

# Inhibition of liver trans-sulphuration pathway by propargylglycine mimics gene expression changes found in the mammary gland of weaned lactating rats: role of glutathione

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In the lactating mammary gland, weaning produces mitochondrial cytochrome *c* release and nuclear DNA fragmentation, as determined by gel electrophoresis. This is followed by a significant decrease in lactation. Weaning for 2 h produces an early induction of the tumour suppressor/transcription factor p53, whereas the oncoprotein c-Jun and c-Jun N-terminal kinase are elevated after 24 h of weaning when compared with controls. The expression of p21<sup>cip1</sup> and p27<sup>kip1</sup>, cyclin-dependent kinase inhibitors, was significantly higher in weaned rats when compared with control lactating rats. All the changes mentioned above also happen in the lactating mammary gland when propargylglycine, an inhibitor of the liver trans-sulphuration pathway, is administered. This effect is partially reversed by *N*-acetylcysteine administration.

The administration of buthionine sulphoximine, an irreversible inhibitor of  $\gamma$ -glutamylcysteine synthetase, to lactating rats produces a decrease in GSH levels and changes in protein concentrations and gene transcripts similar to those in rats with impaired trans-sulphuration pathway. These data suggest that the inter-tissue flux of GSH is an important mechanism of L-cysteine delivery to the lactating mammary gland and emphasize the importance of this physiological event in maintaining the gene expression required to sustain lactation.

**Key words:** apoptosis,  $\gamma$ -cystathionase, L-cysteine, glutathione (GSH), lactation.

## INTRODUCTION

During lactation there are changes in the metabolism of different tissues to ensure a supply of substrates to the mammary gland for milk production. These changes are accompanied by several physiological adaptations, such as hyperphagia, liver and mammary-gland hypertrophy, increased cardiac output and increased blood flow to the gland. In liver,  $\gamma$ -cystathionase expression and activity are significantly higher in lactating rats than in control rats [1,2]. This enzyme catalyses the synthesis of L-cysteine in the liver, and the availability of this amino acid is rate-limiting for GSH synthesis [3]. This tripeptide is then released to plasma and directed to those tissues with high  $\gamma$ -glutamyl-transpeptidase (GGT) activity. During lactation, the mammary gland has a high GGT activity, which, together with the increased blood flow to the gland and the hypertrophy, makes the mammary gland the organ with greatest GSH utilization [2]. This inter-tissue flux of GSH serves as the primary source of L-cysteine to the mammary gland, because the trans-sulphuration pathway is not expressed in this tissue.

Mammary-gland involution is a physiological process that takes place at the end of lactation and it is characterized by (i) metabolic changes, (ii) cessation of milk-protein gene expression, (iii) programmed cell death (PCD) of secretory epithelial cells and (iv) tissue remodelling and preparation of the gland for a new pregnancy. The most important signals in the initiation of this cascade of modifications in the mammary epithelium seem to be milk stasis and hormonal changes when pups stop suckling [4,5].

Oxidants are commonly thought to trigger apoptosis. Recently a link has been shown between GSH oxidation during mammary-gland involution and induction of apoptosis, leading to the speculation that GSH loss may be an important early event in the cell-death pathway [6,7].

To study the role of L-cysteine as a precursor of GSH in the mammary gland during lactation we used rats injected with propargylglycine (PPG), a specific inhibitor of the  $\gamma$ -cystathionase [8]. In these rats the GSH released from the liver is diminished, and so is the delivery of GSH to the lactating mammary gland; this is accompanied by a significant decrease in lactation, even though the ectoenzyme GGT is not inhibited [2,9]. To characterize the changes produced by the injection of PPG, we focused the present study on the genes that appeared to be induced within a few hours of weaning, and we found that most of them were also induced at this time. PPG treatment of lactating rats induces an increase in apoptosis in the mammary gland similar to the results found in weaned rats.

In conclusion, GSH delivery to the lactating mammary gland is essential for the maintenance of lactation, and its decrease leads to apoptosis and involution of the mammary tissue. Treatment of rats with *N*-acetylcysteine (NAC) blunted the effects of PPG on mammary involution. To confirm our hypothesis, another group of rats was treated with buthionine sulphoximine (BSO), a potent and specific inhibitor of  $\gamma$ -glutamylcysteine synthetase. Treatment with BSO decreased GSH levels in several organs, including mammary gland and liver. Decreased concentrations of GSH induced apoptosis in the lactating mammary gland, and changes

Abbreviations used: AP-1, activator protein-1; BSO, buthionine sulphoximine; GGT,  $\gamma$ -glutamyltranspeptidase; NAC, *N*-acetylcysteine; PCD, programmed cell death; (p-) JNK, (phosphorylated) c-Jun N-terminal kinase; PPG, propargylglycine; RT-, reverse transcription; 26S RIB, 26 S ribosomal protein; Stat3, signal transduction and activators of transcription 3.

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in gene expression that were similar to those observed in response to PPG, confirming the importance of GSH for the maintenance of lactation.

## EXPERIMENTAL

### Animals and tissues

Female Wistar rats were kept on individual cages on a 12 h light/12 h dark cycle, had free access to water and were fed *ad libitum*. Rats were cared for and handled in conformance with the National Institutes of Health 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 ten pups. Rats were anaesthetized intraperitoneally with sodium pentobarbital (Abbott Laboratories, S.A., Madrid, Spain; 60 mg · kg body wt.<sup>-1</sup> in 0.9 % NaCl) and the tissues were extracted. Once sampling was finished, sodium pentobarbital (1 ml of a 150 mg/ml solution) was injected through the left femoral vein. This dose was sufficient to stop the heart within 2–3s.

For control lactating rats, inguinal mammary glands were isolated at the peak of lactation (days 12–15). In the case of weaned rats, pups were removed 12 days after delivery to initiate involution; the weaning took place for 2, 4, 8, 24 and 72 h before the animals were killed and then inguinal mammary glands were isolated. At least three rats for each condition were used.

To evaluate the impact of inhibiting the trans-sulphuration pathway in liver on lactation and apoptosis in the mammary gland, lactating rats were treated with PPG and NAC. One group of eight rats on day 12 of lactation was injected with PPG (Sigma Chemical Co., St Louis, MO, U.S.A.) over a period of 3 days (50 mg · day<sup>-1</sup> · kg<sup>-1</sup>, intraperitoneally); a second group of nine rats was treated simultaneously with PPG (50 mg · day<sup>-1</sup> · kg<sup>-1</sup>) and NAC (80 mg · kg<sup>-1</sup> three times a day), obtained from the Pharmacy Unit of our Medical School and was originally from Zambón (Sta Perpetua De Mogoda, Barcelona, Spain); lastly a third group of seven rats at the peak of lactation was injected with NAC only (80 mg · kg<sup>-1</sup>, three times a day) to discard the effects due to this component. To study the effect of PPG on the litter (in case it was transferred through milk consumption) and to rule out the possibility that inhibition of the trans-sulphuration pathway could decrease milk removal, pups were cross-fostered after each daily PPG injection, so that all dams fostered control pups. The experiment was done as follows: 24 h after each administration of PPG, pups were weighed and switched from the PPG-treated rat to a control lactating rat and vice versa.

