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Lipopolysaccharide mediated regulation of neuroendocrine associated proprotein convertases and neuropeptide precursor processing in the rat spleen

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Abstract

Within the secretory pathway, the family of proprotein convertases cleave inactive precursors at paired basic residues to generate a myriad of biologically active peptides. Within the PC family, PC1/3 and PC2 are well known for their preferential expression within neuroendocrine cells. However, various data now indicate their potential expression in immune cells. The aim of our study was two fold: (1) survey PC expression in immune tissues, with emphasis on PC1/3 and PC2 and (2) examine PC expression under conditions that mimic an infectious state using lipopolysaccharide, known to activate immune cells via toll-like receptors. Spatial and temporal analyses of tissues from control and lipopolysaccharide treated rats were carried out using in situ hybridization histochemistry, Northern blot, mass spectrometry and antibacterial assays. Our tissue survey showed the basal expression of all PCs in the lymph nodes, thymus and spleen including PC1/3 and PC2. Focusing on the spleen, basal expression of PC1/3 was seen in the red pulp/marginal zone areas, suggesting expression within macrophages. Lipopolysaccharide treatment produced significant changes in PC1/3 expression and notably an induction in B lymphocytes within germinal centers. Similarly, PC2, which was undetectable in control spleens, was induced in germinal centers following lipopolysaccharide treatment. The PC1/3 and PC2 substrate proenkephalin was also induced following lipopolysaccharide treatment in the marginal zone, where PC1/3 expression was also found. Mass spectrometry analysis of spleen extracts demonstrated the presence of the antibacterial peptide enkelytin. Our studies confirmed that PC1/3 and PC2 expression was not restricted to neurons and endocrine cells, but was also found under basal conditions in both macrophage and lymphocytes. Additionally, plasticity of PC expression in immune cells was observed under conditions that mimic pathogen-like infections, suggesting a mechanistic link through Toll-like receptors. Collectively, these data clearly implicate PCs in immune responses, both innate and acquired.
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Abbreviations: PC, proprotein convertase; POMC, proopiomelanocortin; PENK, proenkephalin; LPS, lipopolysaccharide; PAMPs, pathogen-associated molecular patterns; TLR, toll-like receptor; BSA, bovine serum albumin; DIA, dot immunobinding assay; ACN, acetonitrile; RP-HPLC, reverse phase-high pressure liquid chormatography; TFA, trifluroracetic acid; HCCA, α-cyano-4-hydroxycinnamic acid; MALDI, matrix-assisted laser desorption/ionization; ACTH, adrenocorticotropic hormone; RP, red pulp; WP, white pulp; GC, germinal centers; PALS, periarteriolar lymphocyte sheath; MZ, marginal zone; kb, kilobase.

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1. Intoduction

Proteolytic processing is a post-translational modification by which a cell diversifies and regulates the protein products of its genes. In mammalian species, a family of enzymes known as the subtilisin-like proprotein convertases (PCs) participates in the formation of biologically active products through their endoproteolytic actions (for a review see Ref. (Bergeron et al., 2000)). Cleavage by PCs occurs at single and paired basic residues of precursor proteins and is recognized as an essential step to yield active hormones and neuropeptides. The PC family of enzymes consists of seven distinct members named, furin, PC2, PC1/3, PC4, PACE4, PC5/6 and PC7 (Bergeron et al., 2000). The distribution pattern of each PC has been extensively investigated, mainly in brain, heart, endocrine tissues and reproductive organs (Bergeron et al., 2000; Day et al., 1992, 1993; Schafer et al., 1993). Some of these enzymes, such as furin, PC5/6 and PC7 have a ubiquitous or widespread tissue distribution (Day et al., 1993; Seidah et al., 1996; Lusson et al., 1993). In contrast, PC2 and PC1/3 have an expression pattern that closely follows that of endocrine and neuronal cells (Day et al., 1992; Seidah et al., 1990). This is consistent with the notion that PC2 and PC1/3 process precursors that enter the regulated secretory pathway, mainly hormones and neuropeptides (Steiner, 1998). One insight obtained from distribution studies is that although distinct expression patterns are observed for each PC, it is clear that cells do not express only one PC at a time, but rather express a distinct cocktail of PCs (Bergeron et al., 2000; Day et al., 1992, 1993; Schafer et al., 1993).

The expression of neuropeptides was originally believed to be exclusively associated with endocrine and nervous tissues (Douglass et al., 1984). However, it is now known that expression of various neuropeptide precursors, such as proopiomelanocortin (POMC) (Oates et al., 1988; Mechanick et al., 1992), proenkephalin (PENK) (Rosen et al., 1989; Vindrola et al., 1994; Martin et al., 1987) and endomorphins (Jessop et al., 2000), known to be processed by PCs, is not limited to the neuroendocrine system, and has been demonstrated in various cells of the immune system. Mature forms of the expected neuropeptides have been isolated and characterized in immunocytes, such as peptides with immunoregulatory and signaling functions (Weigent and Blalock, 1997; Blalock, 1985; Stefano, 1996). Recent studies have shown that neuropeptide precursors can also yield peptides with antibacterial activities (Tasiemski et al., 2000, 2002; Lugardon et al., 2000; Strub et al., 1995; Goumon et al., 1996). For example, two PENK-derived peptides with antibacterial activity, enkelytin and peptide B, have been shown to be produced by the immune system after a bacterial challenge (Tasiemski et al., 2000, 2002; Goumon et al., 1998). The processing of the neuroendocrine-associated precursors such as chromogranins A and B can also result in the release of antibacterial peptides, vasostatin-1 (Lugardon et al., 2000) and secretolytin (Strub et al., 1995). Chromogranins A and B, more commonly viewed as markers for endocrine and neuronal cells, were also found to have an immune origin (Tasiemski et al., 2002). The sum of these data lead us to the hypothesis that the same processing enzymes which cleave these substrates in endocrine and neural tissues could also be expressed in immunocytes.

