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Nitration of cathepsin D enhances its proteolytic activity during mammary gland remodelling after lactation

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Proteomic studies in the mammary gland of control lactating and weaned rats have shown that there is an increased pattern of nitrated proteins during weaning when compared with controls. Here we report the novel finding that cathepsin D is nitrated during weaning. The expression and protein levels of this enzyme are increased after 8 h of litter removal and this up-regulation declines 5 days after weaning. However, there is a marked delay in cathepsin D activity since it does not increase until 2 days post-weaning and remains high thereafter. In order to find out whether nitration of cathepsin D regulates its activity, iNOS (inducible nitric oxide synthase)$^{-/-}$ mice were used. The expression and protein levels of this enzyme were similar to WT (wild-type) animals, but the proteolytic activity was significantly reduced during weaning in knockout compared to WT mice. *In vitro* treatment of recombinant human cathepsin D or lactating mammary gland homogenates with relatively low concentrations of peroxynitrite enhances the nitration as well as specific activity of this enzyme. Using MS, it has been shown that the residue Tyr^{168} was nitrated. All of these results show that protein nitration during weaning might be a signalling pathway involved in mammary gland remodelling.

Key words: cathepsin D activity, mammary gland involution, nitration, nitrotyrosine, post-translational modification, weaning.

INTRODUCTION

The mammary gland is a complex, highly dynamic organ that has evolved to provide nutrition and immunological protection for the offspring of mammals [1]. The mammary gland undergoes cycles of proliferation, differentiation and apoptosis with each successive pregnancy/lactation cycle. During mammary gland involution after natural weaning or litter removal, the gland is properly remodelled to a virgin-like state through a process involving apoptosis and tissue remodelling in which the epithelial compartment of the gland regresses and the adipocytes redifferentiate, refilling the adipose compartment. Under conditions of forced weaning, this process is separated into two phases [2,3]; the first phase is characterized by apoptosis of the secretory alveolar epithelium without major changes in gland architecture. This primary apoptotic process is reversible if suckling is resumed and it is mainly controlled by p53. In the second phase, apoptosis is accompanied by activation of MMPs (matrix metalloproteinases) that are involved in the degradation of the extracellular matrix and alveolar basement membrane. This stage is irreversible and p53 independent. This second phase is also associated with redifferentiation of the adipocytes.

Lactating mammary gland involution is initiated on cessation of suckling. A number of mammary-derived signals are currently considered to be possible inducers of mammary epithelial cell apoptosis [2,3]. Indeed, the essential signalling pathways that initiate involution have been extensively studied by genetic and microarray approaches [4–6]. Using genetically modified mice, many different factors that either promote or delay involution have been identified. Among the promoters, Bax, STAT3 (signal transducer and activator of transcription) [3,7], TNFα (tumour necrosis factor α) [8] and retinoids [9,10] are found. Interestingly, the NF-κB (nuclear factor κB) pathway is activated at the early events that trigger apoptosis [3]; 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 litter removal [11]. Downstream targets of this pathway are up-regulated, ensuring the transition to the second phase of involution. In previous studies, we have demonstrated an induction of iNOS [inducible nitric oxide synthase; NOS-2 (nitric oxide synthase-2)] gene expression, regulated by NF-κB, during weaning, resulting in an increase in nitric oxide levels and, subsequently, increased protein nitration [6].

Nitration is a post-translational modification of tyrosine residues of proteins mediated by peroxynitrite ($ONOO^-$). Nitration of tyrosine or tyrosyl groups of a protein modulates protein function and is involved in signal transduction pathways, which lead to an alteration of cellular metabolism and function. In particular, $ONOO^-$-induced protein oxidation often leads to a loss of protein activity [12,13], seldom to activation [14–16]. Because of its recognized significance, there is an increasing interest in identifying nitrated proteins in order to gain a better understanding of their involvement in different biological processes, as is the case for mammary gland remodelling. In the present study, using MS-based proteomic technology, the pattern of nitrated proteins during weaning was analysed and compared with control lactating mammary glands. We have identified cathepsin D (EC 3.4.23.5) within the nitrated proteins found after weaning, and for the first time we describe this post-translational modification on this enzyme.

Cathepsin D is a major aspartic proteinase of endosomes and lysosomes and is a member of the pepsin family of proteinases [17]. It is involved in the turnover of cellular proteins as well as in the selective processing of MHC II antigens, hormones and

Abbreviations used: IEF, isoelectric focusing; iNOS, inducible nitric oxide synthase; KO, knockout; LC, liquid chromatography; MS/MS, tandem MS; NF-κB, nuclear factor κB; RT, reverse transcription; WT, wild-type.

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growth factors, in the range of pH 2.8–5.0. Gene-KO (knockout) studies have demonstrated that cathepsin D-deficient mice die prematurely from massive destruction of lymphoid organs and progressive atrophy of the intestinal mucosa, suggesting essential functions of cathepsin D in tissue homoeostasis [18]. Cathepsin D is synthesized as an inactive 52 kDa pre-pro-enzyme that undergoes sequential proteolytic cleavages to produce the mature form. In lysosomes, the removal of 44 amino acids from the N-terminus yields an active 48 kDa single-chain molecule. This single-chain form is then cleaved by autoprocessing and, mainly, by cysteine proteinases into a mature two-chain enzyme consisting of a light 14 kDa N-terminal domain and a heavy 34 kDa C-terminal domain [19]. Cathepsin D is increased during weaning, playing a key role in protein degradation during mammary gland apoptosis; indeed, cathepsin D is known to be involved in the mitochondrial caspase-dependent intrinsic pathway of cell death [20]. Despite the relevance of this proteinase in physiological and pathological events, little data exist on the factors that modulate its secretion, activity and extracellular function in lactating mammary tissues. Recently, it has been demonstrated that prolactin controls the processing and polarized secretion of cathepsin D in this tissue [21]. On the other hand, interferon-γ activates STAT1, resulting in decreased vacuolar pH, which greatly affects cathepsin D processing and secretion [22]. In regard to cathepsin D activation, we report in this paper the novel finding that cathepsin D is nitrated during weaning; Tyr^{168} is identified as the modified residue. Moreover, it is noteworthy that *in vivo* and *in vitro* nitration of cathepsin D enhances its proteolytic activity. All these results highlight the importance of protein nitration as part of the physiological events that take place at the end of lactation, when the mammary gland is prepared for the next pregnancy/lactation cycle.

EXPERIMENTAL

Animals and tissue extraction

Pregnant Wistar rats (Harlan), WT (wild-type) control mice and $iNOS^{-/-}$ mice (Taconic, Ejby, Denmark) were used. Mice were C57BL/6 and the genotype of the $iNOS^{-/-}$ strain was verified by PCR, using DNA prepared from tail samples taken at the end of the experiments. Animals were kept in individual cages in a controlled environment (12 h light/12 h dark cycle) and they received water and food ad libitum. They were cared for and handled in conformance with the NIH (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) approved the study protocol. After parturition, the litters were maintained with at least ten pups in the case of rats and seven pups in the case of mice.

At the peak of lactation (days 12–14), the rats were divided into different groups: control lactating rats ($n = 8$) or weaned rats from which pups were removed 12 days after delivery to initiate involution. Mammary tissue samples were collected at the indicated times after weaning (at least three rats for each condition were used). For mice, the groups were as follows: control mice at the peak of lactation (days 9–11) or weaned animals from which the litter was removed 6, 24, 48 and 72 h before killing.