Another set of four rats was treated with BSO (Sigma) over a period of 2 days (4 mmol · kg<sup>-1</sup>, twice a day intraperitoneally) to decrease GSH levels.

### GSH determination

The freeze-clamped tissue was powered in liquid nitrogen with a pestle and a mortar, and a portion of the powder (about 1 g) was extracted with 4 vol. (v/w) of 6 % HClO<sub>4</sub> 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 (EC 2.5.1.18) method [10].

### Measurement of $\gamma$ -cystathionase activity

The activity of this enzyme (EC 4.4.1.1) was measured by determining the rate of L-cysteine synthesis from cystathionine [11].

Briefly, the tissue was homogenized in ice-cold 30 mM potassium phosphate buffer, pH 6.9. Homogenates were centrifuged at 28 000 g for 30 min, and the supernatant fluid was assayed. The reaction mixture for the assay of cystathionase activity contained 2 mM L-cystathionine (Sigma), 0.53 mM pyridoxal 5'-phosphate (Sigma) in 0.1 M potassium phosphate buffer, pH 6.9. A 1 ml portion of this cocktail was added to 250  $\mu$ l of sample, which was incubated at 37 °C for 0, 15 and 30 min. Then 125  $\mu$ l of 20 % HClO<sub>4</sub> was added to stop the reaction; under these conditions the rate of formation of cysteine was linear with time. Dithiothreitol (10 mM; Sigma) was added to bring all cysteine into the reduced form, and the amount of cysteine was determined by the spectrophotometric method of Gaitonde [12].

### Measurement of $\gamma$ -GGT activity

$\gamma$ -GGT activity was measured by monitoring the reaction of  $\gamma$ -glutamine *p*-nitroanilide (Sigma) as a substrate of GGT hydrolytic activity and glycylglycine as glutamate acceptor for the transpeptidation reaction, as described previously [13].

### Milk production

Sampson and Jansen [14] developed a simple method that permits estimation of daily milk yield in the well-nourished dam from pup weight and weight gain. The following equation relating pup milk yield to pup weight and weight gain was used to estimate milk production:

$$\text{Yield} = 0.0322 + 0.0667 (\text{weight}) + 0.877 (\text{gain})$$

where yield is daily yield per pup (g/day per pup), weight is pup weight (g) and gain is pup daily weight gain (g/day).

### DNA fragmentation

After weaning, one of the earliest changes denoting cell death is the activation of a calcium-dependent endonuclease and associated DNA fragmentation [15]. DNA was extracted as follows: 0.1 mg of tissue was homogenized with 1 ml of lysis buffer containing 100 mM Tris/HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.4 mg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) and 0.5 % SDS. The solution was incubated at 50 °C for 3 h. The DNA was purified by phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.; Sigma) extraction, precipitated with 95 % (v/v) ethanol, washed, dried and dissolved in 200  $\mu$ l of TE buffer [10 mM Tris/HCl (pH 7.5)/1 mM EDTA]. Then it was treated at 37 °C for 2 h with 0.2 mg/ml DNase-free RNase (Roche) and quantified spectrophotometrically at 260 nm. After electrophoresis on 3 %-(w/v)-agarose gel, the proteins in the gel were revealed by staining with ethidium bromide.

### RNA isolation and reverse-transcription (RT)-PCR

For RT-PCR total RNA was isolated from individual mammary glands using guanidinium thiocyanate/phenol/chloroform extraction [16]. Equal amounts of RNA (2  $\mu$ g) were used in the RT reaction, using oligo(dT)<sub>23</sub> (Sigma) as the primer, to generate the first-strand cDNA and SuperScript™ reverse transcriptase (200 units/ $\mu$ l) (Invitrogen Life Technologies, Frederick, MD, U.S.A.), following the instructions of the manufacturer.

The cDNA was used as a template for amplification in PCR; we used 2  $\mu$ l of the first-strand reaction for PCR. The PCR analysis for the different genes was performed using Biotools DNA polymerase gel-form from B&M Laboratories, S.A. (Madrid, Spain). The conditions for each PCR were determined in

**Table 1**  $\gamma$ -Cystathionase and  $\gamma$ -GGT activities and concentration of GSH in liver and mammary gland of control lactating rats and lactating rats injected with PPG, NAC, or bothResults are means  $\pm$  S.E.M with the numbers of rats in parentheses. Different superscript letters within a row indicate significant differences,  $P < 0.05$ . ND, not detectable.

Organ	Control	+ PPG, 3 days	+ PPG + NAC, 3 days	+ NAC, 3 days
Liver				
GSH ( $\mu\text{mol/g}$ )	5.8 $\pm$ 0.4 (11) <sup>b</sup>	3.2 $\pm$ 0.5 (8) <sup>a</sup>	5.9 $\pm$ 1.1 (9) <sup>b</sup>	7.6 $\pm$ 0.3 (7) <sup>b</sup>
$\gamma$ -Cystathionase [ $\mu\text{mol}/(\text{g} \cdot \text{min})$ ]	0.39 $\pm$ 0.02 (9) <sup>a</sup>	ND	ND	0.37 $\pm$ 0.03 (7) <sup>a</sup>
Lactating mammary gland				
GSH ( $\mu\text{mol/g}$ )	2.3 $\pm$ 0.2 (13) <sup>ab</sup>	1.5 $\pm$ 0.2 (8) <sup>a</sup>	2.3 $\pm$ 0.3 (8) <sup>ab</sup>	2.8 $\pm$ 0.2 (7) <sup>b</sup>
$\gamma$ -Cystathionase ( $\mu\text{mol}/\text{min per g}$ )	ND	ND	ND	ND
$\gamma$ -GGT ( $\mu\text{mol}/\text{min per g}$ )	11.0 $\pm$ 0.3 (4) <sup>a</sup>	10.7 $\pm$ 0.9 (4) <sup>a</sup>	9.0 $\pm$ 0.4 (4) <sup>a</sup>	8.9 $\pm$ 0.5 (4) <sup>a</sup>

preliminary experiments and optimized for each set of primers. Expression levels of the tumour suppressor/transcription factor p53 were determined using the following specific primers (5' to 3'): CACAGTCGGATATGAGCATC and GTCGTCCAGATACT-CAGCAT; primers for p21 (5' to 3') were: GTGAGACACCAG-AGTGCAAGA and ACAGCGATATCGAGACACTCA; specific primers for the oncoprotein c-Jun (5' to 3') were: TGAGTGCA-AGCGGTGTCTTA and TAGTGGTGATGTGCCATTG. To amplify the 26S ribosomal protein (26S RIB) the following primers (5' to 3') were used: AATTCGCTGCACGAAGTGGC and CAGCAGGTCTGAATCGTGGT. The amplification conditions were one cycle at 85 °C for 2 min and one cycle at 94 °C for 2 min. Then for p53, 35 cycles of denaturation (94 °C for 30 s), annealing (58 °C for 30 s) and extension (70 °C for 1 min) with a final extension of 70 °C for 10 min were used; the differences among the different amplifications were with regard to the number of cycles and the annealing temperature so that: for c-Jun, there were 40 cycles and the annealing temperature was 54 °C; for p21 there were 35 cycles and the annealing temperature was 62 °C; and for 26S RIB there were 25 cycles and the annealing temperature was 51 °C. The resulting PCR products were separated by electrophoresis in a 1.5%-(w/v)-agarose gel in TBE (90 mM Tris/90 mM boric acid/2 mM EDTA, pH 8.0) and stained with ethidium bromide. The expected sizes for the PCR products were: 26S RIB (252 bp), p53 (600 bp), c-Jun (461 bp) and p21 (400 bp). In this semi-quantitative method the intensity of the bands was measured by densitometry using the GeneGenius system and the GeneTools analysis software (Syngene, Cambridge, U.K.).

