Weaning induces NOS-2 expression through NF-κB modulation in the lactating mammary gland: importance of GSH

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At the end of lactation the mammary gland undergoes involution, a process characterized by apoptosis of secretory cells and tissue remodelling. To gain insight into this process, we analysed the gene expression profile by oligonucleotide microarrays during lactation and after forced weaning. Up-regulation of inflammatory mediators and acute-phase response genes during weaning was found. Expression of IκBα (inhibitory κBα), a protein known to modulate NF-κB (nuclear factor-κB) nuclear translocation, was significantly up-regulated. On the other hand, there was a time-dependent degradation of IκBα protein levels in response to weaning, suggesting a role for NF-κB. Furthermore, we have demonstrated, using chromatin immunoprecipitation assays, binding of NF-κB to the NOS-2 (inducible nitric oxide synthase) promoter at the early onset of events triggered during weaning. The three isoforms of NOS are constitutively present in the lactating mammary gland; however, while NOS-2 mRNA and protein levels and, consequently, NO production are increased during weaning, NOS-3 protein levels are diminished. Western blot analyses have demonstrated that protein nitration is increased in the mammary gland during weaning, but this is limited to a few specific tyrosine-nitrated proteins. Interestingly, inhibition of GSH synthesis at the peak of lactation partially mimics these findings, highlighting the role of NO production and GSH depletion during involution.

Key words: GSH, involution, lactation, NF-κB, nitric oxide.

INTRODUCTION

Involution of the mammary gland is characterized by an increase in the number of apoptotic events in the epithelia of the lobulo–alveolar compartment soon after weaning [1]. This is followed by proteolytic degradation of the basement membrane and major remodelling of the mammary gland [1]. The changes observed during mammary gland involution are controlled at the transcriptional level by circulating hormones and locally derived factors [2]. Although the molecular signals that initiate involution remain to be identified, changes in the pattern of expression of a number of transcription factors and stress response genes have been observed [3–5]. Furthermore, inflammatory mediators and acute-phase response genes have also been shown to be up-regulated during involution [4,5]. In addition, several transcription factors that are known to regulate the transition from lactation to involution in the mammary gland, including JunB [5], Stat3 (signal transducer and activator of transcription 3) [4,5], Stat5 [3] and NF-κB (nuclear factor-κB) [4], were also found to be differentially expressed after weaning. Nonetheless, these data alone cannot discern the signalling events and molecular mechanisms connecting hormonal stimuli and local factors with the transcription of target genes. In this sense, we have shown that GSH levels in the mammary gland play a prominent role in the maintenance of lactation, and that a decrease leads to apoptosis and involution of the mammary tissue [6,7]. GSH status has been described as being involved in the modulation of NF-κB activity in a wide variety of cell types [8].

As mentioned above, NF-κB is known to be involved in mammary gland involution; furthermore, this transcription factor plays an important role in controlling the immune and inflammatory responses [9]. In mammalian cells, there are several members of the NF-κB/Rel family. The classic form of NF-κB, a heterodimer of the p50 and p65 subunits, is retained in the cytoplasm through interactions with IκB inhibitory proteins. Inducing stimuli lead to the phosphorylation and degradation of IκB, allowing NF-κB to enter the nucleus and regulate gene expression [9,10]. Following its degradation, IκB is transcriptionally up-regulated, since IκB is an NF-κB target gene [9,11]. In the mouse mammary gland during pregnancy, the DNA-binding activity of NF-κB (p50/p65) is increased until day 16 *post coitum* and decreases with the onset of lactation [10,12]. Similarly, the activity of an NF-κB-dependent luciferase reporter gene in transgenic mice was demonstrated to be maximal during pregnancy, decreased to near undetectable levels during lactation, and increased again during involution [13]. This up-regulation of NF-κB led us to question whether NF-κB has a role in the signalling events that trigger involution of the mammary gland, and, if so, what would be the target genes for this transcription factor. Active NF-κB, in turn, is known to be involved in the transcriptional modulation of over 150 genes. Most of the proteins encoded by NF-κB target genes participate in the stress and host immune responses, such as NOS-2 (nitric oxide synthase-2) [14]. In this regard, it is generally believed that modulation of NOS-2 transcription is the most important component of NOS-2 regulation, and that NF-κB seems to be a central target for activators or inhibitors of its expression [15].