The animals were anaesthetized with sodium pentobarbital (60 mg/kg body weight in 0.9 % NaCl intraperitoneal; Abbott Laboratories) and killed immediately after removing the inguinal mammary glands that were snap-frozen in liquid nitrogen and stored at − 80 °C for further analysis.

Antibodies

Depending on the species and the forms of cathepsin D to be detected, different anti-(cathepsin D) antibodies were used in the Western blots. A rabbit polyclonal human cathepsin D antiserum (sc-10725; Santa Cruz Biotechnology) was used to determine the expression of pro-cathepsin D and mature single-chain forms of cathepsin D in rats and mice. This antibody was also used for cathepsin D immunoprecipitation. The sc-10725 antibody is made against an N-terminal peptide of the mature single chain and should thus be able to detect the pro form, the single-chain form and the light-chain of the double-chain form, which includes the N-terminus. Nonetheless, we could only detect the pro and the mature single-chain forms. For this reason, other anti-(cathepsin D) antibodies were used: a rat monoclonal mouse cathepsin D antiserum (clone 204712) from R&D Systems was used in blots from mice homogenates to visualize the mature double-chain forms of cathepsin D, both the heavy and light chains. Finally, a mouse monoclonal anti-(human cathepsin D) antibody (clone BC011) from Calbiochem was used to determine the expression of recombinant cathepsin D from human liver and in the immunoblots performed after immunoprecipitation of cathepsin D. To corroborate '*in vitro*' nitration with $ONOO^-$, a mouse monoclonal anti-nitrotyrosine antibody (clone 1A6) was used (Upstate Biotechnology).

Immunoblotting

Protein expression was evaluated by using a standard immunoblotting procedure. Mammary gland tissues were homogenated in a buffer containing detergents and protease inhibitors as described previously [23]. A 20 μg portion of whole extract proteins, quantified by the Bradford assay, were denatured with Laemmli sample buffer, separated by PAGE on a 12 % gel (Bio-Rad Laboratories) and then electroblotted on to a nitrocellulose membrane (Protran®; Whatman Biosystems). After the addition of the corresponding primary antibody, immunocomplexes were revealed by using a peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), as appropriate, and subsequent peroxidase-induced ECL® (enhanced chemiluminescence) reaction (GE Healthcare).

Immunoprecipitation

Tissue lysates obtained from lactating and weaned mammary glands were precleared with Protein A–Sepharose and Protein G–Sepharose (50:50, v/v) (GE Healthcare) for 1 h at 4 °C and centrifuged at 12 000 ***g*** for 5 min. The supernatant was incubated first with the anti-(cathepsin D) antibody from Santa Cruz Biotechnology overnight at 4 °C, and then with Protein A–Sepharose and Protein G–Sepharose (50:50, v/v) for 4 h at 4 °C on a rotating device. Pellets were collected by centrifugation at 14 000 ***g*** for 1 min at 4 °C. The supernatants were discarded, and each pellet was subsequently washed three times with 50 mM Tris/HCl (pH 8.0). After washing the Sepharose, the immunocomplexes were eluted by boiling in the SDS-gel sample buffer for 5 min and then subjected to SDS/PAGE immunoblotting as described previously. Representative results of three separate experiments are shown.

Pepstatin A affinity purification of cathepsin D

Homogenates from weaned mammary gland (500 μg of whole extract proteins) were incubated for 15 h at 4 °C with pepstatin A–agarose-coated beads (Sigma) in 20 mM citrate phosphate buffer (pH 7.0) following the method of Follo et al. [24]. Then, the mixture was centrifuged at 20 000 ***g*** for 5 min and the pellet was

washed with 20 mM citrate phosphate buffer (pH 7.0). Finally, the pellet was resuspended and denatured in 30 μl of sample loading buffer prior to Western blot analysis.

Two-dimensional electrophoresis

Mammary gland tissue from each condition was homogenized in rehydration buffer from Bio-Rad Laboratories [8 M urea, 2 % CHAPS, 50 mM DTT (dithiothreitol), 0.2 % Bio-Lyte® 3/10 ampholyte and 0.001 % Bromophenol Blue]. After cleaning up the samples with a ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories), 500 μg of tissue lysates was loaded on to precast immobilized IPG strips (immobilized pH gradient strips; pH 5–8; Bio-Rad Laboratories) and subjected to IEF (isoelectric focusing) two-dimensional PAGE. The IEF conditions were those recommended by the manufacturer for the type of strip used. Second-dimension gels contained 8 % acrylamide. Simultaneously, proteins were resolved by two-dimensional PAGE and then electrophoretically transferred on to nitrocellulose membranes (Protran®) or visualized using silver or Coomassie Blue staining. Membranes were probed with a 1:1000 dilution of anti-nitrotyrosine antibody (Upstate Biotechnology) as described above. Spots corresponding to nitrated proteins were excised manually from Coomassie Blue-stained gels and identified by MS.

Protein identification by MS

Protein preparation and tryptic digestion

Protein samples corresponding to weaned mammary gland lysates recovered from two-dimensional gels were precipitated with 20 % TCA (trichloroacetic acid) on ice and, after centrifugation at 20 000 ***g*** for 30 min, the pellet was washed with cold acetone. The same procedure was performed in the case of human cathepsin D (25 μg) treated with 20 μM $ONOO^-$ or with 1.2 M NaOH, in order to identify the nitration site of the enzyme. Precipitated proteins were resuspended in 25 mM ammonium bicarbonate and digested with 12.5 ng/μl trypsin for 12 h at 37 °C.

LC (liquid chromatography)–ESI (electrospray ionization)-MS/MS (tandem MS) analysis

MS/MS analysis was performed as previously described [25]. Microcapillary reversed-phase LC was performed with a CapLC™ (Waters) capillary system. Reversed-phase separation of tryptic digests was performed with an Atlantis, C_{18}, 3 μm, 75 μm × 10 cm Nano Ease™ fused silica capillary column (Waters) equilibrated in 5 % acetonitrile and 0.2 % formic acid. After injection of 6 μl of sample, the column was washed for 5 min with the same buffer and the peptides were eluted using a linear gradient of 5–50 % acetonitrile in 45 min at a constant flow rate of 0.2 μl/min. The column was coupled online to a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80 °C and the spray voltage was 1.8–2.2 kV. MS/MS data were collected in an automated data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by CID (collision-induced dissociation) using an isolation width of 2.0 and a relative collision energy of 35 V. Data processing was performed with MassLynx 4.1. Database searching was carried out with ProteinLynx Global Server 2.1 (Waters) and Phenyx 2.2 (GeneBio) against Uniprot knowledgebase release 12.3 consisting of UniprotKB/Swiss-Prot release 54.3 and UniprotKB/TrEMBL release 37.3 with 285335 and 4932421 entries respectively. The search was enzymatically constrained for trypsin and allowed for one missed cleavage site. Further search parameters were as follows: no restriction on molecular mass and pI; fixed modification, carbamidomethylation of cysteine; and variable modification, oxidation of methionine. Data were then manually inspected and the results were only accepted when the molecular mass and pI of the identified protein were coincident with the electrophoretic mobility of the corresponding spot in two dimensions.