### Immunoblot analysis

Tissues were homogenized in 10 ml of ice-cold buffer A [20 mM Hepes (pH 7.9)/20% (v/v) glycerol/250 mM KCl/2 mM MgCl<sub>2</sub>/0.2 mM EDTA/0.5 mM dithiothreitol/1 mM PMSF/5 mM NaF/0.5 mM Na<sub>3</sub>VO<sub>4</sub>/0.1% Triton X-100] in the presence of protease inhibitors (5  $\mu\text{l}/\text{ml}$  of buffer of protease inhibitor cocktail from Sigma)/g of tissue. The resulting homogenate was centrifuged at 500 000 g for 15 min at 4 °C.

To analyse cytochrome *c* release from mitochondria, cytosol extracts were obtained from mammary tissue by differential centrifugation as follows: tissues were homogenized in 10 ml of ice-cold buffer B [10 mM Hepes (pH 7.9)/10 mM KCl/2 mM MgCl<sub>2</sub>/0.5 mM dithiothreitol/1 mM PMSF/5 mM NaF/0.5 mM Na<sub>3</sub>VO<sub>4</sub>/0.1% Triton X-100] in the presence of protease inhibitors/g of tissue. Then the homogenate was centrifuged at 800 g for 10 min at 4 °C. The resulting supernatant was centrifuged at 20 000 g for 15 min at 4 °C. The pellet was discarded and the supernatant was used to analyse cytosol proteins.

Mammary homogenates were normalized for protein concentration using the BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.). Equal amounts of protein (25  $\mu\text{g}$ ) were then boiled in sample buffer for 5 min and separated by SDS/PAGE. After electrophoresis, the proteins were transferred to 0.2- $\mu\text{m}$ -pore-size nitrocellulose. For all antibodies, the membranes were incubated in blocking solution [5% (w/v) non-fat dry milk with 0.05% (v/v) Tween 20], for 1 h at room temperature with shaking; following three washes with TTBS [25 mM Tris/HCl (pH 7.5)/0.15 M NaCl/0.1% (v/v) Tween 20], blots were incubated with primary antibodies [p53, p21 and p27 monoclonal mouse antibodies were purchased from Calbiochem, La Jolla, CA, U.S.A.; cytochrome *c*, phosphorylated c-Jun N-terminal kinase (p-JNK) and c-Jun were from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] in TTBS for 1 h at room temperature. Blots were washed again with TTBS and incubated with the secondary antibody conjugated to horseradish peroxidase (Santa Cruz) for 60 min at room temperature. Finally, blots were washed with TTBS and detection was carried out using the chemiluminescent luminol reagent (Supersignal West Pico; Pierce). The intensity of the bands was measured by densitometry using the GeneGenius system and the GeneTools analysis software (Syngene).

### Statistics

In Tables 1 and 2 below and in the bar graphs of Figures 3, 4, 5 and 6 below, ANOVA was performed. The homogeneity of the variances was analysed by the Levene test. The null hypothesis was accepted for all the 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

#### $\gamma$ -Cystathionase and $\gamma$ -GGT activities and GSH concentration in liver and mammary gland from lactating rats, PPG-treated lactating rats and PPG-treated rats injected with NAC

As seen in Table 1, liver  $\gamma$ -cystathionase activity was undetectable in PPG-treated lactating rats when compared with control lactating rats because this compound is an irreversible inhibitor of the enzyme. When PPG and NAC were injected simultaneously, the activity remained undetectable; the injection of NAC into control lactating rats did not change the enzyme activity in liver. The  $\gamma$ -cystathionase activity in lactating mammary gland was undetectable under all situations studied, confirming that the transsulphuration pathway is not expressed in this tissue.

**Table 2** Daily milk production in lactating rats under different experimental conditions

(a) Daily milk production in untreated lactating rats and lactating rats injected with PPG, NAC, or both. Results are means  $\pm$  S.E.M.. There were ten pups/dam. Different superscript letters within a row indicate significant differences,  $P < 0.05$ . (b) Daily milk production in lactating rats and lactating rats injected with PPG after cross-fostering of pups. Results are means  $\pm$  S.E.M. with the number of rats in parentheses. Different superscript letters within a row indicate significant differences,  $P < 0.05$ . ND, not detectable.

(a)

Dam	Milk production (ml/pup)			
	Control (5)	+ PPG (5)	+ PPG + NAC (5)	+ NAC (6)
Previous day	3.0 $\pm$ 0.1 <sup>a</sup>	3.3 $\pm$ 0.3 <sup>a</sup>	2.9 $\pm$ 0.1 <sup>a</sup>	3.0 $\pm$ 0.2 <sup>a</sup>
24 h	3.2 $\pm$ 0.1 <sup>a</sup>	2.2 $\pm$ 0.3 <sup>a</sup>	2.4 $\pm$ 0.2 <sup>a</sup>	3.1 $\pm$ 0.2 <sup>a</sup>
48 h	3.2 $\pm$ 0.1 <sup>ab</sup>	2.1 $\pm$ 0.1 <sup>a</sup>	2.3 $\pm$ 0.4 <sup>a</sup>	3.3 $\pm$ 0.2 <sup>b</sup>
72 h	3.3 $\pm$ 0.2 <sup>b</sup>	1.3 $\pm$ 0.3 <sup>a</sup>	2.2 $\pm$ 0.4 <sup>ab</sup>	3.2 $\pm$ 0.2 <sup>b</sup>

(b)

Dam	Milk production (ml/pup)	
	Control (3)	+PPG (3)
Previous day	2.9 $\pm$ 0.2 <sup>a</sup>	2.8 $\pm$ 0.2 <sup>a</sup>
24 h	3.5 $\pm$ 0.2 <sup>a</sup>	2.1 $\pm$ 0.5 <sup>a</sup>
48 h	3.3 $\pm$ 0.2 <sup>b</sup>	1.1 $\pm$ 0.4 <sup>a</sup>
72 h	4.2 $\pm$ 0.4 <sup>b</sup>	0.8 $\pm$ 0.2 <sup>a</sup>

The  $\gamma$ -GGT activity in the lactating mammary gland was not affected by the administration of PPG (Table 1), but was significantly lower after the removal of the pups (results not shown).

Liver GSH concentration was significantly lower in PPG-treated lactating rats than in control lactating rats. The administration of NAC to PPG-treated lactating rats restored the concentration of liver GSH to control values (Table 1). The GSH concentration in the mammary gland of PPG-treated rats was significantly lower than in control lactating rats and in rats injected with NAC (Table 1).