Nitric oxide (NO) regulates several physiological events [16] by means of rapid responses, such as blood flow, blood pressure and smooth muscle contraction. NO has also been shown to act

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as a signal that regulates gene expression, apoptosis, cell cycle arrest and differentiation [17]. NO is synthesized from L-arginine and O_2 by NOS. Three distinct isoforms have been identified [18]: neuronal NOS (NOS-1), inducible NOS (NOS-2) and endothelial NOS (NOS-3). The concentration and source of NO are the major factors determining its biological effects. In fact, while basal NO production by constitutive NOS isoforms (NOS-1 and NOS-3) has a physiological role, at high concentrations the reactive species derived from NO mediate the deleterious effects of this molecule [19]. The potential consequence of producing large amounts of NO may be local cytotoxicity, favouring processes such as apoptosis, which is characterized by up-regulation of the tumour suppressor p53, cytochrome *c* release from mitochondria, activation of caspases, changes in the expression of pro- and anti-apoptotic Bcl-2 family proteins, chromatin condensation and DNA fragmentation [20].

The presence of NOS in the lactating mammary gland has been demonstrated by immunohistochemistry [21]. In various experimental models, high levels of exogenous NO induce apoptosis via a caspase/cytochrome *c*-dependent mechanism [22]. In the present paper we explore some of the molecular mechanisms behind the initial events triggered during involution. We demonstrate that, during weaning, NF-κB translocates to the nucleus, where it binds to the NOS-2 promoter, inducing its expression, which explains the increase in NO production observed after litter removal. Since we have already demonstrated that GSH is essential for the maintenance of lactation, we hypothesize that GSH might play an important role in the modulation of NO production.

EXPERIMENTAL

Animals and tissue extraction

Pregnant Wistar rats were kept in individual cages in a controlled environment (12 h light/12 h dark cycle) and received water and food *ad libitum*. The rats were cared for and handled in accordance with the NIH guidelines and the Guiding Principles for Research Involving Animals and Humans approved by the Council of The American Physiological Society. The Research Committee of the School of Medicine (University of Valencia, Valencia, Spain) approved the study protocol.

Following parturition, litters were maintained with at least 10 pups. At day 12 of lactation, the rats were divided into five groups: control lactating rats ($n = 6$) at the peak of lactation (days 12–15); two groups of weaned rats, where pups were removed 12 days after delivery to initiate involution; the weaning took place 8 and 24 h before the rats were killed ($n \geqslant 3$ for each condition); rats at the peak of lactation that had been weaned for 24 h followed by resuckling for another 24 h ($n = 3$); and lactating rats ($n = 4$) treated with BSO (buthionine sulphoximine; Sigma Chemical Co.) over a period of 2 days (4 mmol/kg, twice a day intraperitoneally) to lower GSH levels.

The rats were anaesthetized with sodium pentobarbital (60 mg/kg body wt. in 0.9 % NaCl, intraperitoneal; Abbott Laboratories) and killed immediately after removal of the inguinal mammary glands.

GSH determination

The freeze-clamped tissue was powered in liquid nitrogen, and aliquots of 1 g were extracted with 4 vol. (v/w) of 6 % $HClO_4$ by homogenization with a motor-driven Teflon homogenizer. The extract was centrifuged at 1500 **g** for 10 min to remove proteins, and the final supernatant was used to measure GSH using the glutathione S-transferase method [23].

Isolation of acini

Acini were prepared by a modification of the procedure of Katz et al. [24]. For full details, see Robinson and Williamson [25].

RNA extraction and PCR analysis

Total RNA from mammary tissue or isolated acini was extracted using TRIzol reagent (Invitrogen Life Technologies). Quantitative RT-PCR (reverse transcription–PCR) was performed in one step using the TTh DNA polymerase kit (Roche Diagnostics). The mRNA expression was studied by real-time PCR (iCycler iQ real-time PCR detection system) using specific oligonucleotides for NOS-2 (5′-TGTGACACACAGCGCTA-CAA-3′ and 5′-TGTTGAAGGCGTAGCTGAAC-3′) and GAP-DH (glyceraldehyde-3-phosphate dehydrogenase) (5′-GGGTCA-TCATCTCTGCACCT-3′ and 5′-GGTCATAAGTCCCTCCAC-GA-3′). mRNA detection was carried out by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. GAPDH expression was used as an internal control. The threshold cycle (C_T) was determined, and then relative gene expression was expressed as follows:

$$\text{Change in expression (fold)} = 2^{-\Delta(\Delta CT)}$$

where $\Delta C_T = C_T \text{ (target)} - C_T \text{ (housekeeping)}$, and $\Delta(\Delta C_T) = \Delta C_T \text{ (treated)} - \Delta C_T \text{ (control)}$.