RNA isolation and quantitative RT (reverse transcription)–PCR

RNA was purified using TRIzol® reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA (5 ng) was reverse-transcribed and the cDNA products amplified by QPCR (quantitative PCR) using the GeneAmp Fast PCR Master Mix (Applied Biosystems). All reactions were carried out in triplicate. Quantitative real-time PCR was run in the 7900HT Fast Real-time PCR System. Predeveloped Taqman primers specific for cathepsin D and 18S were purchased from Applied Biosystems. Results were normalized according to 18S quantification in the same sample reaction. Gene expression values were obtained by employing the following equation:

$$\text{Relative amount} = 2^{-\Delta Ct}$$

where ΔC_t stands for the difference between C_t values from the gene of interest and 18S.

Enzymatic assay of cathepsin D

Cathepsin D protease activity was measured in the samples using the cathepsin D assay kit from Sigma following the method of Yasuda et al. [26]. Briefly, 5 μl of tissue homogenates (1 mg of protein/ml) from lactating rats and mice or human cathepsin D (2.4 μg/ml; Sigma) was added to the assay buffer (pH 3.5), followed by the addition of the quenched fluorimetric substrate 7-methoxycoumarin-4-acetic acid at a final concentration of 20 μM. The fluorescence released by the action of the enzyme was measured at 37 °C in a fluorimetric plate reader during 60 min of incubation. To prove that the substrate was hydrolysed by cathepsin D only, the specific inhibitor pepstatin A from Sigma was added to parallel samples at a final concentration of 0.2 mg/ml to inhibit the cathepsin D activity (results not shown). Samples were assayed at least three times in triplicate. Results are given as means ± S.E.M.

In vitro protein nitration

$ONOO^-$ was purchased from Calbiochem, suspended in 1.2 M NaOH and stored in an oxygen-free atmosphere at −80 °C until use. Prior to experimentation, the concentration of $ONOO^-$ was measured by the increase in molar absorption coefficient (ε) at 302 nm (ε_{302} 1.670 $M^{-1} \cdot cm^{-1}$) in 1.2 M NaOH [27]. A 20 μg portion of recombinant cathepsin D from human liver (Sigma) or 50 μg of protein from control lactating mammary gland homogenates were reacted with 0–500 μM $ONOO^-$ in the assay buffer (pH 3.5; from the cathepsin D assay kit) in the presence or absence of 30 μM (−) epicatechin (Sigma) in a total volume of 50 μl. The reaction was performed by placing a small aliquot of $ONOO^-$ into a test tube containing the sample, immediately followed by a 20 min incubation. In control test tubes, the samples were treated with equal volumes of 1.2 M NaOH (vehicle). Aliquots were taken for Western blot analysis and determination of enzymatic activity.

Statistics

Results are reported as means ± S.E.M. In Figures 4(A) and 5(A), a Student's *t* test with the Bonferroni correction was used for comparison of two groups. This analysis was also used in

Figures 3(A) and 3(B) to compare WT and KO mice at the same points of weaning. A difference was considered to be statistically significant when $P < 0.05$. For the rest of the Figures, statistical significance was evaluated using one-way ANOVA. The homogeneity of the variances was analysed by the Levene test; in those cases where the variances were unequal, the data were adequately transformed before ANOVA. The null hypothesis was accepted for all of 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 a Tukey's test at $P < 0.05$.

RESULTS AND DISCUSSION

Nitroproteomic analysis of lactating and weaned mammary gland from rats

In our previous work, using chromatin immunoprecipitation assays, it was demonstrated that NF-κB binding to the iNOS promoter at the early onset of events was triggered during weaning, resulting in the current transcription of the gene. This induction resulted in overproduction of nitric oxide during weaning, which led to an increase in the protein nitration pattern; however, this post-translational modification was limited to a few specific proteins [6].

The aim of the present study was to elucidate the functional consequences of protein nitration in our physiological model. Previous reports indicate that the nitration of protein tyrosine residues can alter protein function, either decreasing [12,13] or increasing [14–16] the catalytic activity. To identify the nitrated proteins in the weaned mammary gland, proteomic studies were performed. Figure 1 shows representative silver-stained gels (Figure 1A) and nitrated proteins (Figure 1B) of whole tissue extracts from lactating and 72 h weaned mammary glands. As seen in Figure 1(B), protein nitration is increased during weaning. Nitrated proteins were identified using a combination of procedures that included two-dimensional PAGE immunoblotting with specific antibodies against nitrotyrosine residues and MS of proteins recovered from two-dimensional gels. A total of 20 proteins in the molecular mass range of 15–75 kDa and with a pI between 5 and 8 were identified by two-dimensional IEF and MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS). Since milk stasis plays an important role in the process of involution of the mammary gland after weaning, we were interested in the complete protein profile after weaning and therefore we did not use milk-depleted mammary tissue for our proteomic analysis. As a consequence, we were unable to detect low abundance proteins, and therefore we cannot rule out the possibility that more proteins could be nitrated after weaning. The most general conclusion from the analysis of the protein map shown is a relative abundance of albumin, immunoglobulin chains and casein fragments (results not shown), which is not surprising, due to milk stasis in the weaned mammary gland. Nevertheless, it is possible that these fragments of nitrated albumin could play a more important role than one might think at first. Indeed, it has been described that the injection of small peptides derived from milk into the mammary gland accelerates the process of involution [28]. However, a 48 kDa nitrated protein was identified, with coverage of 2.5 %, as cathepsin D in mammary gland 72 h after weaning.

Immunoprecipitation with anti-(cathepsin D) antibody, followed by immunoblotting against nitrotyrosine residues, further confirmed the nitration of this enzyme after 72 h weaning. Two different forms of cathepsin D immunoprecipitated from the lactating mammary gland homogenates were nitrated. One is the mature single-chain cathepsin D above the 43 kDa molecular mass markers. The other corresponds to the heavy chain (34 kDa) from the mature double-chain form (Figure 1C, left-hand panel). Since it has been described that the mature single chain accumulates in endosomes, whereas the mature double chain is mainly found in lysosomes [21], the results presented here suggest that nitration takes place along the endosomal–lysosomal compartment. In the right-hand panel, equal loading of both samples was assessed by loading on to the gel 1 % of control or weaned mammary gland homogenate (input). Besides, to corroborate that cathepsin D was immunoprecipitated, Western blotting against cathepsin D was performed on the immunoprecipitate from 72 h weaned mammary gland (Figure 1C, right-hand panel).

Since the relevance of cathepsin D nitration was critical to our findings, we wanted to ensure that this post-translational modification took place in our experimental model. Thus another approach to purify cathepsin D from our samples was used. Pepstatin A is an inhibitor of cathepsin D that fits into the active site of the enzyme; we used pepstatin A-coated agarose beads to efficiently precipitate cathepsin D from weaned mammary gland homogenates. Those beads were then incubated with 72 h weaned mammary gland homogenates, extensively washed and the bound fractions were analysed by Western blotting, as described in the Experimental section. The detection of cathepsin D is shown in Figure 1(D) and the same membrane was reprobed with anti-nitrotyrosine antibody, which also immunoreacted with the same bands. This fact further demonstrated that cathepsin D becomes nitrated during weaning.