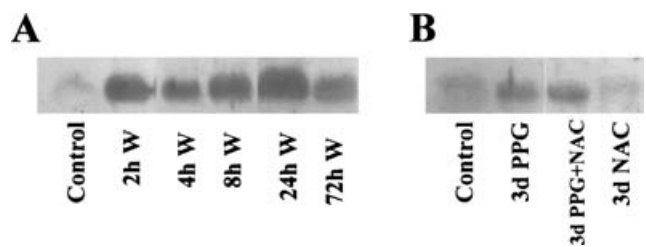
#### Inhibition of liver $\gamma$ -cystathionase activity decreases lactation and is reversed by NAC

Table 2(a) shows that inhibition of liver  $\gamma$ -cystathionase activity by PPG injection over a period of 3 days produced an inhibition of lactation that was reflected in low milk production compared with control lactating rats. This difference was significant 48 h after injection of PPG. When rats were injected simultaneously with PPG and NAC over a period of 72 h, this decrease was prevented. NAC injection to control lactating rats did not modify milk production [2] (See Table 2a).

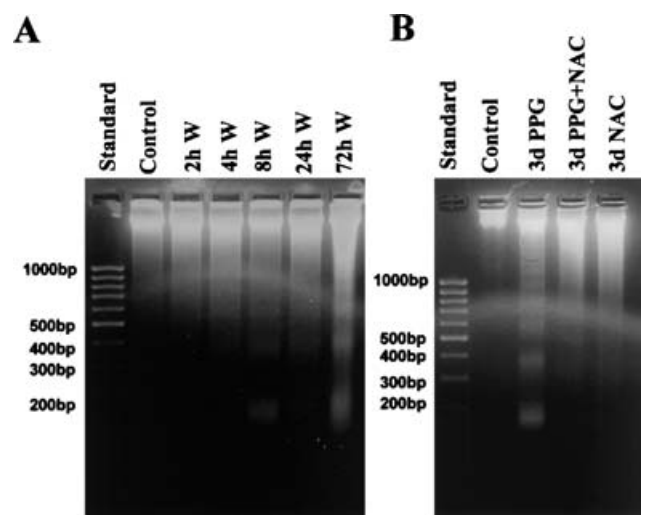
To evaluate the possibility that inhibition of the trans-sulphuration pathway could decrease growth and vitality in pups and consequently they could provide reduced suckling stimuli and milk removal, cross-fostering of pups was done so that all dams fostered control pups. This cross-fostering did not impel the decrease in milk production, as shown in Table 2(b).

#### Apoptosis is induced in lactating mammary gland by litter removal or by the inhibition of liver trans-sulphuration pathway by PPG treatment

Release of cytochrome *c* from mitochondria is a common marker of apoptosis [15]. Figure 1 shows that weaning for 2 h resulted in the release of this protein to cytosol. Similar results were found when analysing cytosol extracts from mammary tissue of PPG-

**Figure 1** Protein concentrations of cytochrome *c* in cytosolic extracts from mammary-gland homogenates

(A) At different times after the removal of pups [2, 4, 8, 24 and 72 h of weaning ('2h W' etc.)], mammary tissue was isolated and cytosol extracts were obtained. Western immunoblot analysis of cytochrome *c* was performed. (B) Cytosol extracts from lactating mammary gland of PPG-, PPG + NAC- and NAC-treated rats were prepared for the same immunoblot analysis. This Figure shows a representative experiment. 3d, 3 days.

**Figure 2** DNA fragmentation in the mammary gland

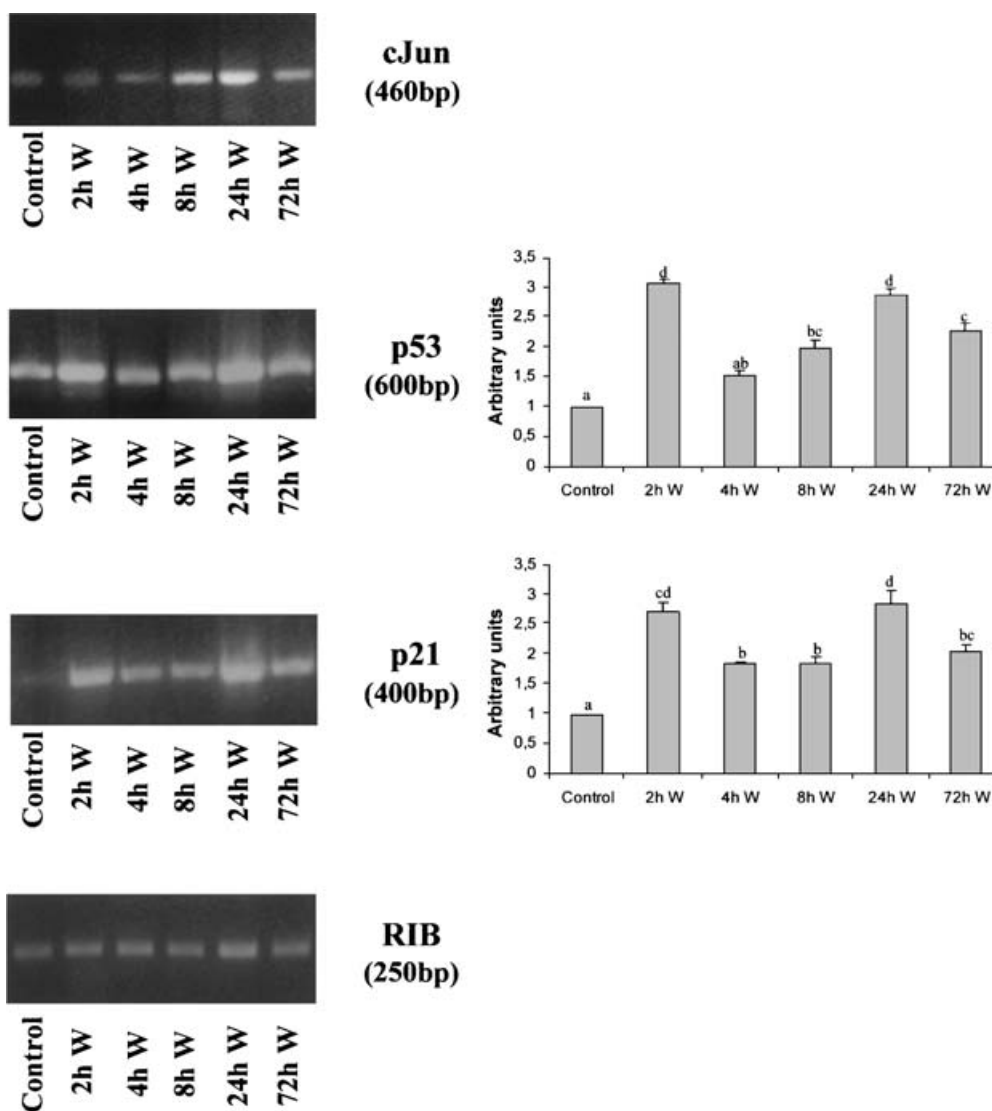
Agarose-gel electrophoresis of DNA from (A) mammary gland after different times of litter removal [control lactating rats and rats weaned for 2, 4, 8, 24 or 72 h ('2h W' etc.)] or (B) from lactating mammary tissue obtained from lactating rats injected with PPG, NAC, or both, over a period of 3 days. This Figure shows a representative experiment.

treated rats. It is noteworthy that NAC administration to PPG-treated rats did not reverse the trend of cytochrome *c* release.