ChIP (chromatin immunoprecipitation) assays

Chromatin from mammary gland acini was fixed and immuno-precipitated according to Borrás et al. [26]. Briefly, isolated acini were treated with 1 % (v/v) formaldehyde for 8 min to cross-link the chromatin, and the reaction was stopped by adding glycine to a final concentration of 0.125 M. After centrifugation at 1500 **g** for 5 min, the cell pellet was resuspended in cell lysis buffer (85 mM KCl, 0.5 % Nonidet P40, 5 mM Hepes, pH 8.0) supplemented with a protease inhibitor cocktail (Sigma), incubated on ice for 15 min and centrifuged at 3500 **g** for 5 min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10 mM EDTA, 1 % SDS, 50 mM Tris/HCl, pH 8.1) at a ratio of 1:1 (v/w) relative to the initial tissue weight, incubated on ice for 10 min, aliquotted in 1 ml fractions and stored at $-80\,^\circ$C until use for ChIP assay.

Cross-linked chromatin (1 ml of each sample) was sonicated on ice with 10 pulses of 10 s at 40 % amplitude in a Vibra-Cell VCX-500 sonicator. The average size of the chromatin fragments obtained was ∼ 500 bp. The sonified chromatin was centrifuged at 14 000 **g** for 10 min and the supernatants, containing soluble chromatin fragments, were diluted 10-fold with dilution buffer (165 mM NaCl, 0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.0) supplemented with protease inhibitor cocktail. The diluted chromatin fractions were precleared by adding 30 μl/ml Protein A/G–agarose (Amersham Biosciences; previously blocked for 1 h with 100 μg/ml λ-DNA, 500 μg/ml tRNA and 1 mg/ml BSA) and kept for 4 h at 4 °C on a rotating plate. The suspensions were then centrifuged at 14 000 **g** for 30 s to remove non-specifically bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg of DNA) were taken, incubated with 2 μg of specific antibodies against the NF-κB p65 subunit and an antibody against RNA polymerase II (both from Santa Cruz Biotechnology), and left overnight at 4 °C under rotation. The samples were then incubated with 50 μl of blocked Protein A/G–agarose under rotation for an additional period of 4 h. The immunocomplex was recovered by centrifugation at 14 000 **g** for 30 s and washed as described previously [27]. An aliquot of the cross-linked chromatin was treated as above, but in the absence of the antibody (No Ab fraction); the first supernatant,

after preclearing with Protein A/G–agarose, was saved as the Input fraction. The immunoselected chromatin was eluted from the Protein A/G–agarose in two consecutive steps by adding 100 μl of elution buffer (1 % SDS, 100 mM NaHSO$_3$) each time, with 30 s of vigorous vortexing. The two supernatants were combined [IP (immunoprecipitated) fraction] and incubated at 65 °C overnight to reverse formaldehyde cross-links. The DNA from all samples was purified with a PCR purification kit (Qiagen) and used for PCR analysis of the target genes.

PCR analysis of immunoprecipitated chromatin

After DNA purification, the Input, IP and No Ab fractions were analysed by PCR with the appropriate primer pairs to amplify products of 180–300 bp in length, corresponding to either the promoter or the coding regions of the target genes. For the analysis, 1:5000 dilutions of the Input and 1:30 dilutions of the IP and No Ab fractions were used. Primers for PCR analysis were as follows: NOS-2 (promoter region), 5′-AGCGGCTC-CATGACTCTCA and 5′-TGCACCCAAACACCAAGGT-3′; α-actin (promoter region), 5′-AGGGACTCTAGTGCCCAACACC-3′ and 5′-CCCACCTCCACCCTACCTGC-3′; NOS-2 (coding region), 5′-ACTGGACCACCGCTGTCAGG-3′ and 5′-CCTG-CTTTGCCACTTGCCAG-3′.

PCR fragments were size-fractionated by 2 % (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Preparation of samples for GeneChip hybridization

Mammary glands from two different groups of rats were selected for this study: those after 12 days of lactation and those at 8 h after forced weaning on day 12 of lactation. Samples for chip hybridization were prepared according to protocols supplied by Affymetrix. Briefly, after isolation of acini from mammary glands under various conditions, total RNA was extracted using TRIzol reagent followed by additional column purification (Rneasy; Quiagen).