Effect of weaning on the expression and activity of cathepsin D in rat lactating mammary gland

Since cathepsin D proteolytic activity is generally involved in the apoptotic process, and forced weaning is known to induce apoptosis in lactating mammary tissue [2], cathepsin D mRNA and protein levels were studied at different times of weaning. Western blot analysis of lactating and weaned mammary gland lysates revealed two major cathepsin D-related bands that correspond to the enzymatically inactive precursor of $\sim$52 kDa and the mature single-chain form of $\sim$48 kDa. Figure 2(A) shows that the amount of the latter (mature single chain) is increased after 8 h of pup removal and remains elevated 3 days later, being barely detectable thereafter. This increase in protein levels correlates with the accumulation of cathepsin D mRNA, measured by real-time RT–PCR, which is maximal at 8 and 24 h after weaning (Figure 2B).

An enhancement of enzyme activity was expected due to the fact that the amount of mature single-chain cathepsin D increased with weaning (Figure 2A). However, when measuring cathepsin D proteolytic activity, it is noteworthy that it was not increased until 48 h after pup removal (Figure 2C). Although previous authors have reported a slight decrease in the proteolytic activity of this enzyme in rats weaned for 24 h [29], this was followed by an additional increase with maximal activity 4 days later [30]. This delay in the activation of the enzyme when compared with its protein levels suggests that a post-translational modification of cathepsin D might be regulating its activity in our experimental model. In fact, we have described the physiological nitration of this proteinase during weaning (Figure 1C), and we hypothesize that this nitration could be involved in the modulation of cathepsin D activity.

Evaluation of the expression and activity of cathepsin D in iNOS$^{-/-}$ and WT mice

In order to study the possible role of nitric oxide in the regulation of cathepsin D activity, studies were performed using KO mice for

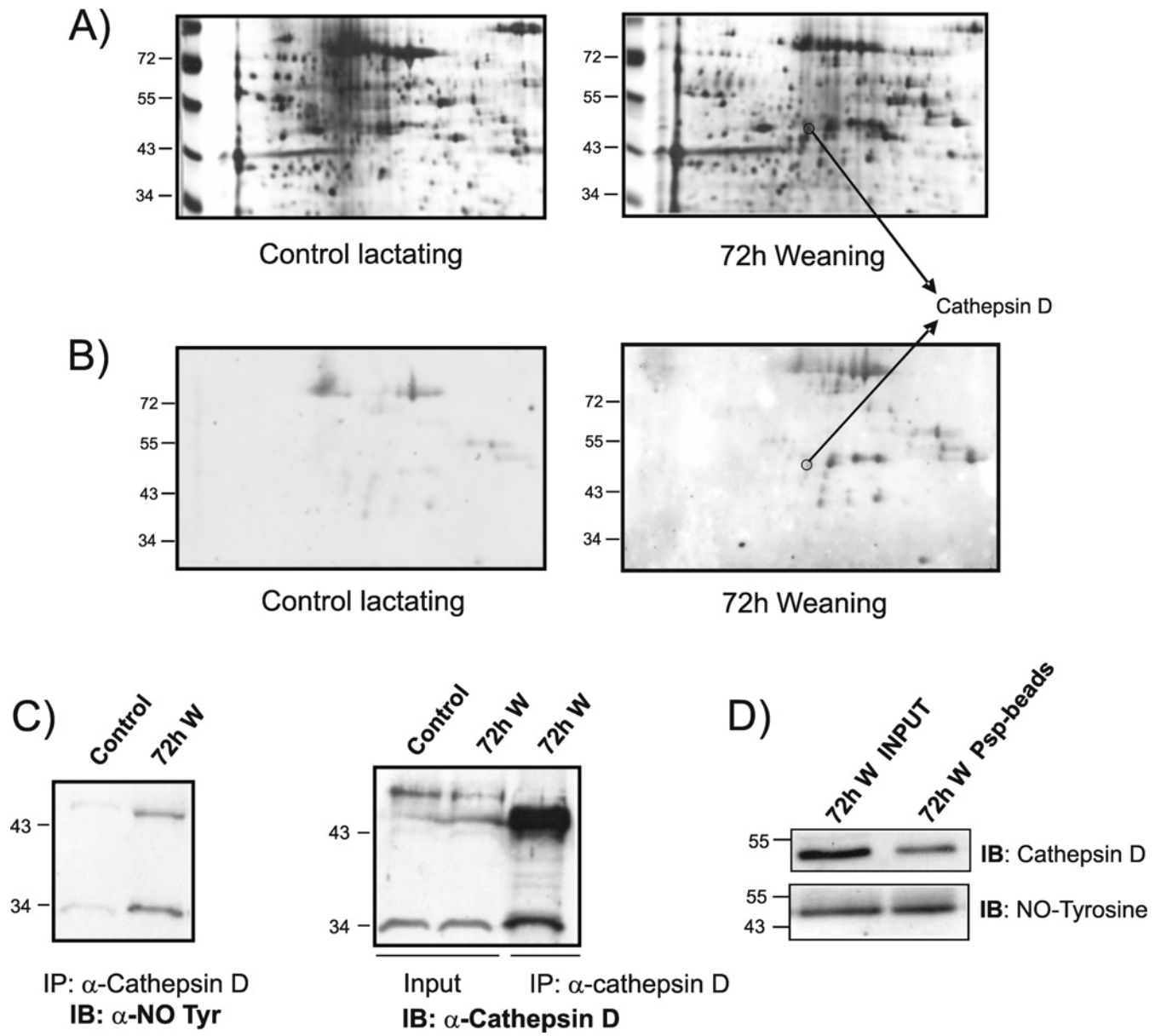

Figure 1 Cathepsin D nitration in mammary glands from weaned rats

Protein expression profiles of control lactating and 72 h weaned mammary glands separated by IEF two-dimensional PAGE and silver stained (**A**) or staining of nitrated proteins by immunoblotting with anti-nitrotyrosine antibody (**B**). The pH increases from left to right on the abscissa and the molecular mass decreases from top to bottom on the ordinate. Molecular mass markers (kDa) are on the left of the gel. (**C**) Tissue homogenates from control lactating or 72 h weaned ('72 h W') mammary glands were immunoprecipitated (IP) by anti-(cathepsin D) antibody (Santa Cruz Biotechnology), and the precipitates were then immunoblotted (IB) with anti-nitrotyrosine antibody (Upstate Biotechnology) or anti-(cathepsin D) antibody (Calbiochem). The position of the molecular mass markers (kDa) is shown on the left. (**D**) Mammary gland homogenates from 72 h weaned rats ('72 h W') were incubated with pepstatin A-coated agarose beads. After elution, the bound fraction was analysed by Western blotting, including an equal amount of starting homogenate (named Input). The membrane was immunoblotted (IB) with anti-(cathepsin D) antibody (Calbiochem) and then stripped and reprobed with anti-nitrotyrosine to verify protein nitration.

the iNOS gene. Those mice have an apparently normal lactation cycle, and no clear differences were seen between iNOS$^{-/-}$ and WT mice with respect to milk production and histological structures of the mammary gland (results not shown). As seen in Figure 3(A), the activity of cathepsin D in weaned glands of WT and KO mice was increased after 2 and 3 days post-weaning. Interestingly, when measuring cathepsin D activity, we found that mice deficient in iNOS showed decreased protease activity throughout involution compared with WT animals (Figure 3A).