At present, the few studies that have investigated PCs expression in the immune system have focused on isolated cells. PC1/3 expression has been shown in a human monocyte cell line (LaMendola et al., 1997) or in differentiated macrophages (Vindrola et al., 1994; LaMendola et al., 1997) whereas PC2 was detected in polymorphonuclear leukocytes (Vindrola et al., 1994). Recently, PC1/3 and PC2 expression have been demonstrated in rat immunocytes of the blood and within inflamed tissue (Mousa et al., 2004). While these studies showed expression of PCs, they did not give much indication as to the potential roles of PCs in immune function. Little is known about PC activity in immune effectors organs, such as spleen, thymus and lymph nodes. In order to provide insights as to PCs functions in immune cells, we focused on two specific aims. First, we determined the in vivo expression pattern of PCs mRNA within tissues of the immune system under resting conditions. Secondly, we mimicked bacterial-like infection conditions by using lipopolysaccharide (LPS), a pathogen-associated molecular pattern (PAMP) ligand acting through toll-like receptor 4 (TLR4) (Pasare and Medzhitov, 2005), and examined PCs expression and PENK, as well as antibacterial activity. The data showed that PCs expression is highly regulated in immune cells following LPS challenge and demonstrated the need for precursor processing in immune defense mechanisms.

2. Materials and methods

2.1. Animal and tissue preparation

Adult male Wistar rats (250–300 g; Charles River, St Constant, Qc, Canada) were used throughout the experiments. Rats ($n=3$/experiments) were injected intraperitonially with either LPS serotype 055:B5 (Sigma, St Louis, MO) at dose of 150 μg/100 g or saline for control. Rats were killed by decapitation, tissues (mesenterial lymph nodes, lung, thymus and spleen) were rapidly removed and frozen in 2-methylbutane pre-cooled to −35 °C. All procedures were conducted in accordance with the guidelines of the Medical Research Council of Canada. The extracted tissues were stored at −80 °C until cryo-sectioning. Frozen 10 μm sections ($n=100$/tissue) were cut on a Reichert cryostat (Leica Corp., Depew, NY), thaw-mounted on polylysine-coated glass slides and stored at −80 °C until further processing.

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2.2. cRNA probes synthesis

All experiments carried out in these studies used antisense cRNA probes and sense probes for negative control. Probes were synthesized by in vitro transcription of linearized plasmid DNA and labeling was done by incorporation of radioactive [^{35}S]UTP/[^{35}S]CTP (Amersham Pharmacia Biotech, Arlington Heights, IL) (Day et al., 1992). All probes used have been described before: furin (Day et al., 1993), PC2 (Seidah et al., 1994), PC1/3 (Lanoue and Day, 2001), PACE4 (Dong et al., 1995), PC5/6 (Lusson et al., 1993), PC7 (Seidah et al., 1996) and PENK (Schafer et al., 1990).

2.3. In situ hybridization

The in situ hybridization studies were carried out using antisense and sense labeled cRNA probes with [^{35}S]UTP/[^{35}S] CTP-labeled cRNA probes. Radioactive probes were diluted to 33×10^3 dpm/μl. Following hybridization with PCs or PENK probes, the sections were submitted to standard in situ hybridization procedures (Day et al., 1992) followed by X-ray autoradiography for 3–7 days, resulting in low resolution images. These slides were also dipped in NTB2 emulsion (Eastman Kodak Co. Rochester, NY) to obtain cellular resolution. The sections hybridized were counterstained with cresyl violet, cleared in xylene, and mounted with Permount histological mounting medium (Fisher Scientific, Pittsburgh, PA).

2.4. Northern blot analysis

Total RNA was extracted from tissues using a guanidium isothiocyanate methodology, which includes a lithium chloride precipitation step (Day et al., 1992). The RNA (5 μg) was separated on an agarose gel containing 6% formaldehyde and blotted on Nytran Plus membranes (Schleicher and Schuell, Inc., Keene, NH). Prehybridization was carried out for 2 h at 65 °C in hybridization buffer containing 5% SDS, 0.4 M sodium phosphate buffer, 1 mM EDTA, 1 mg/mL BSA and 50% formamide. The purified [^{32}P] UTP-labeled cRNA probe was then added to the prehybridization buffer and incubated with the Nitran membrane overnight at 65 °C. Filters were washed in 0.1× sodium citrate, 0.1% SDS, 1 mM EDTA at 72 °C for 2 h and exposed to X-ray film (XAR-5 film with intensifying screens; Eastman Kodak Co.) at −80 °C for 48 h.