GeneChip hybridization and data analysis

Samples of total RNA (5 μg) from each condition were used for the production of biotinylated cRNA according to the manufacturer's protocol (Affymetrix). The quality of the labelled cRNA was confirmed by hybridization to an Affymetrix test chip (Test3-chip). All experiments were performed in triplicate with RNA from three individual rats. Labelled cRNA was hybridized to an Affymetrix RG-U34A chip. The raw data were analysed by Microarray Suite 5.0 software.

Each of the three experiments using tissue from the weaned rats was compared with each of the three experiments using tissue from rats at day 12 of lactation (used as a baseline), to produce a total of nine comparisons. Only probe sets that produced at least seven out of nine change values of $P \leqslant 0.0024$ were considered as showing increased expression (or $P \geqslant 0.9976$ for decreased expression). Only genes with a fold change in expression of $\geqslant 1.5$ were selected, and classified according to the biological process category or first subcategory in which they are involved, following the criteria of the Gene Ontology Consortium [28].

Protein extraction and immunoblotting analysis

The freeze-clamped tissue (0.1 g) was homogenized in 1 ml of extraction buffer as described previously [6]. The lysates were normalized for protein concentration using the BCA protein assay reagent (Pierce Chemical Co.). Equal amounts of protein (10 μg) were loaded in SDS/PAGE gels and analysed by Western blot as described [6]. The antibodies used were: rabbit polyclonal anti-

NOS-1 (Affinity Bioreagents; ABR), rabbit polyclonal anti-NOS-2 and anti-NOS-3 (both from Santa Cruz Biotechnology); mouse monoclonal anti-nitrotyrosine (Calbiochem); rabbit polyclonal anti-IκBα and anti-IκBβ (both from Santa Cruz); and mouse monoclonal α-tubulin (Santa Cruz).

Nitrite determination

The assay used was that described by Misko et al. [29] for the fluorimetric determination of nitrite. The method is based on the reaction of 2,3-diaminonaphthalene (Sigma Chemical Co.) with nitrite under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent product. Samples were prepared by homogenizing 0.1 g of tissue in 10 ml of 50 mM Tris/HCl, pH 7.5, followed by centrifugation at 19 000 g for 10 min. An aliquot of 10 μl of freshly prepared 2,3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) were added to 100 μl of the supernatant and mixed immediately. After a 10 min incubation at 20 °C protected from light, the reaction was stopped with 5 μl of 2.8 M NaOH. Formation of the 2,3-diaminonaphthotriazole was measured using a fluorescent plate reader, with excitation at 365 nm and emission read at 450 nm. White opaque 96-well plates (Bibby Sterilin Ltd.) were used for optimal measurement of fluorescence intensity.

Statistical analysis

ANOVA was performed to analyse the data in Figures 3, 4 and 5. The homogeneity of the variances was analysed by the Levene test; in those cases in which the variances were unequal, the data were adequately transformed before ANOVA. The null hypothesis was accepted for all values of these sets in which the F-value was non-significant at $P > 0.05$. The data for which the F-value was significant were examined by Tukey's test at $P < 0.05$.

RESULTS

Expression profile analysis in mammary glands of rats at the peak of lactation and 8 h after weaning

In order to gain a better understanding of the signalling events triggered during the early onset of involution, we performed a transcript analysis using Affymetrix oligonucleotide microarrays at the peak of lactation and 8 h after litter withdrawal on day 12 of lactation (Figure 1A; see also Supplementary Table 1 and Supplemetary Figure 1 at http://www.BiochemJ.org/bj/391/bj3910581add.htm). Analysis of gene expression profiles revealed a decrease in the expression of genes involved in the metabolism of glucose and lipogenesis *de novo* during involution of the mammary gland; these results correlate with metabolic studies done in the 1970s, in which a decrease in lipogenesis was described in the mammary gland after lactation [30]. Among the genes that were up-regulated during weaning, those encoding inflammatory mediators and acute-phase proteins were identified, in accordance with previous reports [4,5]. As expected, expression of genes involved in cell death, such as those encoding Stat3, caspase-6 and Smac/Diablo, was increased during weaning, whereas genes encoding anti-apoptotic proteins (Dad-1, Bcl-2, etc.) were down-regulated.