Since nitric oxide could be involved in the regulation of cathepsin D expression, we analysed gene expression levels of this enzyme by real-time PCR. However, when analysing cathepsin D mRNA expression, no statistical differences were found between WT and iNOS$^{-/-}$ mice at each experimental point studied (Figure 3B). Furthermore, the increased pattern of expression seen in rats (Figure 2B) was similar to that observed in both types of mice, in which cathepsin D expression was increased at 1, 2 and 3 days post-weaning (Figure 3B).

On the other hand, nitric oxide could also be involved in protein stability or translational regulation; therefore cathepsin D protein levels were studied in WT and iNOS$^{-/-}$ mice. Western blot analysis demonstrated that there was no change in the protein

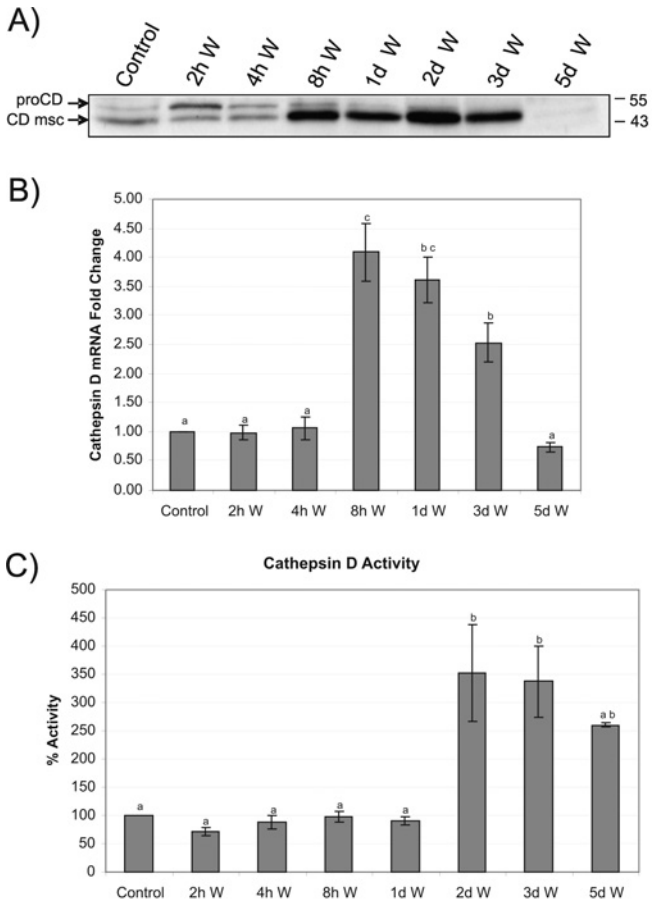

Figure 2 Characterization of mammary gland cathepsin D from control lactating and weaned rats

(**A**) Proteins from mammary gland homogenates were analysed by SDS/PAGE and treated for immunoblotting as described in the Experimental section. Two immunoreactive bands, corresponding to pro-cathepsin D (proCD) and mature single-chain cathepsin D (CD msc) forms of cathepsin D were visualized in control and weaned rats using the anti-(cathepsin D) antibody from Santa Cruz Biotechnology. The position of the molecular mass markers (kDa) is shown on the right. (**B**) Cathepsin D mRNA expression in rat mammary tissue at the peak of lactation (control) and at different times of weaning, measured by real-time RT–PCR. 18S was used as internal control (see the Experimental section). Results are means ± S.E.M. for three independent experiments. (**C**) Cathepsin D activity in mammary gland from control lactating and weaned rats by measurement of the hydrolysis of the fluorigenic substrate 7-methoxycoumarin-4-acetic acid as described in the Experimental section ($n = 4$ per group). In (**B** and **C**), ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $P < 0.05$; the letter 'a' always represents the lowest value within the group. W, weaning.

levels of cathepsin D in KO compared with WT mice at the same experimental time points. In both types of mice, the protein levels increased during weaning in a time-dependent manner. This increase was maximal for the pro-cathepsin D (~52 kDa) 2 days after weaning. The mature single chain (Figure 3C, CD msc) was cleaved yielding the mature double-chain form of cathepsin D (heavy and light chains were detected by the antibody, giving immunoreactive bands of 30 and 14 kDa respectively). In conclusion, the results obtained with iNOS$^{-/-}$ mice emphasize the importance of nitric oxide in cathepsin D activity regulation.

Effect of *in vitro* ONOO$^-$-induced nitration on cathepsin D activity: studies in rat mammary gland homogenates and in human recombinant cathepsin D

Mammary gland homogenates from control lactating rats were incubated with 0–500 μM ONOO$^-$. Cathepsin D activity was 50 % higher at 20 μM ONOO$^-$, compared with the control, but this increase was abolished in the presence of (−) epicatechin (Figure 4A). The flavonoid epicatechin has been shown to interfere with protein tyrosine nitration by intercepting the tyrosyl radical [31]. However, at concentrations higher than 50 μM ONOO$^-$, there was a linear decrease in activity (results not shown). This observation can be attributed to the simultaneous oxidative effects of ONOO$^-$ on a variety of amino acid residues in addition to nitration of aromatic amino acids [32]. Since we wanted to study nitration within a physiological range, further characterization of the effects of high concentrations of ONOO$^-$ was not pursued.

Western blotting with anti-nitrotyrosine antibodies showed that protein nitration was enhanced after the incubation with 20 μM ONOO$^-$ and was undetectable either in control lactating rats treated with vehicle or with ONOO$^-$ and epicatechin, a nitration inhibitor (Figure 4B). In order to assess whether cathepsin D was nitrated after the incubation with 20 μM ONOO$^-$, immunoprecipitation of control and nitrated samples with anti-(cathepsin D) antibody was performed (Figure 4C). Cathepsin D was immunoprecipitated from tissue lysates incubated with vehicle or ONOO$^-$, and analysed for tyrosine nitration by Western blotting. The results showed that nitrated cathepsin D was increased after ONOO$^-$-induced nitration (Figure 4C, right-hand panel).

Although we have described for the first time cathepsin D nitration and this post-translational modification seems to correlate with increased catalytic activity, other proteins, also affected by nitric oxide, could be modulating cathepsin D activity indirectly. To gain a better understanding of the mechanisms by which protein nitration might affect cathepsin D activity, *in vitro* experiments with recombinant cathepsin D from human liver were performed. As seen in Figure 5(A), the presence of ONOO$^-$ produced an increase in human cathepsin D activity. Epicatechin (30 μM) completely abolished tyrosine nitration of recombinant human cathepsin D by 20 μM ONOO$^-$ (Figure 5B). Moreover, epicatechin completely prevented the induction of cathepsin D activity by ONOO$^-$ (Figure 5A), corroborating that this enzymatic activity is specifically modulated by tyrosine nitration.

The next question raised by the present study is how nitration of this amino acid can affect cathepsin D activity. As already mentioned, immature cathepsin D can be partially autoactivated, leading to the mature form of the enzyme; therefore a specific nitration could be somehow accelerating or interfering with the autoprocessing of the immature form of cathepsin D. However, our experiments with WT and KO animals show the same pattern of proteolytic cathepsin D cleavage (Figure 4C). Moreover, it has been shown that pro-cathepsin D requires lysosomal cysteine proteinases for the final processing of its pro part and we have shown that recombinant human cathepsin D undergoes *in vitro* nitration and activation in a cysteine protease-free buffer. Therefore we can exclude the autoprocessing as the mechanism for the observed nitration-induced activity of cathepsin D.