2.5. Polyclonal antisera

Polyclonal anti-enkephalins (Met–EnkRF, Met–Enk RGL, Leu–Enk) used in dot immunobinding assay (DIA) were kindly provided by Iris Lindberg (New Orleans, USA). The specificity of the antisera has been described (Lindberg and Dahl, 1981; Lindberg and White, 1986; Lindberg and Thomas, 1990).

2.6. Antibacterial assays

The antibacterial activity was tested by liquid growth inhibition assay as described by Bulet et al. (1991). Briefly, 10 μL of the interest product was incubated in microtiter plates with 100 μL of a mid-logarithmic phase culture of *Micrococcus luteus* (strain A270) or *E. coli* (strain D31) with a starting absorbance of 0.001 (1.2×10^6 cells/ml) at 620 nm in poor broth medium. Microbial growth was assessed by the increase in A620 nm after a 16 h incubation at 30 °C for *M. luteus* and 37 °C for *E. coli*.

2.7. Dot immunobinding assay (DIA)

The 40% acetonitrile (ACN) reversed-phase HPLC (RP-HPLC) eluted fractions were freeze dried before resuspending in 100 μL of water. The procedure was conducted according to Salzet et al. (1994). One microliter of the 100 μL total solution was spotted onto a nitrocellulose membrane and was incubated with the enkephalin antisera (1/1000). Bound antibodies were detected by a goat anti-rabbit IgG conjugated to horseradish peroxidase by using a chemoluminescence kit (ECL; Amersham, Saclay, France).

2.8. Purification procedure

Frozen rat spleens from control or injected animals were individually submitted to acidic extraction (acetic acid 1 N:20 mL for 5 g of tissue) before sonification and bath under gentle stirring overnight at 4 °C. Centrifugation (10,000 *g*, 30 min, 4 °C) was then used to clarify supernatants, which were loaded onto 2 g Sep-Pak C18 Vac cartridges (Waters Inc) equilibrated in water acidified with 0.05% of trifluroracetic acid (TFA). After washing with acidified water, three successive elution steps were performed with 10%, 40% and 80% ACN in acidified water. The obtained fractions were then concentrated by evaporation and reconstituted in respectively 100 μL, 500 μL and 1 mL of pure water (depending on the material quantity) for the 10%, 40% and 80% ACN fractions. Ten microliters were tested for antimicrobial activity as described above. Only 40% fractions were active and submitted to RP-HPLC. All the HPLC steps were carried out on a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector. Column effluent was monitored by UV absorption at 220 and 280 nm. Aliquots of the 40% Sep-Pak fractions were subjected to reversed phase HPLC on a Ultasphere C18 column (250 × 4.6 mm, Macherey Nagel). Elution was performed with a linear gradient of 0–60% ACN in acidified water (0.5% TFA) over 60 min at a flow rate of 0.5 mL/min. Fractions corresponding to absorbance peaks were collected in polypropylene tubes, dried, reconstituted in water and tested for antimicrobial activity and antibody recognition by DIA before analyzed in mass spectrometry.

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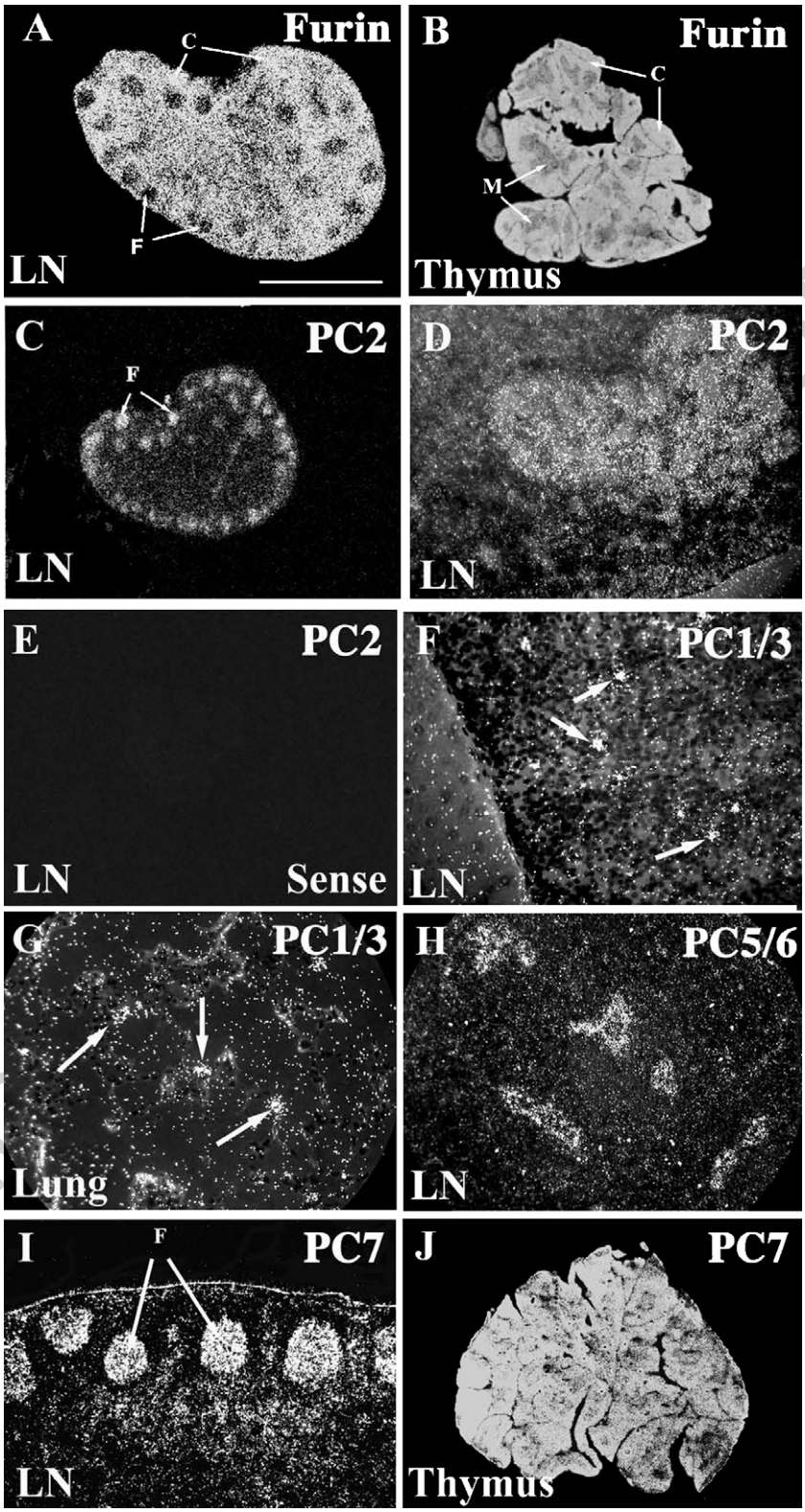

