Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem*. 2004; 279(31):32848–32857. [PubMed: 15148322]
- Yin XM. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. *Gene*. 2006; 369:7–19. [PubMed: 16446060]
- Zhao X, Lacasse P. Mammary tissue damage during bovine mastitis: causes and control. *J Anim Sci*. 2008; 86(13 Suppl):57–65.

FIGURE 1.

Schematic of doxycycline inducible transgenic mouse model. Two transgenics are crossed which: 1) express the reverse tetracycline transactivator (rtTA) driven by the mouse mammary tumor virus (MMTV) promoter, and 2) express a constitutively active IKK2 upstream of the tet operon (tet O) promoter. In the double transgenic, termed IKMV, the rtTA protein binds the tet-O promoter in the presence of doxycycline (dox) and drives IKK2 expression.

FIGURE 2.

Characterization of IKMV transgenics at 1 day post wean (1dpw). a) Expression of FLAG-tagged IKK2 transgene in mammary glands from control and IKMV mice treated with dox (0.5g/L for 48 hours). Protein expression was analyzed by western blot of whole cell mammary gland extracts (n=4). Western blot for actin was performed as a loading control. b) Expression of FLAG-tagged IKK2 in tissues from an IKMV mouse. Protein expression was analyzed by western blot of whole cell tissue extracts. Western blot for actin was performed as a loading control. c) EMSA using an oligonucleotide probe specific for the consensus NF- κ B binding site in nuclear extracts from mammary glands from control and IKMV mice (control n=3, IKMV n=5). One hundred-fold excess of cold probe was added to demonstrate binding specificity.

FIGURE 3.

IKMV mammary glands display a more rapid involution at 1dpw. a) Hematoxylin and eosin staining of mammary gland tissue sections from control and IKMV mice at 1dpw, dox (0.5g/L) was administered at day 8 of lactation, pups weaned at day 9 and mammary glands collected at day 10. b) Expression of cleaved Caspase-3 and Beta-casein in mammary glands from control and IKMV mice at 1dpw. Protein expression was analyzed by western blot of whole cell tissue extracts (n=3 mice per group). Western blot for actin was performed as a loading control. c) Real time RT-PCR analyses performed on RNA from control and IKMV mammary glands at 1dpw to determine expression of Beta-casein. Levels are arbitrary units and are normalized to GAPDH (n=4, *p<0.05).

FIGURE 4.

Increased expression of apoptotic proteins is observed in IKMV mammary glands at 1dpw. a) Real time RT-PCR analyses performed on RNA from control and IKMV mammary glands to determine expression of TNF- α , TWEAK, Bcl-2 and Bid at 1dpw, dox (0.5g/L) was administered at day 8 of lactation, pups weaned at day 9 and mammary glands collected at day 10. Levels are arbitrary units and are normalized to GAPDH (n=4, *** p<0.0005, **p<0.005, *p<0.05). b) Expression of Bcl-2 and Bid in mammary glands from control and IKMV mice at 1dpw. Protein expression was analyzed by western blot of whole cell tissue extracts (n=3 mice per group). Western blot for actin was performed as a loading control.

FIGURE 5.

Involution is initiated when NF- κ B is activated during lactation. a) Hematoxylin and eosin staining of mammary gland tissue sections from control and IKMV treated with dox (0.5g/L) from day 8 until collection at day 10 of lactation. b) Expression of cleaved Caspase-3 and Beta-casein in mammary glands from control and IKMV mice treated with dox. Protein expression was analyzed by western blot of whole cell tissue extracts (n=3). Western blot for actin was performed as a loading control. c) Real time RT-PCR analyses performed on RNA from control and IKMV mammary glands to determine expression of Beta-casein. Levels are arbitrary units and are normalized to GAPDH (n=4, **p<0.005).

FIGURE 6.

NF- κ B activation during lactation up-regulates apoptotic protein expression. a) Real time RT-PCR analyses performed on RNA from control and IKMV mammary glands treated with dox (0.5g/L) from day 8 until collection at day 10 of lactation to determine expression of TNF- α , TWEAK, Bcl-2 and Bid. Levels are arbitrary units and are normalized to GAPDH (n=4, *** p<0.0005). b) Expression of Bcl-2 and Bid in mammary glands from control and IKMV mice. Protein expression was analyzed by western blot of whole cell tissue extracts (n=3 mice per group). Western blot for actin was performed as a loading control.

FIGURE 7.

LPS treatment of lactating mammary glands leads to NF- κ B activation and milk loss. a) To test efficiency of delivery via the nipple, a mouse was injected with 50 μ l of trypan blue. The gland was removed and fixed in formalin on a glass slide. b) NGL reporter transgenics were injected with PBS into the left mammary gland as a control and LPS (5 μ g) into the right mammary gland at day 8 of lactation. Mice were imaged for luciferase activity at 6 hours after injection. Effect of LPS on luciferase activity was expressed as a percentage of the luciferase activity of the corresponding PBS control gland from the same animal. Data are represented as mean $\pm$ S.E. luciferase activity expressed as a percentage of the PBS control gland. * indicates $p < 0.05$, significantly different from control; $n = 4$ per group. c) Mammary glands were harvested at 24 hours after injection and levels of beta-casein were determined.

Protein expression was analyzed by Western blot of whole cell mammary gland extracts. Densitometry was performed on Western blots and levels normalized to Actin loading control. Data are represented as mean $\pm$ S.E. band density expressed as a percentage of the PBS control gland from the same animal. * indicates $p < 0.05$, significantly different from control; $n = 4$ per group.

FIGURE 8.

Expression of dominant NF- κ B inhibitor in mammary epithelium blocks mammary NF- κ B activation and prevents LPS-induced milk loss. a) Expression of myc-tagged DN-I κ B α transgene in mammary glands from DN MV and control mice treated with dox (0.5g/L) for 48hrs. Protein expression was analyzed by western blot of whole cell mammary gland extracts (n=4). Western blot for actin was performed as a loading control. b) Mammary glands were collected from DN MV mice crossed with NGL reporters (DN MG) mice and NGL controls treated with dox (0.5g/L) from day 12 to day 14 of pregnancy. Tissue luciferase activity was determined as a measure of NF- κ B activity. Data are represented as mean $\pm$ S.E. luciferase activity normalized to protein concentration. * indicates p < 0.05, significantly different from control; n=3. c) Lactating DN MV transgenics were treated with dox (0.5g/L) from day 7 of lactation then injected with PBS into the left mammary gland as a control and LPS (5 μ g) into the right mammary gland on day 8. Mammary glands were harvested at 24 hours after injection and levels of beta-casein were determined. Protein expression was analyzed by Western blot of whole cell mammary gland extracts.

Densitometry was performed on Western blots and levels normalized to actin loading control. Data are represented as mean $\pm$ S.E. band density expressed as a percentage of the PBS control gland; n=4.