These results suggest a conserved nitration domain among species, since both rat and human cathepsin D can be nitrated *in vitro* by ONOO$^-$ (Figures 4C and 5B). Moreover, the fact that this nitration induced an increase in enzyme activity compared with non-nitrated cathepsin D suggests that nitric oxide can modulate cathepsin D activity in a direct manner (Figures 5A). One could argue that the protein context surrounding cathepsin D or the conformation of cathepsin D *in vivo* could be other than that favouring the nitration. Nevertheless, we have shown that cathepsin D from extracts of control lactating mammary gland can also be nitrated *in vitro* (Figure 4), as demonstrated for the recombinant protein, and that this nitration induces an increase in the enzymatic activity (Figure 4A). On the whole, our experiments

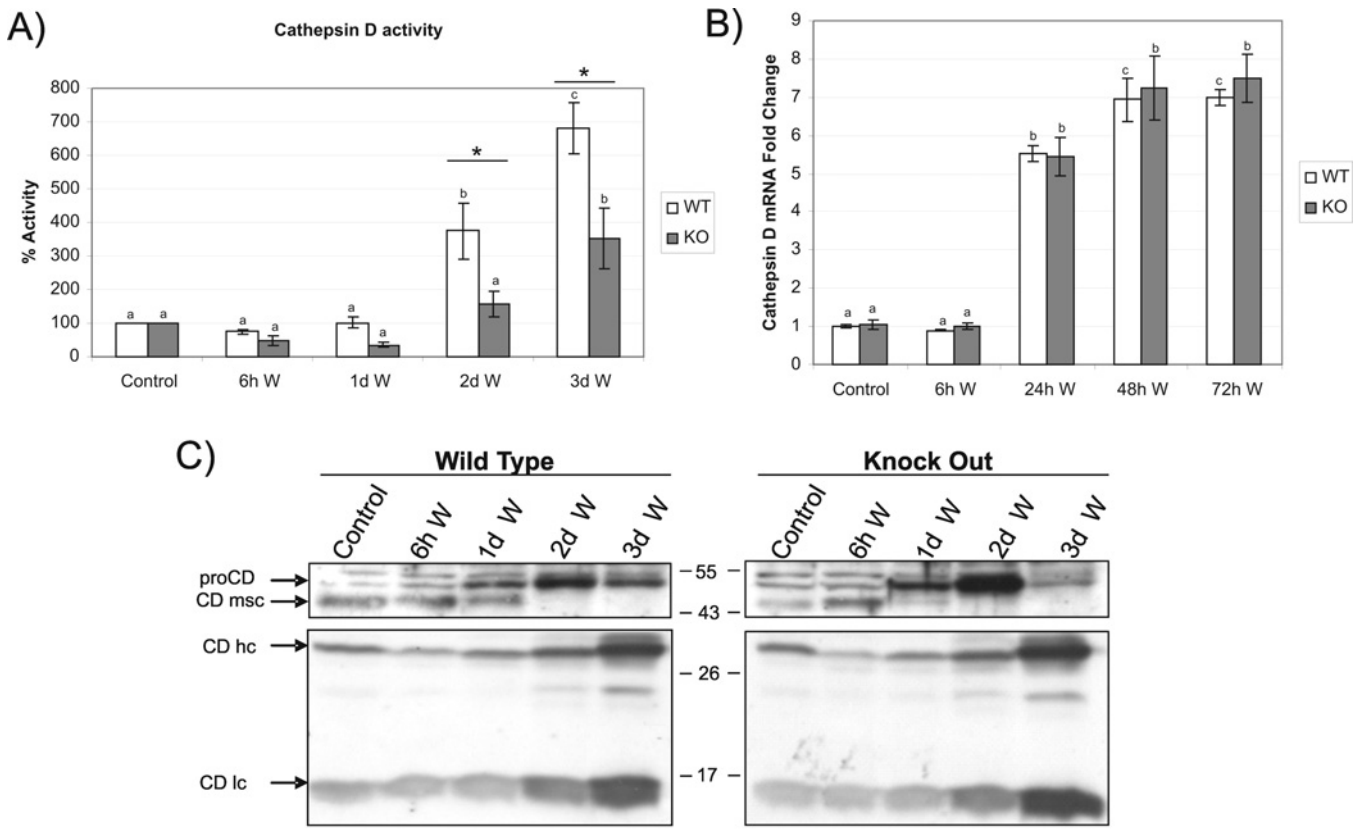

Figure 3 Expression of cathepsin D and proteolytic activity in iNOS$^{-/-}$ mice

(**A**) Lactating and weaned mammary glands were collected from WT and inducible iNOS-null mice (KO) and analysed for cathepsin D activity ($n = 4$ per group). (**B**) Gene expression of cathepsin D from WT and iNOS$^{-/-}$ mice during lactation and weaning ($n = 4$ per group). In (**A** and **B**), to compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $P < 0.05$; the letter 'a' always represents the lowest value within the group. A Student's *t* test with the Bonferroni correction was used for comparison between WT and KO at each experimental time point; $^*P < 0.05$. (**C**) Western blot analysis of cathepsin D at the peak of lactation (control) and during mammary gland involution (weaning) in WT and iNOS$^{-/-}$ mice. The blot shows the presence of precursor (proCD) and mature single-chain (CD msc) forms of cathepsin D recognized with the anti-(cathepsin D) antibody from Santa Cruz Biotechnology. Since this antibody does not immunoreact with the mature double chain, the two degradation products of ~30 kDa (CD heavy chain) and ~14 kDa (CD light chain) are recognized with the anti-(cathepsin D) antibody from R&D Systems. The blot shows a representative example from one individual mouse from each group. The molecular forms of cathepsin D are indicated on the left and the molecular mass markers (kDa) in the middle of both blots. W, weaning.

show that a low degree of nitration increases cathepsin D catalytic activity, not only in recombinant human cathepsin D, but also in tissue homogenates, suggesting that direct nitration of this enzyme is responsible for the enhanced activity observed.

Identification of the nitration site

It has been shown that tyrosine nitration can dramatically induce profound changes in protein structure and function due to a shift in the p*K*a of the tyrosine hydroxy group [33]. In order to identify the tyrosine residue targeted by $ONOO^-$, tryptic digests of nitrated and non-nitrated human recombinant cathepsin D were characterized by MS. The doubly charged ion with *m/z* 823.92 was assigned as the nitrated form of peptide L^{159}VDQNIFSFYLSR^{171}. All y ions from y4 showed the extra 45 Da, suggesting the nitration of Tyr^{168}. In contrast, no nitration was detected when tryptic digests from control cathepsin D were analysed. In particular, a doubly charged ion with *m/z* 801.42 was assigned as the non-nitrated L^{159}VDQNIFSFYLSR^{171} peptide (Figure 6).

Nevertheless, although we could sequence most of the tyrosine residues present in the protein (13 tyrosine residues out of 19), we were unable to sequence more than 40 % of human cathepsin D and therefore we cannot rule out the possibility that other tyrosine residues could also be nitrated. As shown in Figure 7, Tyr^{168} is found in the heavy chain of cathepsin D and is highly conserved among species and other proteases, such as human renin, porcine pepsin and bovine chymosin. In fact, detailed protein sequence analysis reveals a possible nitration domain with the sequence F-S-F/V-**Y**-X-X-R/S. Further studies would be needed in order to determine whether other proteinases with this conserved domain can also undertake an *in vivo* and *in vitro* nitration.