Fig. 1. Distribution of PCs in rat immune related tissues by in situ hybridization. Low power autoradiographic X-ray (A, B, C, E, I and J) and cellular resolution (D, F, G and H) images showing furin (A and B), PC2 (C, D and E), PC1/3 (F and G), PC5/6 (H) and PC7 (I and J) mRNA expression in various tissues. (D) Higher magnification of PC2 expression in the follicles of mesenterial lymph nodes. The emulsion-dipped sections of lymph nodes (F) and lung (G) revealed labeling of PC1/3 respectively in the follicles or on the walls of the alveoli (arrows). In the lymph nodes (H), PC5/6 transcripts were localized along the walls of the sinuses. (E) No positive labeling was observed in control section labeled with PC2 sense probe. LN, lymph nodes, C, cortex; F, follicles; M, medulla. Bar, 4 mm.

2.9. Characterization procedure

Samples were analyzed using α-cyano-4-hydroxycinnamic acid (HCCA) as matrix. To get a better homogeneity of the dried samples and thus a better spot to spot reproducibility during the analyses, two layers preparation was chosen for the MALDI experiments. Briefly, 1 mL of HCCA in acetone (15 mg/mL) was applied on the MALDI target. After evaporation and preparation of a thin layer of matrix, 1 mL of samples and immediately after 1 mL of classical HCCA solution (10 mg in TFA 0.1%/ACN 3 : 7, v/v) were applied on top of the first layer and allow to dry at room temperature. Analyses were carried out on a Voyager Elite (Perseptive BioSystems, Framingham, MA) MALDI-time of flight mass spectrometer equipped with a N_2 laser (2 ns pulse duration, 2 Hz repetition rate). Mass spectra were recorded in the positive linear mode using delayed extraction. Laser fluence was set at a value just above the threshold for ion production. Calibration was realized with four external calibrants covering the mass range of interest (Substance P, ACTH 18–39, ACTH 7–38 and bovine insulin) prepared in the same way as the samples.

HPLC fractions recognized by the antibodies or presenting an antibacterial activity are of special interest for this study and corresponding mass spectra were more carefully looked out for these fractions. In order to unambiguously identify the differentially expressed peptides, structural information was collected through MS^n fragmentation studies using ESI-Ion trap instrument. The experiments were performed on an Esquire 3000 instrument (Bruker Gmbh, Bremen, Germany) running in the nanospray mode. To obtain more or less charged ions, two different solvents were used for ESI. The analyte was directly mixed with an equal ratio of ACN or with an equal ratio of formic acid 1% in ACN. Three milliliters of the mixture was then loaded into the nanospray silver coated needle (Protana). To get a stable spray the capillary voltage was generally set between 750–800 V. The different parameters of the instrument were set to obtain the best intensity for the interesting ions. For MS^2 experiments, helium was the collision gas and the energy was set in a range from 0.9 V to 1.5 V depending on the mass and the charge of the ion. All the collected data were treated using the software integrated to the instrument.