It is interesting to note that IκBα mRNA was significantly up-regulated during weaning (Figure 1B). Since IκBα gene expression is usually up-regulated after degradation of the protein, due to the fact that IκBα is a target gene of activated NF-κB [10], our results indirectly suggested that NF-κB was activated after weaning. Therefore we determined whether weaning induced IκB protein degradation. In general, IκBα is rapidly degraded after cellular activation, whereas other IκB proteins (IκBβ and IκBε)
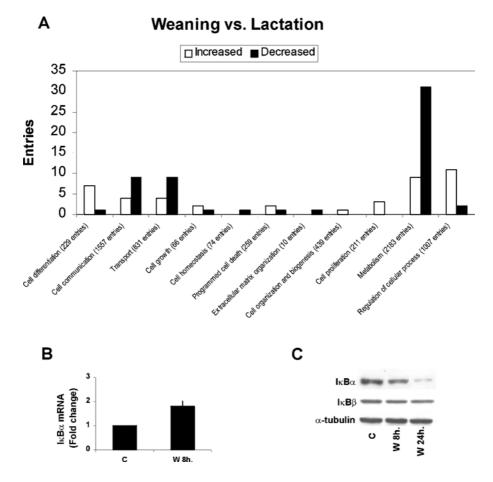
Figure 1   Differential gene expression in mammary glands from control lactating rats and after weaning

(**A**) Three complementary microarray experiments show differential gene expression in mammary glands from rats after weaning compared with that in control lactating glands. Genes down- and up-regulated after weaning are represented by solid and empty bars respectively. Genes with a fold change in expression of $\geqslant 1.5$ were classified according to the category of biological process in which they are involved, following the criteria of the Gene Ontology Consortium [26]. The number of entries in a category relative to the total number of entries in the RG-U34A chip is given in parentheses. (**B**) The profile of IκBα expression was studied in control mammary glands (C) and 8 h after weaning (W 8h.); the values are normalized mean probe intensities for the three microarray experiments. (**C**) Effects of weaning on IκBα and IκBβ protein levels. Western blot analysis of equal amounts (20 μg) of protein derived from control lactating mammary gland (C) and mammary glands 8 h (W 8h.) or 24 h (W 24h.) after weaning were probed with anti-IκBα and anti-IκBβ antibodies as described in the Experimental section.

are degraded with slower kinetics. As shown in Figure 1(C), we found that IκBα degradation was induced as early as 8 h after litter removal, and was maximal at 24 h. However, IκBβ protein levels did not change during weaning, as this protein is more stable.

Active NF-κB binds to the NOS-2 promoter, modulating its transcription in the mammary gland after weaning

NF-κB is activated in mammary epithelium by targeted degradation of its repressor IκBα. To determine the *in vivo* nuclear protein binding of NF-κB to the NOS-2 promoter region, ChIP assays were performed with an affinity-purified antibody directed against p65 (Figure 2A). DNA was extracted from the Input, IP and No Ab fractions; equal amounts from each fraction were amplified using primers specific for the NOS-2 promoter region. Binding was determined by the relative intensity of ethidium bromide fluorescence when compared with the input control. Our data show that binding of NF-κB to the NOS-2 promoter was evident at 8 h after weaning, whereas it was almost undetectable during lactation. Binding to the α-actin promoter (used as a negative control) was not observed (Figure 2A), indicating specific binding of NF-κB to the NOS-2 promoter.

In order to determine if NF-κB binding is involved in the transcription of NOS-2, the same cross-linked chromatin samples

used for the ChIP assay with the anti-p65 antibody were also used for ChIP with an antibody against RNA polymerase II [27]. Briefly, the chromatin was immunoprecipitated with anti-(RNA polymerase II) antibody and specific primers of the coding region of the desired gene were used for DNA amplification. A positive result means that the gene is being currently transcribed. As shown in Figure 2(B), no signal was observed using mammary gland from lactating rats; however, after weaning, the RNA polymerase was bound to the coding region of the NOS-2 gene. Binding to α-actin (used as a negative control) was not observed (Figure 2B), indicating specific binding of RNA polymerase II to the NOS-2 gene.

The presence of NF-κB bound to the NOS-2 promoter during weaning suggests that this transcription factor modulates the expression of NOS-2, and the concomitant binding of the RNA polymerase II to the coding region reflects the current transcription of the NOS-2 gene.