The important role played by pH in the modulation of cathepsin D activity has been extensively documented [22,34,35]. Indeed, while an acidic pH leads to a fully active enzyme, at pH 7.5 a molecular bond between specific tyrosine residues and the catalytic aspartate residues stabilizes the protein structure in an inactive conformation [36]. Although Tyr^{168} is 17.76 Å (1 Å = 0.1 nm) away from the active site and such a distance is not compatible with a direct hydrogen bond with the catalytic residues, it would be conceivable that the nitration of Tyr^{168} could favour an irreversible active conformation of the enzyme by blocking a molecular bond crucial for cathepsin D folding. Moreover, an inhibitory domain has been localized at the N-terminus of cathepsin D [36]; at pH 7.5 the mature N-terminus is repositioned into the active site cleft, interacting with the catalytic residues. Since we cannot exclude the possibility of nitration in

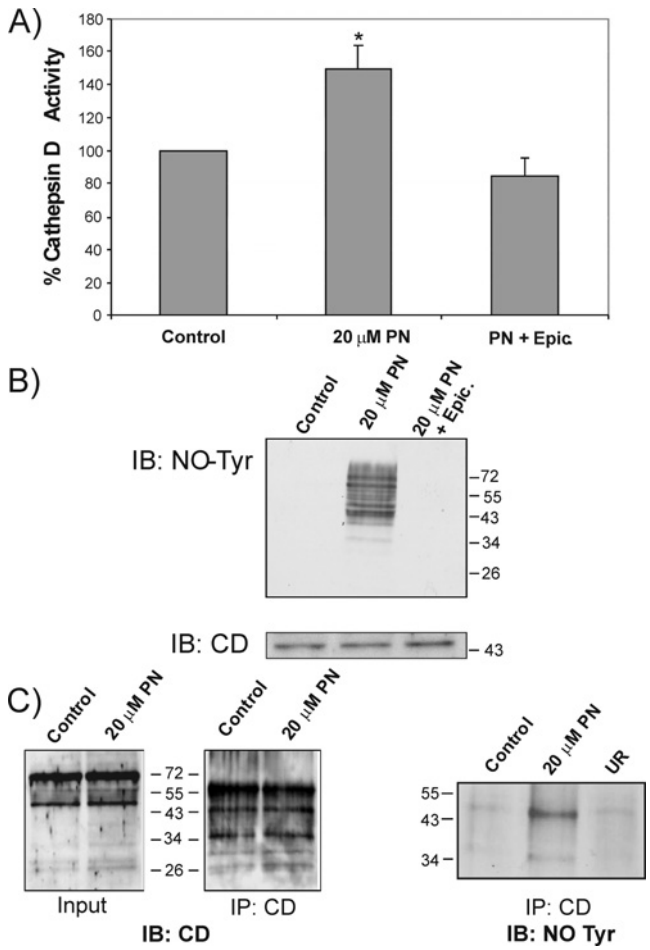

Figure 4 ***In vitro*** **nitration of lactating mammary gland homogenates**

Lactating mammary gland lysates were incubated with 0 or 20 μM $ONOO^-$ (PN) in the presence or absence of (−) epicatechin. (**A**) The graph shows the increase in cathepsin D activity in the nitrated sample (20 μM PN) when compared with control (treated with vehicle) or lactating mammary gland homogenate treated simultaneously with PN and epicatechin (PN + Epic.). The increase in cathepsin D activity induced by PN was statistically significant. $^*P < 0.05$. (**B**) A representative Western blot against tyrosine-nitrated residues or cathepsin D (with the Santa Cruz Biotechnology antibody) in samples from mammary tissue after treatment with $ONOO^-$ is shown. (**C**) Tissue homogenates from control lactating mammary glands treated with vehicle (control) or with $ONOO^-$ (20 μM PN) were immunoprecipitated (IP) by anti-(cathepsin D) antibody (Santa Cruz Biotechnology), and the precipitates were then immunoblotted (IB) with anti-nitrotyrosine antibody (right-hand panel) or anti-(cathepsin D) antibody from Calbiochem (left-hand panel). Molecular mass markers (kDa) are indicated on each blot. CD, cathepsin D; NO Tyr, anti-nitrotyrosine antibody, UR, unrelated.

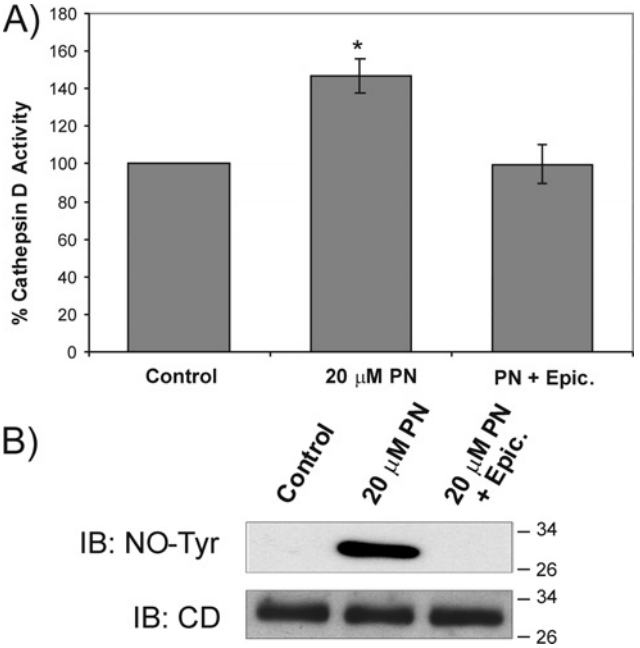

Figure 5 **$ONOO^-$-induced activation of cathepsin D**

Commercial human cathepsin D reacted with 0 or 20 μM $ONOO^-$ (PN) in the presence or absence of (−) epicatechin (Epic.). This recombinant cathepsin D from human liver corresponds mainly to the heavy chain of the mature cathepsin D form (34 kDa). Samples were analysed for tyrosine nitration or cathepsin D activity. (**A**) Effect of 20 μM $ONOO^-$ on human cathepsin D activity. A significant increase ($^*P < 0.05$) is observed after the addition of PN. (**B**) Cathepsin D samples from human liver, treated with vehicle (control), $ONOO^-$ (PN) and $ONOO^-$ and epicatechin (PN + Epic.), were analysed for tyrosine-nitrated and overall cathepsin D [using the mouse monoclonal anti-(human cathepsin D) antibody (CD) from Calbiochem] by Western blotting. Immunoblots (IB) are representative of three separate experiments and the position of the molecular mass markers (kDa) is shown on the right. NO-Tyr, anti-nitrotyrosine antibody.

other non-identified tyrosine residues, the *in vivo* nitration of a specific tyrosine at this site could block the binding of the inhibitory propeptide and allow free access of the substrate.