Lactating rats were weaned for 2, 4, 8, 24 and 72 h. Failure of milk removal from the gland induced PCD after 24 h of pup removal. As Figure 2(A) shows, this PCD was characterized by an increased activation of the apoptosis-specific endonuclease which led to a ladder-type DNA pattern of nuclear apoptosis in the mammary gland. Figure 2(B) shows that, when control lactating rats were injected with PPG over a period of 3 days, an apoptotic pattern similar to that seen for mammary gland after weaning was obtained. The simultaneous administration of NAC and PPG to control lactating rats over a period of 3 days showed a lower rate of apoptosis when compared with rats injected with PPG alone. NAC by itself did not produce any change at all, and the pattern was the same as that shown by control lactating rats.

#### Effect of weaning on JNK, c-Jun, p53, p21 and p27 expression in rat mammary gland

p53 plays a pivotal role during weaning; the amount of p53 mRNA and protein levels were studied in the mammary gland from



**Figure 3** RT-PCR analysis of c-Jun, p53 and p21 in mammary gland during weaning

RT-PCR analysis of whole RNA isolated from mammary gland was used to examine the transcription of c-Jun, p53 and p21 from control lactating rats (control) and at different times of weaning (W). Three rats for each condition were employed for semi-quantitative analysis. The graphs show the ratio of each gene to 26S RIB; results are means  $\pm$  S.E.M. Different superscript letters within a row indicate significant differences;  $P < 0.05$ .

control lactating rats and rats under different times of weaning. As Figures 3 and 4 show, the steady-state mRNA levels of p53 and protein concentrations respectively increased more than threefold 2 h after weaning when compared with controls. Interestingly this increase was not constant; at 4 and 8 h after weaning the fold change was lower than at 2 h, and then, at 24 h after weaning, the levels rose again.

The transcript levels of c-Jun, which is upstream of p53, did not change after 4 h of weaning (Figure 3), but the amounts of c-Jun and JNK (a kinase that directly regulates c-Jun) protein were significantly increased 24 h after the removal of pups when compared with control values and remained elevated at 72 h after weaning (Figure 4).

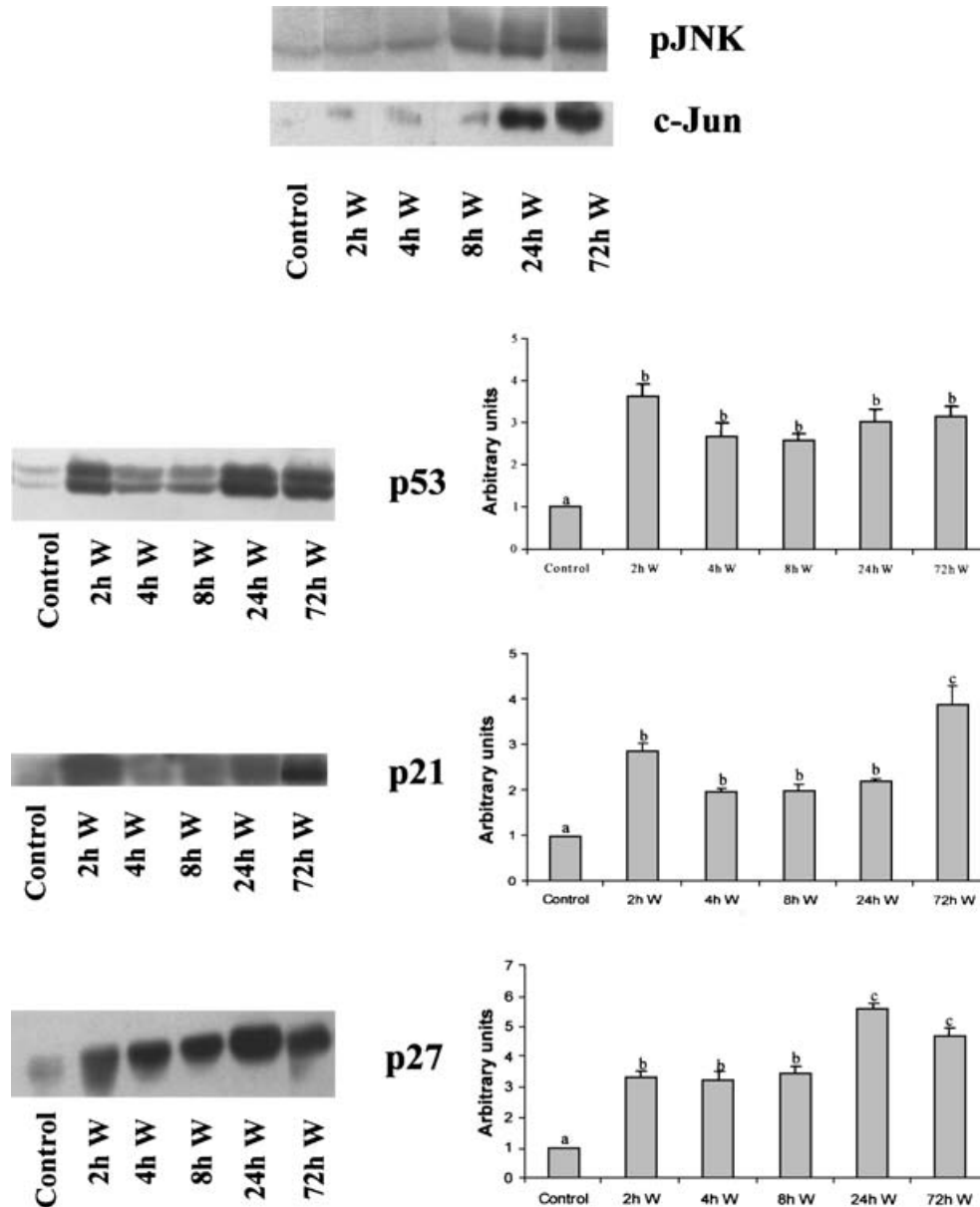
p53 is known to induce the expression of the p21<sup>cip1</sup>, which belongs to a family of proteins that regulate cell-cycle arrest [17]. The steady-state amounts of mRNA (Figure 3) and protein (Figure 4) of p21<sup>cip1</sup> were significantly increased during weaning when compared with control lactating rats. Since p21<sup>cip1</sup> is a target

of p53, it was not surprising to find that there was also a fluctuation in the increase of this gene; as with p53, there was a pronounced increase 2 h after weaning; weaning then for 4 and 8 h resulted in a lower fold change of p21<sup>cip1</sup>; it increased again at 24 and 72 h.

Similar results were obtained when the protein p27<sup>kip1</sup>, another cyclin-dependent kinase inhibitor from the same family, was studied. As seen in Figure 4, the levels of this protein increased when the weaning took place.