Inducible NOS mRNA expression is increased during weaning and after GSH depletion

Our results suggested a possible induction of NOS-2 expression in the mammary gland following weaning. In order to determine whether weaning modulates NOS-2 expression, real-time
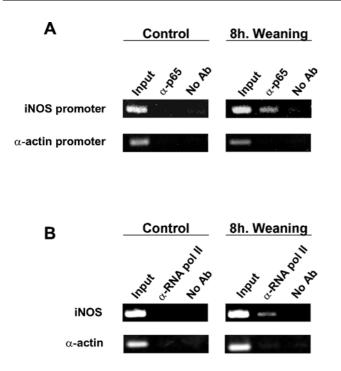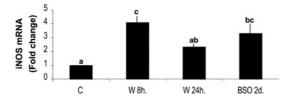
Figure 2   Analysis of NOS-2 gene expression in mammary glands from lactating rats and 8 h after weaning: *in vivo* NF-κB binding to the promoter and current transcription of the gene

Immunoprecipitation of formaldehyde-cross-linked chromatin was carried out using samples from control mammary glands (lactating) and 8 h after weaning with specific antibodies against the p65 subunit (**A**) or RNA polymerase II (**B**). Immunoprecipitates were aliquotted and subsequently analysed by PCR with specific primers for either the NOS-2 promoter (**A**) or the NOS-2 coding region to study the current transcription of the gene (RNA polymerase II ChIP assay; **B**). Total chromatin (Input) and samples containing no antibody (No Ab) were included in the PCR reactions. The PCR products obtained with oligonucleotides specific for the α-actin promoter and coding region were included as negative controls. Results are representative of three independent experiments.

Figure 3   NOS-2 mRNA expression in the mammary gland

Expression of NOS-2 (iNOS) mRNA in mammary glands from control lactating rats, rats after weaning and BSO-treated lactating rats was determined by real-time RT-PCR. GAPDH was used as an internal control (see the Experimental section). Results are means ± S.E.M. from three independent experiments. ANOVA was performed for statistical analysis; different superscript letters indicate significant differences ($P < 0.05$), and the letter 'a' always represents the lowest value within the group.

RT-PCR was performed using mammary tissue from lactating and weaned rats. NOS-2 mRNA expression was increased 4-fold at 8 h after weaning, and remained significantly increased in rats 24 h after weaning (Figure 3).

We have shown that GSH depletion triggers mammary gland involution, reproducing the changes observed after weaning [6]. To study further the molecular mechanisms of NOS-2 induction during involution, we analysed the effect of GSH on NOS-2 expression by real-time RT-PCR in lactating rats treated with BSO, a potent inhibitor of γ-glutamylcysteine synthetase. Administration of BSO over a period of 2 days inhibited GSH synthesis, resulting in a significant decrease in GSH levels in the mammary
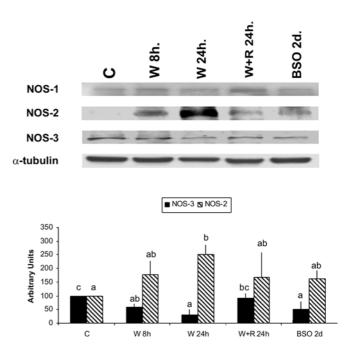

Figure 4   Effects of weaning and BSO treatment on the levels of NOS-1, NOS-2 and NOS-3 in the mammary gland

Western blot analysis was performed using whole protein extracts (50 μg) derived from the mammary tissue of control lactating rats (C), rats 8 and 24 h after weaning (W 8h. and W 24h. respectively), rats 24 h after weaning followed by resuckling for a further 24 h (W + R 24h.) and rats treated with BSO over a period of 2 days (BSO 2d.). Extracts were probed with specific antibodies against NOS-1, NOS-2 and NOS-3 and with anti-α-tubulin. Results are means ± S.E.M. for three independent experiments. Different superscript letters indicate significant differences ($P < 0.05$); the letter 'a' always represents the lowest value within the group.

gland (control, 2.17 ± 0.15 μmol/g of tissue; BSO-treated, 0.32 ± 0.05 μmol/g; means ± S.E.M.). Interestingly, depletion of GSH with BSO induced a 3-fold increase in NOS-2 mRNA expression, reaching similar levels to those observed 8 h after weaning (Figure 3).