Physiological and medical implications

All in all, in the future it would be very interesting to further investigate the mechanisms of modulation of cathepsin D activity induced by both nitration and nitrosylation, as well as the possibility of finding more tyrosine and/or cysteine residues modified by nitric oxide. The relevance of these findings is granted by the fact that cathepsin D is known to be involved in pathological processes, such as inflammation, tumour progression and metastasis. Indeed, cathepsin D is an independent marker of poor prognosis in human breast cancer [37,38]. Recently published studies have demonstrated a direct relationship between thrombin and cathepsin D up-regulation that would enhance angiogenesis, tumour growth and metastasis [39]. Regarding cathepsin D activity, it is 8–16 times higher in the cytosol of mammary cancer cells than in normal cells [40]. A direct relationship between cathepsin D activity and the metastatic potential of tumours has been demonstrated [41], although a mutated cathepsin D devoid of its catalytic activity also stimulates the growth of cancer cells, suggesting a novel mechanism by which cathepsin D could act as a mitogen in tumour progression [42]. Nevertheless, little is known about the molecular mechanisms involved in the modulation of cathepsin D activity in all of these processes, or how tyrosine nitration could affect protein function.

In summary, mammary gland involution is physiological, orderly and developmentally programmed. Thus the involuting mammary gland is an ideal physiological model to search for genes involved in growth arrest, differentiation and apoptosis. These processes are known to play an important role in cancer and other pathologies; therefore those genes relevant to involution in the normal mammary gland may be highly relevant in breast cancer. A pilot study using cDNA microarrays has demonstrated that through the analysis of differentially expressed genes during early mammary gland involution, it may be possible to identify 'novel' genes relevant to human breast cancer [43]. This can also be applied to cathepsin D; the activation of this protein plays a key role in mammary gland involution, but more importantly, the involuting mammary gland offers an excellent experimental

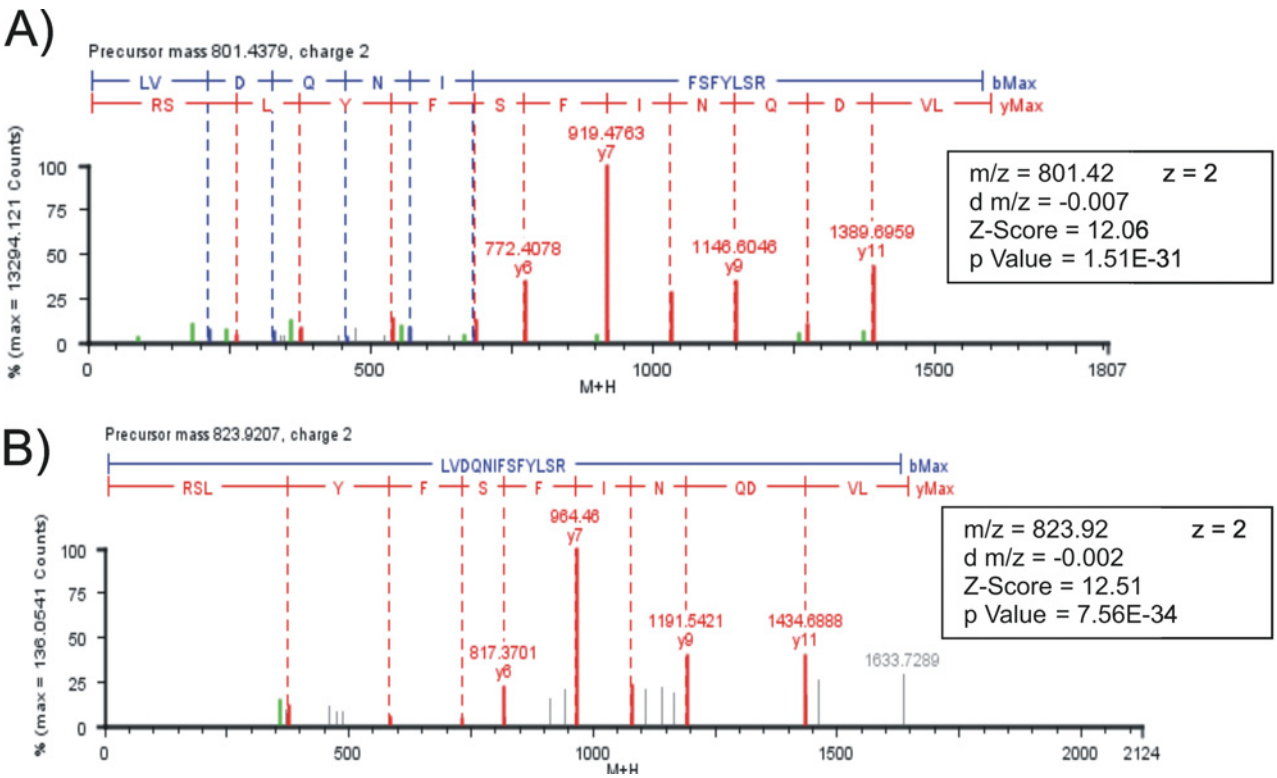

Figure 6 Identification of the modified amino acid by MS

Representative MS/MS spectra of tryptic digests from human cathepsin D treated with (**A**) vehicle or (**B**) $ONOO^-$ are shown. The mass/charge value of 823.92 for the peptide L^{159}VDQNIFSFYLSR^{171} from cathepsin D treated with $ONOO^-$ contains a +45 Da adduct when compared with the vehicle-treated enzyme (*m/z* 801.42), suggesting the nitration of Tyr^{168}.

132 DGILGMAYPRISVNNVLPVFDNLMQQKLVDQNIFSFYLSRDPDAQPGGELMLGGT 186 CD human
194 DGILGMGYPHISVNNVLPVFDNLMQQKLVDKNIFSFYLNRDPEGQPGGELMLGGT 248 CD mouse
191 DGILGMGYPFISVNKVLPVFDNLMKQKLVEKNIFSFYLNRDPTGQPGGELMLGGT 245 CD rat

194 DGILGLGYPSLAVGGVTPVFDNMMAQNLVDLPMFSVYMSSNPEGGAGSELIFGGY 250 CE human
181 DGILGLGYPSLAAGGVTPVFDNMMAQNLVALPMFSVYLSSDPQGGSGSELTFGGY 237 CE mouse
182 DGILGLGYPSLAVGGVTPVFDNMMAQNLVALPMFSVYLSSDPQGGSGSELTFGGY 238 CE rat

187 EFDGVVGMGFIEQAIGRVTPIFDNIISQGVLKEDVFSFYYNRDSENSQSLGGQIVLG 245 REN human

176 DGILGMAYPSLASEYSIPVFDNMMNRHLVAQDLFSVYMDRNGQESMLTLGAIDPSY 233 CHY bovine

177 DGILGLAYPSISASGATPVFDNLWDQGLVSQDLFSVYLSSNDDSGSVVLLGGIDSSY 233 PEP porcine

Figure 7 Sequence alignments for the mammalian aspartic proteases: cathepsin D, cathepsin E, renin, pepsin and chymosin

The possible nitration domain, which is highly conserved among different proteinases, is shown in boldface in grey. Abbreviations: CD, cathepsin D; CE, cathepsin E; REN, renin; CHY, chymosin; PEP, pepsin. Sequence sources: cathepsin D: Swiss-Prot entries CATD_HUMAN, MOUSE and RAT; cathepsin E: Swiss-Prot entries CATE_HUMAN, MOUSE and RAT; renin: Swiss-Prot entry RENI_HUMAN; chymosin: Swiss-Prot entry CHYM_BOVIN; and pepsin: Swiss-Prot entry PEPA_PIG.

model for studying the mechanisms of cathepsin D activation that could also apply for breast cancer development.

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