#### Effect of the inhibition of liver trans-sulphuration pathway by PPG on JNK, c-Jun, p53, p21 and p27 expression in rat lactating mammary gland

p53 was analysed in lactating rats injected with PPG over a period of 3 days and in lactating rats injected with PPG and NAC over a period of 3 days. The amounts of p53 mRNA (Figure 5) and protein (Figure 6) in the mammary gland from PPG-treated rats were increased twofold when compared with rats at the peak of



**Figure 4** Western-blot analysis of p-JNK, c-Jun, p53, p21 and p27 from weaned mammary gland

Western-blot analysis of proteins extracted from mammary glands (50  $\mu$ g of protein/lane). Total extract from control lactating rats at the peak of lactation (control) and after different periods of involution [2, 4, 8, 24 and 72 h weaning ('2h W' etc.)] were analysed by Western blot for phosphorylated JNK, c-Jun, p53 and the Cip/Kip proteins p21<sup>cip1</sup> and p27<sup>kip1</sup>. Results are means  $\pm$  S.E.M. Different superscript letters within a row indicate significant differences,  $P < 0.05$ .

lactation. This pattern was similar to that exhibited by weaned-pup glands. The administration of NAC to PPG-treated rats prevented the increase of p53 transcripts, although the amount of protein was slightly higher than in controls.

Administration of PPG over a period of 3 days to lactating rats also increased the transcription of c-Jun (Figure 5), but when NAC was administered simultaneously, the increase was prevented. Protein amounts of JNK and c-Jun were higher after PPG administration (Figure 6). Administration of NAC to PPG-treated rats partially prevented the increase in the levels of these proteins.

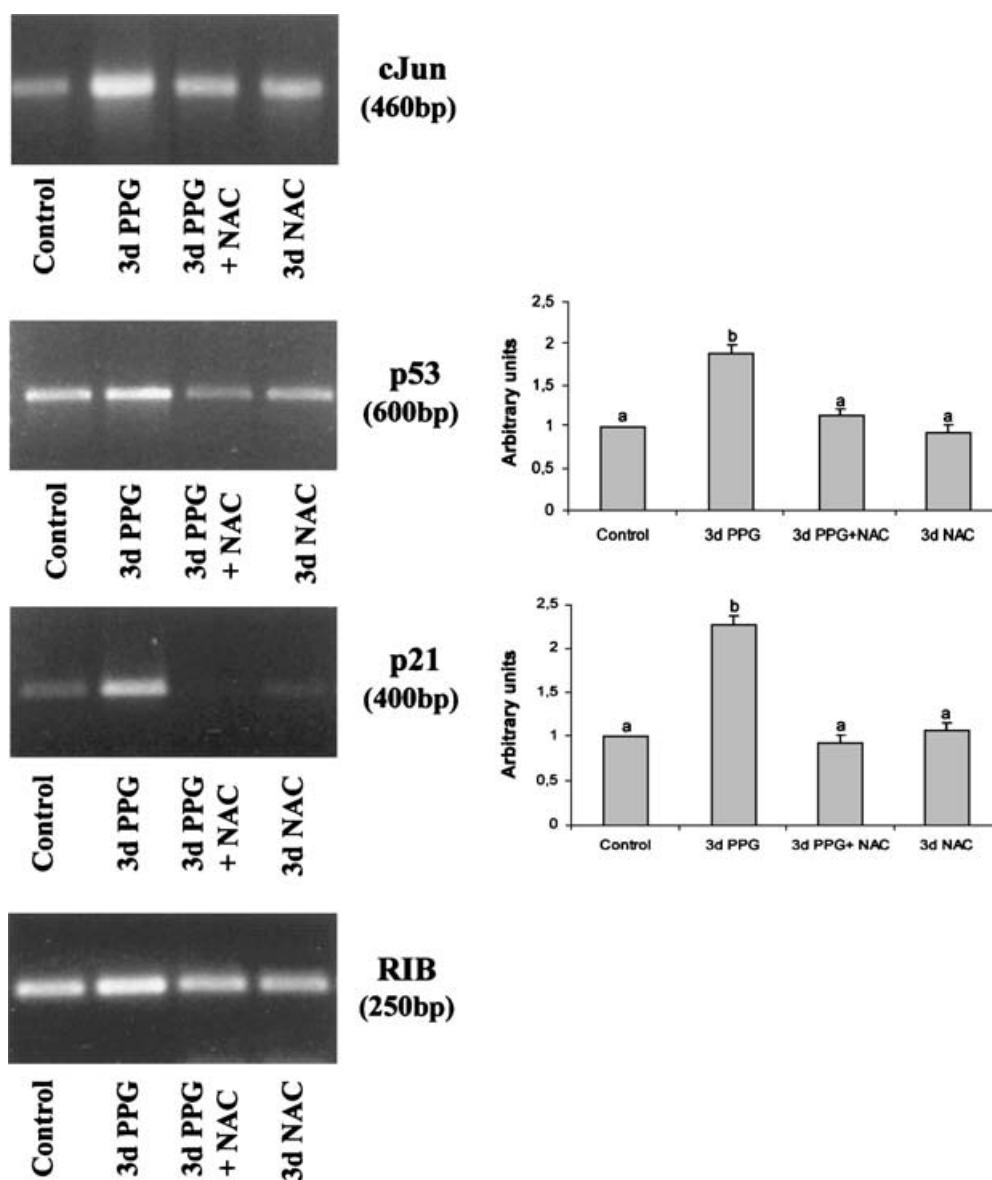
When studying the steady-state mRNA (Figure 5) and the protein levels (Figure 6) of p21<sup>cip1</sup> in PPG-treated rats, the results showed that inhibition of the trans-sulphuration pathway also

produced an increase in p21<sup>cip1</sup> expression similar to that shown by weaned rats. The administration of NAC appeared to maintain p21<sup>cip1</sup> transcripts at control levels, although protein levels were higher than those of controls.

Treatment of rats with PPG over a period of 3 days also resulted in higher levels of p27<sup>kip1</sup> protein (Figure 6), but the administration of NAC partially reversed this increase.

#### **BSO injection to rats at the peak of lactation reduces GSH availability to the mammary gland, reproducing gene and protein changes seen for PPG treatment**

BSO is a potent and specific inhibitor of  $\gamma$ -glutamylcysteine synthetase; administration of BSO over a period of 2 days



**Figure 5** RT-PCR analysis of c-Jun, p53 and p21 in control lactating rats and rats treated with PPG, NAC or both

Total RNA was isolated from lactating tissue (control) or mammary gland from rats treated with PPG, PPG + NAC or NAC for 3 days and amplified by RT-PCR as described in the Experimental section. The graphs show the ratio of p53 and p21 to 26S RIB for each experimental condition. The results shown are representative of three independent experiments for the semi-quantitative analysis; results are means  $\pm$  S.E.M. Different superscript letters within a row indicate significant differences,  $P < 0.05$ .

resulted in a significant decrease in GSH level in the liver [control  $5.8 \pm 0.4 \mu\text{mol/g}$  of tissue (11) and BSO-treated rats:  $1.1 \pm 0.1$  ( $P < 0.05$ )  $\mu\text{mol/g}$  of tissue (3)] and in mammary gland [Control values were  $2.3 \pm 0.2 \mu\text{mol/g}$  of tissue (12) and BSO-treated rats  $0.4 \pm 0.1$  ( $P < 0.05$ )  $\mu\text{mol/g}$  of tissue (3)]. Results are expressed as means  $\pm$  S.E.M.