Effects of weaning and BSO treatment on NOS protein levels in the mammary gland

Since it is known that the cellular half-life of NOS-2 is relatively short [31], it was important to assess whether the changes observed in NOS-2 mRNA expression correlated with protein levels. The three isoforms were studied by Western blot in mammary glands from rats at the peak of lactation (control), rats at different times after weaning and BSO-treated rats. NOS-2 protein levels were increased in a time-dependent manner in the mammary glands of rats 8 and 24 h after weaning; there was also an increase in NOS-2 protein levels in BSO-treated rats, similar to the values found 8 h after weaning (Figure 4). In contrast, the protein levels of the NOS-3 isoform were diminished during weaning and after treatment with BSO. Interestingly, the changes observed during weaning were partially reversed towards control values after resuckling for 24 h (Figure 4).

Elevated nitrite production during weaning and following GSH depletion

Even though NOS-2 is thought to be regulated primarily at the transcriptional level, other mechanisms of regulation have been described [8,31]. Therefore NO produced and secreted by the lactating mammary gland was estimated by measuring the nitrite
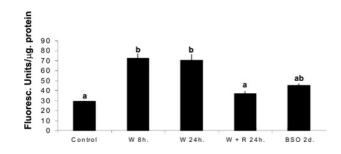
Figure 5   Determination of NO production in the mammary gland under various experimental conditions

NO production in the mammary gland in control lactating rats (C), rats 8 and 24 h after weaning (W 8 h. and W 24 h. respectively), rats 24 h after weaning followed by resuckling for a further 24 h (W + R 24 h.) and BSO-treated rats (BSO 2d.) was determined by measuring the levels of nitrite in the mammary gland for at least three rats from each experimental group. Different superscript letters indicate significant differences ($P < 0.05$); the letter 'a' always represents the lowest value within the group.
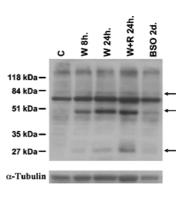

Figure 6   Nitration of proteins in the rat mammary gland

Total proteins (50 μg) extracted from the mammary tissue of control lactating rats (C), rats 8 and 24 h after weaning (W 8 h. and W 24 h. respectively), rats 24 h after weaning followed by resuckling for a further 24 h (W + R 24 h.) and rats treated with BSO over a period of 2 days (BSO 2d.) were resolved in 10 % (w/v) polyacrylamide gels by reducing SDS/PAGE and examined by Western blot analysis with a monoclonal antibody against nitrotyrosine. Approximate sizes (kDa) were estimated using pre-stained molecular-mass markers (left). The arrows indicate proteins that are clearly nitrated on tyrosine. The Figure is representative of three independent experiments.

converted from NO. The amounts of NO produced by mammary glands 8 and 24 h after weaning showed a 2-fold increase compared with the NO produced by mammary glands from control lactating rats. This increase in NO production during weaning reverted almost to control values after resuckling for 24 h (Figure 5). Treatment of lactating rats with BSO partially reproduced the increase in NO formation found during weaning. This enhancement of NO production in mammary glands following weaning and from BSO-treated rats could account for the observed up-regulation of the NOS-2 isoform (Figure 4).

Appearance of nitrotyrosine-containing proteins in the mammary gland during involution

As a consequence of NO overproduction, there will be an increase in reactive species derived from NO. Submicromolar concentrations of NO can react with superoxide radical to generate the potent oxidizing agent peroxynitrite, which can directly attack various biological targets. In addition, peroxynitrite readily nitrates tyrosine residues in proteins, producing a post-translational modification that might alter protein function, modifying signal transduction. Western blot analysis of total tissue extracts from mammary gland demonstrated that nitrotyrosine residues were increased after weaning, although this increase was limited to a few specific tyrosine-nitrated proteins of different molecular masses (see arrows in Figure 6). Interestingly, in this case, the tyrosine nitration was not reversed after resuckling. The protein nitration seen in mammary glands from rats at the peak of lactation treated for 2 days with BSO was similar to that observed in rats weaned for 8 h (Figure 6).