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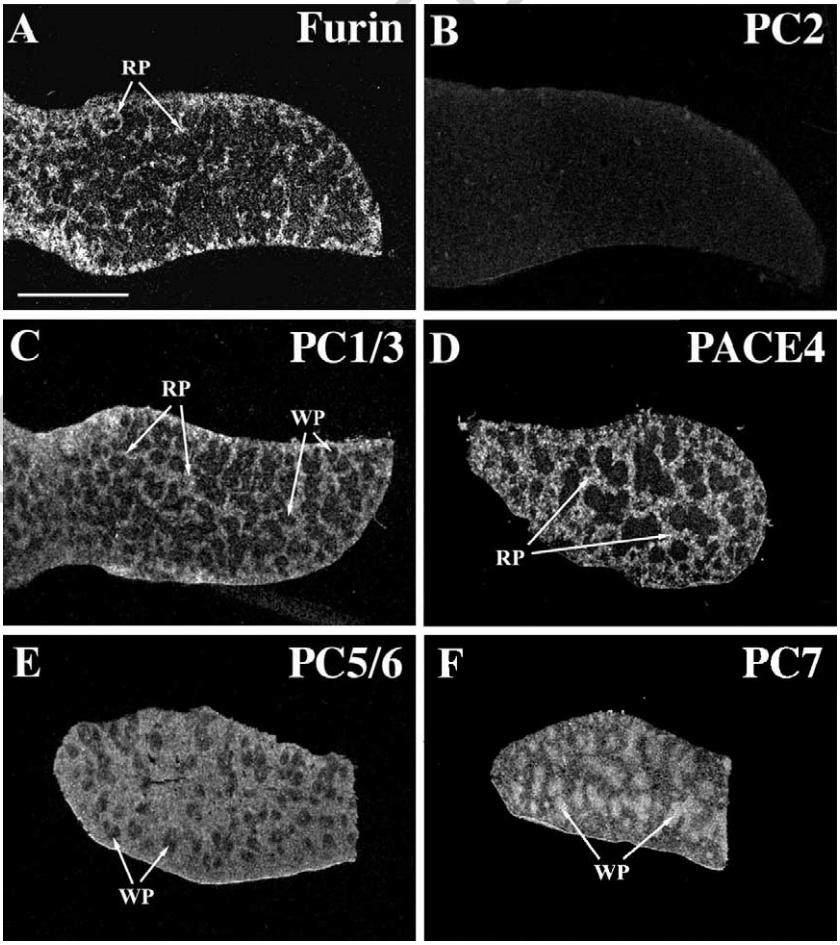

Fig. 2. Comparative distribution of PCs in rat spleen. Autoradiograms showing distribution of mRNA coding for (A) furin, (B) PC2, (C) PC1/3, (D) PACE4, (E) PC5/6 and (F) PC7 in the spleen. RP, red pulp; WP, white pulp. Bar, 4 mm.

3. Results

3.1. Basal expression of PCs in the lung, lymph nodes and thymus

We first conducted a survey of PCs mRNA expression patterns in immune related tissues using in situ hybridization. Different expression patterns were observed for each PC, except for PC4, that we could not detect any kind of expression, consistent with its preferential expression in testicular germ cells (Seidah et al., 1992). All further studies thus exclude PC4. For the other PCs, we observed a widespread and complex distribution in immune related tissues (Fig. 1). In agreement with its previously described ubiquitous distribution (Day et al., 1993), furin was highly expressed in the cortex and medulla of mesenterial lymph nodes (Fig. 1A). The cortex is characterized by a dense accumulation of lymphocytes. The medulla consists of lymphatic tissue cords interspersed with sinuses. While the expression of furin was less abundant in the follicles, it was still detectable in this zone. Numerous lymphocyte subpopulations populate the lymph nodes and furin expression