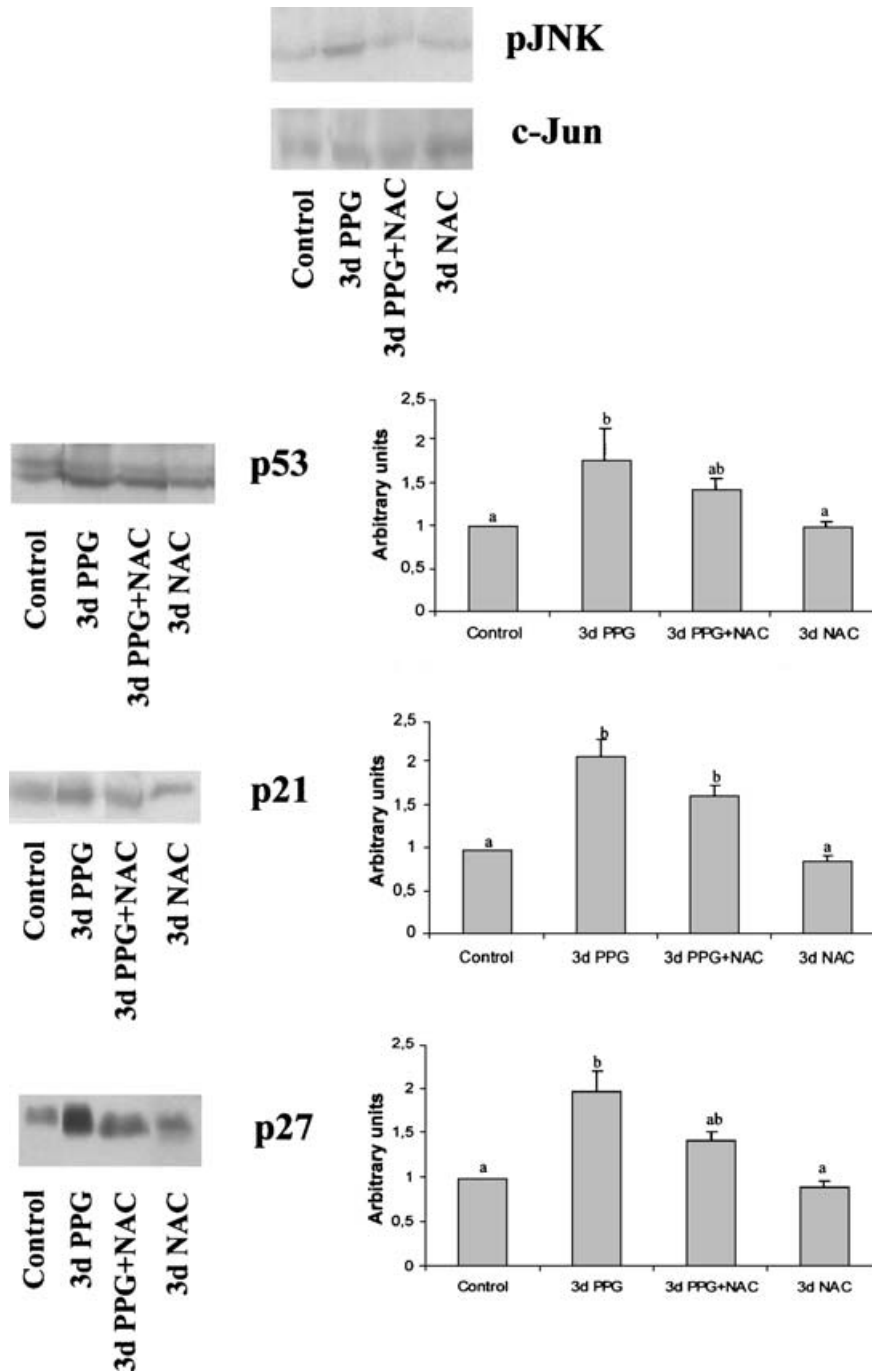
The  $\gamma$ -GGT activity in the lactating mammary gland was not affected by the administration of BSO (results not shown).

Figure 7(A) shows that, in lactating rats injected with BSO, the mammary gland undergoes apoptosis because the endonuclease has been activated and a ladder pattern is seen when isolating DNA from the mammary tissue. Figure 7(B) shows that the changes in the different genes commented above were also reproduced when GSH was depleted using BSO. Finally, in Figure 7(C), Western-blot analyses show similar changes.

## DISCUSSION

Milk production by the lactating mammary gland is dependent on a complex interplay of lactogenic hormones and autocrine effects due to suckling of the litter. Programmed cell death in the mammary gland is associated with the expression of several growth-arrest genes [18,19]. The Cip/Kip proteins (p21 and p27), which inhibit all cyclin-cyclin-dependent kinase complexes arresting the cell cycle [20], are overexpressed during weaning and in rats injected with PPG and BSO, which shows that a low availability of GSH to the gland of lactating rats is responsible in part for the decline in lactation.

Other gene products which are found to be activated during the early phase of involution are JNK and c-Jun (see Figures 3 and 4). An involvement of JNK in the activation of apoptosis has been



**Figure 6** Immunoblot analysis of p-JNK, c-Jun, p53, p21 and p27 in mammary tissue from control lactating rats and rats treated with PPG, NAC or both

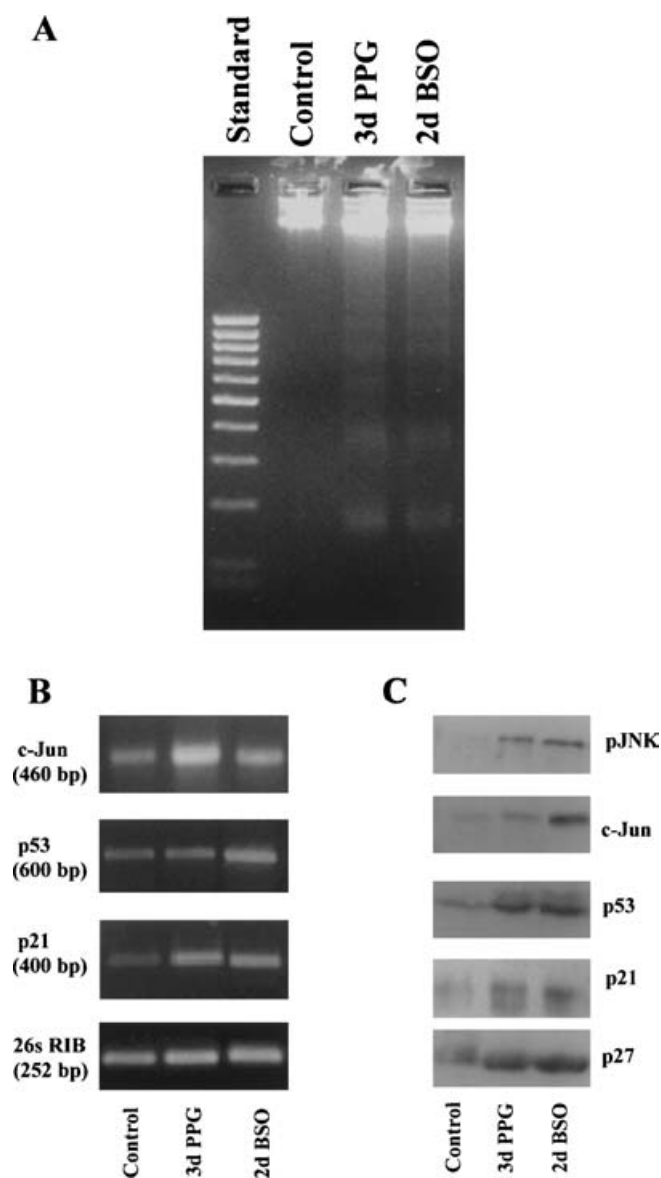
Total extracts (50  $\mu$ g of protein/lane) from mammary tissue of control rats, mammary PPG-, PPG + NAC- or NAC-treated rats, were electrophoresed and immunoblotted with specific antibodies for pJNK, c-Jun, p53, p21 and p27. The graphs show the amount of each protein under the different conditions studied. Results are means  $\pm$  S.E.M. for three different experiments. Different superscript letters within a row indicate significant differences,  $P < 0.05$ .