DISCUSSION

The mammary gland undergoes a highly regulated cascade of events, going from cell growth to differentiation to apoptosis during each pregnancy/lactation cycle. This complex evolution makes this tissue an excellent model in which to study the regulation of these events, which should be tightly controlled. It is now widely accepted that NF-κB is essential for proper development of the mammary tissue [3,13,32]. The activity of this transcription factor is elevated during pregnancy and involution, but not during lactation [13]. In fact, activation of NF-κB is one of the most rapid transcription factor responses in the involution of the mammary gland, with detectable DNA binding within 2 h after cessation of suckling [12]. Our results show that, 8 h after

weaning, there was degradation of the major NF-κB inhibitor, IκBα (Figure 1C), followed by activation of NF-κB in the mammary gland. Activated NF-κB translocates to the nucleus, where it binds to specific target genes. We found a 2-fold increase in the expression of IκBα (Figure 1B), which would modulate the NF-κB response [10].

A plethora of mouse models have been generated in the search for key regulators and cell signalling pathways involved in mammary gland involution [33], although so far these molecular mechanisms remain to be fully understood. In the present investigation we have studied the expression of NOS-2, which is known to have NF-κB binding sites on its promoter [34], and propose that NO is one of the signalling molecules connecting hormonal stimuli and milk stasis to biological responses during involution. Our data show that NF-κB is bound to the NOS-2 promoter during weaning, resulting in the actual transcription of the gene, as demonstrated by binding of RNA polymerase II to the coding region of the NOS-2 gene (Figure 2). This result has been confirmed by real-time PCR, which showed a 4-fold increase in the expression of the NOS-2 gene in the mammary glands of rats 8 h after weaning and a 2-fold increase 24 h after weaning (Figure 3).

The presence of NOS activity has been reported in immuno-histochemical studies in human [35], goat [36] and rat [37] mammary glands, where the three isoforms were present in the epithelial cells of alveoli and lactiferous ducts. We postulate that NO is generated at a low rate constitutively in the mammary gland during lactation, mainly by the NOS-3 isoform. This NO produced at low concentrations is physiological, and may be involved in mammary gland differentiation and in the regulation of blood flow to the mammary gland during lactation. However, the protein levels of this isoform in the mammary gland had declined 24 h after weaning, whereas NOS-2 levels were increased (Figure 4). Down-regulation of NOS-3 and an increase in NOS-2 expression in the rat mammary gland after treatment with lipopolysaccharide [37], in rat glomerular cells [38] and in cultured bovine coronary venular endothelial cells [39] has been reported previously. Our data suggest that this switch in the regulation of the two isoforms is directly related to the involution of the mammary gland, a tissue remodelling process that involves apoptosis of mammary epithelial cells. Overproduction of NO by NOS-2 might trigger the apoptotic process, inducing p53 activation [40], or result

in the post-translational modification of various proteins, e.g S-nitrosylation or nitration of tyrosine and tryptophan residues [41].

The process of involution that begins after litter removal is known to be reversible if pups start suckling again within a short period of time. However, some of the changes are irreversible if weaning takes place for longer periods. In fact, lactation will be maintaned in the mammary gland only by those cells that have not entered apoptosis at the moment when the resuckling stimuli appear. In the present study, NOS-2 mRNA expression, protein levels of the NOS isoforms and NO production that occurred during weaning were reversed by resuckling to control values. However, it is noteworthy that protein nitration did not revert to control values after resuckling, since this protein modification is irreversible. This is why we still found nitrated proteins under these conditions, similar to the results observed 24 h after weaning.

Since GSH depletion induced NOS-2 expression to levels similar to those induced by weaning, and the molecular switch between NOS-3 and NOS-2 was partially reproduced by treatment with BSO, we postulate that GSH status could be behind the molecular mechanisms leading to NOS-2 induction and/or NOS-3 repression. We have demonstrated previously that GSH is crucial for the maintenance of lactation [6,7]. In fact, a decrease in GSH levels *in vivo* induces an increase in the expression of p53, p21, p27, c-Jun and p-JNK (c-Jun N-terminal kinase), and results in epithelial cell death by apoptosis [6,42]. All of these changes mimic those found in the lactating mammary gland when pups are removed, being responsible for mammary gland involution [6]. It is interesting to note that NO increases during weaning and after GSH depletion; this could be a potential signal that triggers involution during weaning and in BSO-treated lactating rats, lending further support to the importance of maintaining GSH levels within the physiological range in the lactating mammary gland.

Although we propose a novel mechanism underlying the initial events leading to involution of the mammary gland, further investigation is required to provide information regarding the relevance of these findings in the context of apoptosis and the role of NO in the signalling events triggered after weaning.

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