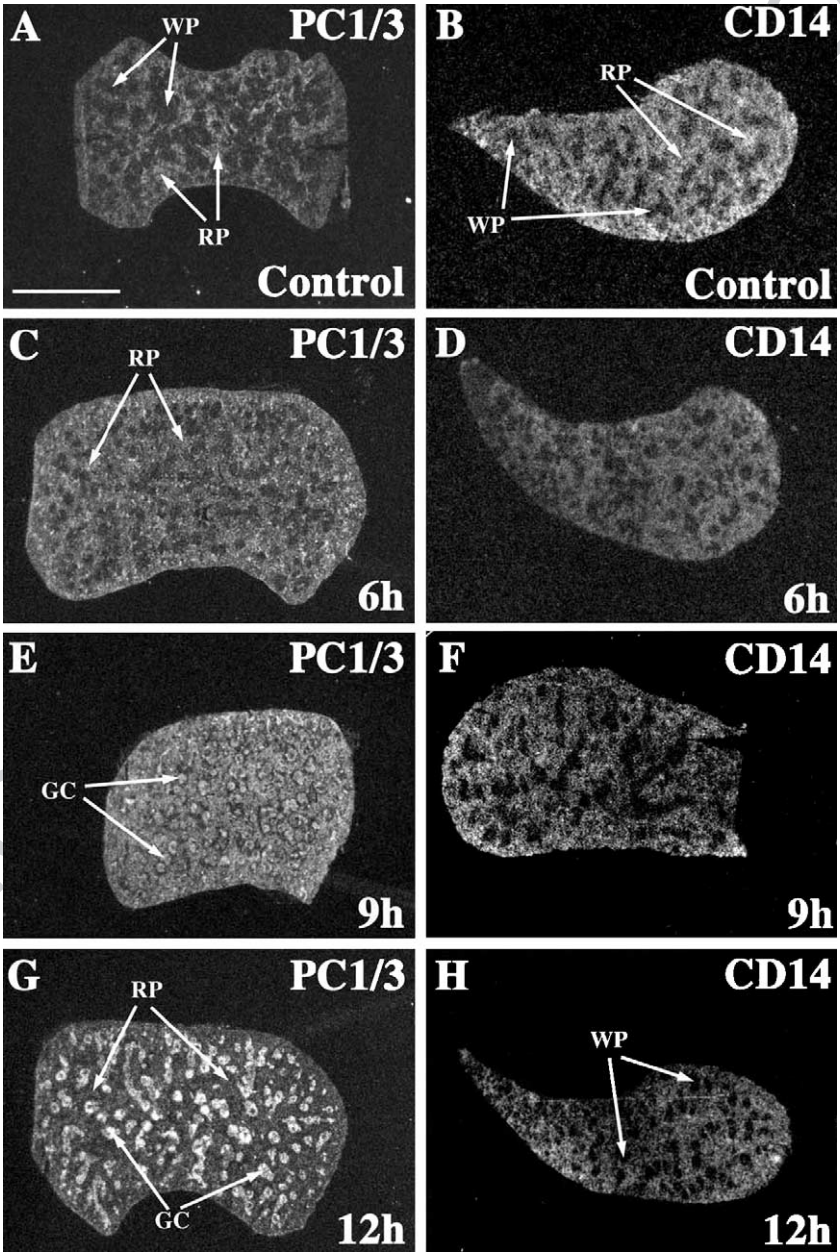

Fig. 3. Induction of PC1/3 mRNA expression by LPS in rat spleen. Autoradiograms showing distribution of mRNA coding for PC1/3(A, C, E and G) and CD14 (B, D, F and H) in the rat spleen. (A) PC1/3 expression in control spleen had a similar distribution to the macrophage marker CD14 (B). In (C, E and G) is the time course of PC1/3 mRNA expression of rats treated with LPS for 6, 9 and 12 h, respectively. Note that the pattern of PC1/3 expression changed from mainly in the red pulp to that of the white pulp. (D, F and H) No change in CD14 mRNA labeling was seen following LPS treatment. WP, white pulp; RP, red pulp. Bar, 6 mm.

represents an important component in all these cell types. In the thymus (Fig. 1B), high levels of furin gene expression were observed both in the cortex and medulla, although slightly higher in the cortex. The thymic cortex contains numerous densely packed lymphocytes without forming lymphatic nodules. In contrast, the medulla contains fewer lymphocytes but more epithelial reticular cells. The thymus is a site of T cell differentiation and high levels of furin expression imply it potentially important role. The observed variations in furin expression levels were consistent with previous observations in other tissues (Schafer et al., 1993; Day et al., 1993).

PC1/3 and PC2 have been considered as neuroendocrine-specific convertases, since they are preferentially expressed in neurons and endocrine cells. In the present study we observed PC2 mRNA expression in lymph nodes follicles (Fig. 1C and D), which contain densely packed lymphocytes, mainly lymphoblasts and B lymphocytes (De Wolf-Peeters and Delabie, 1993). PC1/3 was also expressed in the lymph nodes, in scattered cells within the dense paracortical regions, where scattered macrophages are normally found (Fig. 1E). Consistent with this observation, PC1/3 expression in the lung (Fig. 1F) was seen in alveolar walls, a localization indicative of free macrophages.

PC5/6 and PC7 were surveyed in the lymph nodes and the thymus. High levels of PC5/6 transcripts were observed in the cells lining lymph nodes sinuses (Fig. 1H). High PC7 mRNA levels were in the lymph node follicles (Fig. 1I) and in the thymus with an identical distribution pattern to that of furin (Fig. 1J and A).

3.2. PC mRNA regulation in the spleen

In Fig. 2 are representative sections of a comparative distribution of PCs mRNAs in the rat spleen. Furin, PC1/3, PACE4, and PC5/6 mRNAs (Fig. 2A, C, D and E, respectively) were expressed in the red pulp (Sharp et al., 1998), a highly heterogeneous compartment consisting of extravasated blood cells including macrophages, some lymphocytes, a spongy framework of reticular cells, arterial capillaries and a vast system of venous sinuses. Consistent with the known ubiquitous expression of furin mRNA (Day et al., 1993), its expression in the white pulp (WP) was also noted, although at much lower levels. No PC2 expression was observed in the spleen under static conditions (Fig. 2B). In agreement with previous studies showing high levels of PC7 in lymphoid-associated tissues (Bergeron et al., 2000; Seidah et al., 1996); PC7 mRNA was highly expressed distributed in the white pulp of spleen, a compartment containing macrophages, reticular dendritic cells, lymphocytes and lymphoblasts (Fig. 2F).

In order to mimic an infectious state, we used a bacterial-like challenge by treating rats with a single dose of LPS. In this context of immune system activation, we focused on the effects on PC2 and PC1/3 gene expression in the spleen at 3, 6, 9 and 12 h post-injection. General markers of macrophages (CD14) and B cells (CD20) were also examined under the same conditions. Regional differences in the spleen were analyzed and included WP, germinal centers (GC), periarteriolar lymphocyte sheath (PALS), marginal zone (MZ) and RP. Representative sections showing the effects of LPS on PC2 and PC1/3 expression in the spleen