previously documented [21]. c-Jun represents a major target for JNK, and phosphorylation of c-Jun by JNK enhances its activity. Activator protein-1 (AP-1) can trigger both pro- or anti-apoptotic signals depending on the buffer composition, the cell types or the death-inducing treatments [22]; the role of c-Jun in apoptosis is thus complex, a function for AP-1 in apoptosis has initially been proposed based on the observation that c-fos and c-jun mRNAs are rapidly induced in cytokine-dependent lymphoid cells upon

growth-factor withdrawal [23]. The results of all of these studies are in agreement with the increased expression found in JNK and c-Jun in the weaned-pup glands and in the glands from PPG-treated rats and BSO-treated rats.

Weaning also produces an induction of p53 expression, resulting in cell arrest and apoptosis [24]; nevertheless, it has been shown, using p53-deficient mice, that the involution of the mammary epithelium takes place, but with a delay of several days.





**Figure 7** DNA fragmentation: RT-PCR analysis for c-Jun, p53 and p21 and Western blot of p-JNK, c-Jun, p53, p27 and p21 in lactating rats treated over a period of 2 days with BSO

(A) Agarose-gel electrophoresis of DNA from control lactating mammary gland, mammary tissue obtained from rats injected with PPG over a period of 3 days and from mammary gland obtained from rats treated with BSO over a period of 2 days. (B) Whole RNA isolated from mammary gland was used for RT-PCR analysis of c-Jun, p53 and p21 from control lactating rats, from rats treated with PPG and from rats treated over a period of 2 days with BSO. (C) Western-blot analysis of proteins extracted from mammary glands (50  $\mu$ g of protein/lane) from control lactating rats, rats treated with PPG and rats treated with BSO, was done for phosphorylated JNK, c-Jun, p53 and the Cip/Kip proteins p21<sup>kip1</sup> and p27<sup>kip1</sup>.

In this type of null mice, the stromal proteases are induced in the mammary gland by 5 days post-weaning, providing a p53-independent mechanism for epithelial involution [24]. In the present study, p53 levels (mRNA and protein) are high 2 h after weaning begins (Figures 3 and 4), showing that this gene might be responsible for the early changes at the onset of weaning. It is noteworthy that the increased expression of p53 (mRNA and protein levels) at the first hour of weaning is much higher at 2 and 24 h than at 4 and 8 h after weaning. This behaviour of p53 gene product remains to be investigated.

Using mice that contain a homozygous deletion of the oxytocin gene, milk is retained in the alveolar lumens of the glands, owing to the failure of milk ejection, although the pups remain suckling. In this situation, PCD starts as a result of local factors, even in the presence of systemic lactogenic hormones [4]. The possible signals that bring about the increased p53 expression in the first hours of weaning could be one of the following: (i) a decrease in plasma lactogenic hormones after removal of pups, (ii) a lower rate of delivery of hormones to the gland when compared with control lactating rats, because lactating rats weaned for 5 h have a marked fall in mammary-gland blood flow [25] that decreases the levels of receptors for prolactin and oestrogens [26], and (iii) milk accumulation. There are three stages of involution; the first one is regulated locally by milk stasis, is p53-dependent and is reversible. In this phase, apoptosis is promoted by the transcription factor Stat3 (signal transduction and activators of transcription 3) and transforming growth factor  $\beta$ 3. Stat3 may induce apoptosis by up-regulating a known promoter of apoptosis, insulin-like growth factor-binding protein-5, and by down-regulating Stat5a [27]. The second stage is also characterized by apoptosis, but it is mediated by lactogenic hormones; finally, the third stage is a biosynthetic phase in which mammary stroma is remodelled and repopulated with adipose tissue [28].

There is *in vitro* evidence showing that GSH utilization by the lactating mammary gland is mediated by  $\gamma$ -GGT. Using as an experimental model bovine mammary acini, it has been shown that the label from [<sup>35</sup>S]GSH is incorporated into the acid-precipitable proteins when substrates are provided for protein synthesis. The inclusion of a GGT inhibitor decreased the incorporation of <sup>35</sup>S into protein [29]. There is an inverse relationship between the activities of GGT and  $\gamma$ -cystathionase in different mammalian tissues [30]; the lactating mammary gland has a very high activity of GGT and a non-detectable activity of  $\gamma$ -cystathionase. During weaning, the activity of the GGT in the mammary gland is significantly lower than in control lactating glands [31], and thus GSH utilization and L-cysteine availability should decline during mammary involution.

It has been shown *in vivo* that a high availability of GSH to the lactating mammary gland is important to maintain lactation [2]; this happens because there is an increase GSH release by the liver, the gland increases its size and the blood flow is also increased, which assures a net supply of GSH to the gland. The signals responsible for the high release of total glutathione by the liver of lactating rats are the increase in protein intake, which modulates the expression and activity of hepatic  $\gamma$ -cystathionase, and the liver hypertrophy, which is unrelated to protein intake and is due to physiological changes [2]. In the present work it is clearly shown that, when GSH delivery to the gland is impaired, the mammary gland undergoes similar changes to those that happen following weaning. This emphasizes the importance of GSH availability to GGT to provide a supply of L-cysteine needed to keep the lactation and explains that the changes induced in the gland when the liver trans-sulphuration pathway is inhibited with PPG are partially reversed with NAC. It is noteworthy that the apoptotic process evaluated by DNA fragmentation is induced when the supply of L-cysteine as GSH is diminished and is partially reversed when PPG-treated rats are injected simultaneously with NAC. The experiments performed in BSO-treated rats show the same results as PPG-treated rats. This points out the importance of GSH homeostasis in maintaining lactation.

Our results highlight that a decrease in GSH levels *in vivo* induces apoptosis in the mammary gland. Different experimental models have shown previously similar results using *in vitro* models, but as far as we know this correlation found *in vitro* has never been shown in whole tissue [32]. Recently, it has

been shown that GSSG (oxidized glutathione) levels in lactating mammary gland are higher during involution than at the peak of lactation; a direct relationship between mtDNA damage and the GSSG/GSH ratio has been revealed, but glutathione oxidation precedes DNA fragmentation [6]. However, it remains unclear whether high glutathione levels are essential to protect the tissue against oxidative stress. Alternatively, glutathione could play other undefined metabolic roles during the apoptotic process.

This work emphasizes the physiological importance of the inter-tissue flux of GSH found in the lactating mother, because it allows a delivery of L-cysteine to the mammary gland that is required to maintain gene expression in this tissue.

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