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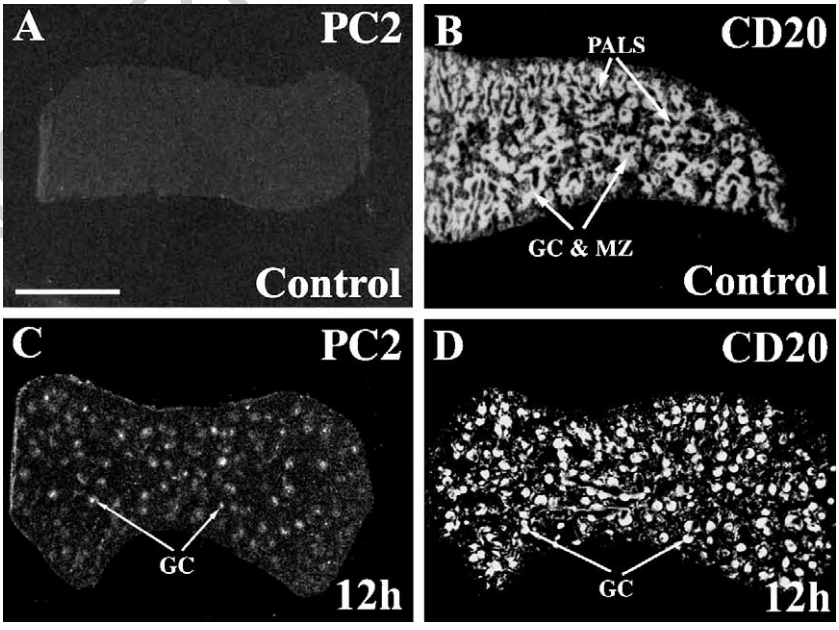

Fig. 4. Induction of PC2 mRNA expression by LPS in rat spleen. PC2 and CD20 mRNA distribution as revealed by in situ hybridization in spleens of (A and C) control and (B and D) LPS treated rats. Twelve hours after LPS administration, PC2 mRNA expression was induced in the germinal center of the white pulp (B), in a similar distribution than the B cell marker CD20 (D). (C) In control animals, CD20 expression was seen in the white pulp, but not in the germinal centers. GC, germinal center; WP, white pulp. Bar, 6 mm.

are shown in Figs. 3–6. The sum of these data are summarized in Table 1.

In the spleen of control animals, PC1/3 mRNA was principally expressed in the RP (Fig. 3A) and this localization pattern, although more restricted, was very similar to that of CD14 (Fig. 3B). CD14 expression was found almost equally in the RP and MZ, while PC1/3 was found more predominantly in the RP (Table 1). This data suggests the expression of PC1/3 within RP macrophages. Following 6 h of LPS treatment (Fig. 3C), PC1/3 expression was still observed in the RP areas, but was now more widely distributed to include the MZ, in a pattern that was almost identical to that of CD14. At the 6 h time point, CD14 mRNA expression and distribution was unchanged as compared to the control (Fig. 3D compared to B). Dramatic changes in PC1/3 expression were observed at the 9 h time point (Fig. 3E). In addition to the RP/MZ distribution, PC1/3 mRNA expression was now observed within the GC (Fig. 3E), a densely packed compartment situated in the middle of the WP characterized by active lymphocyte production and containing few macrophages. At this time point CD14 expression was still unchanged (Fig. 3F). Finally, at the 12

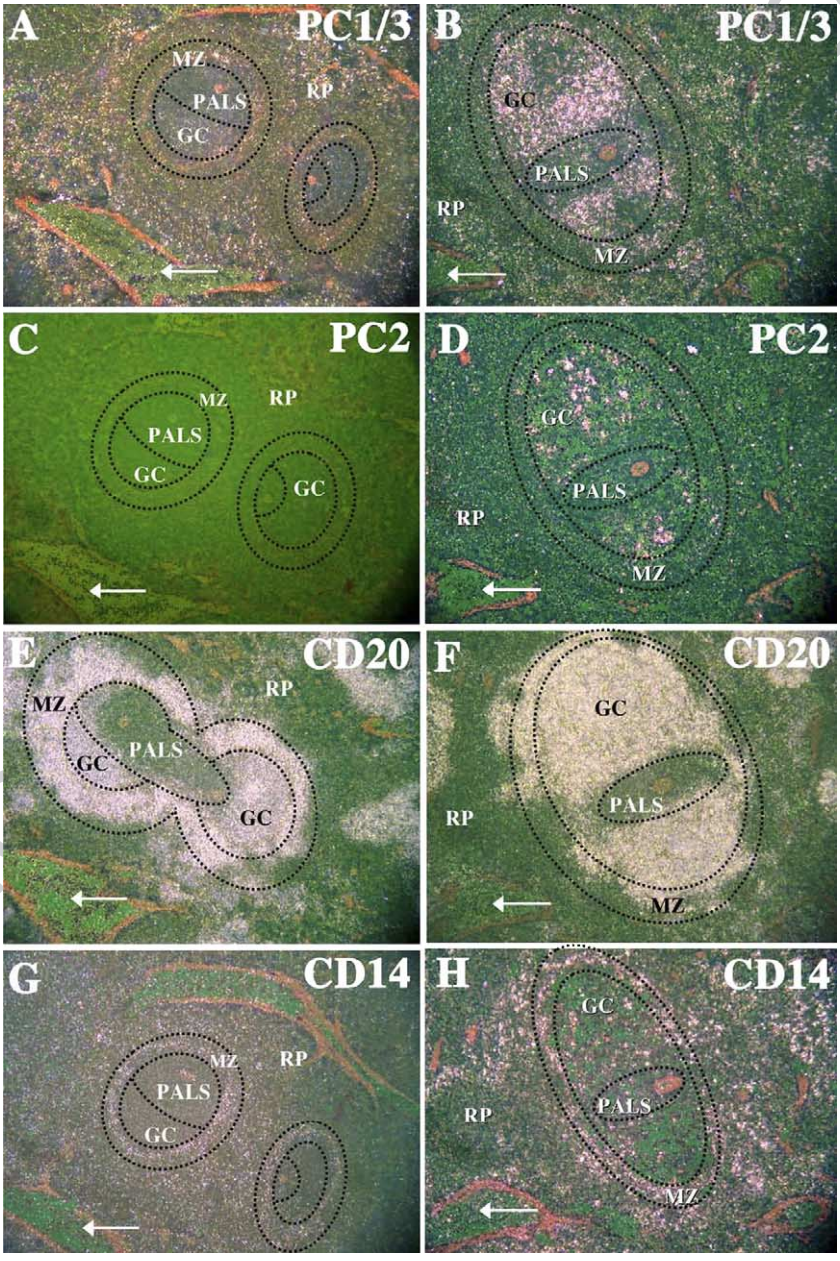

Fig. 5. Regional changes observed in PCs mRNA expression and CD14 and CD20 markers, before and after LPS. In A, C, E and D are adjacent spleen sections of control rats showing the normal distribution pattern of (A) PC1/3, (B) PC2, (C) CD20 and (D) CD14. In B, D, F, and H are distribution of (B) PC1/3, (D) PC2, (F) CD20 and (H) CD14 after LPS treatment, also on adjacent sections. PC1/3 is normal expressed in the red pulp, however after LPS treatment is found in the GC and MZ, while the CD14 marker is very poorly expressed in the GC. PC2 mRNA is induced after LPS and is observed solely in the GC where CD20 is strongly expressed.

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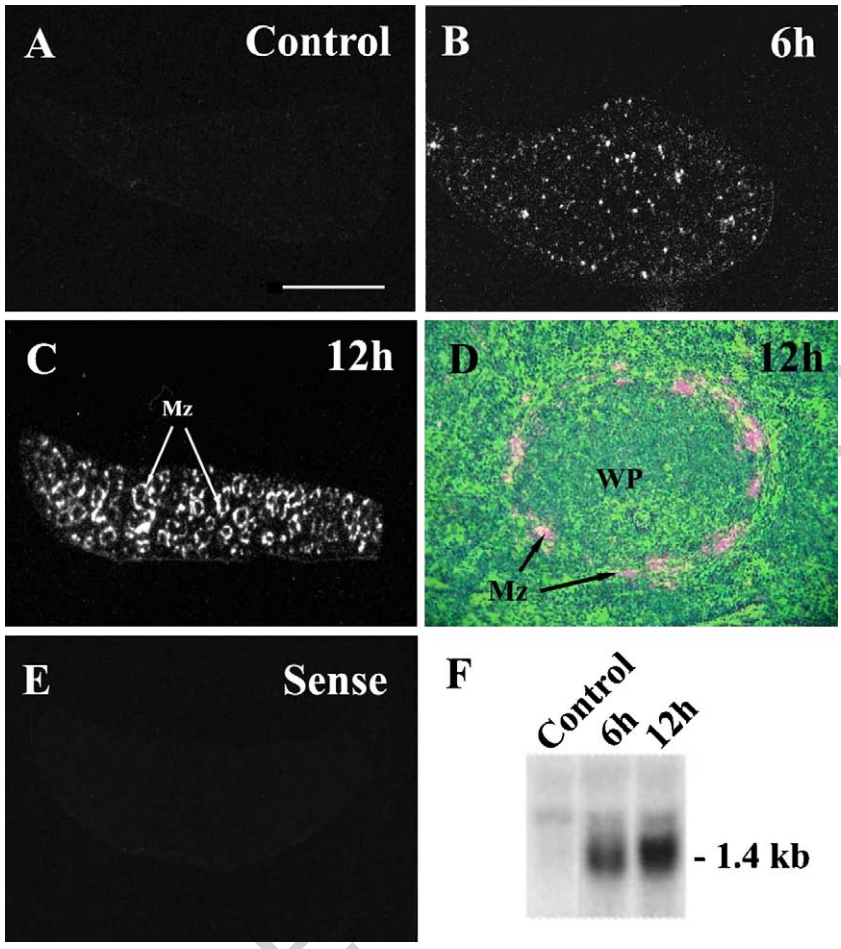

Fig. 6. Induction of PENK mRNA expression by LPS in rat spleen. Autoradiographic (A, B, C and E) and cellular resolution (D) images showing PENK mRNA distribution in spleen. (A) PENK mRNA expression was undetectable in the spleen of control animals. Increased expression levels were observed after (B) 6 and (C) 12 h following LPS injection. (D) The cells labeled after 12 h were located in the marginal zone. (E) No positive labeling was observed in control sections labeled with PENK sense probe. (F) Increased PENK mRNA levels were observed by Northern blot analysis. Corresponding 18S ribosomal analysis determined that equal loading of each lane was obtained (data not shown). Molecular weight markers are shown on the right. WP, white pulp; Mz, marginal zone. Bar, 6 mm.

h time point (Fig. 3G), PC1/3 expression had a completely different pattern from its initial distribution seen in the controls (Fig. 3A). The strongest PC1/3 expression was observed within the GC region of the WP, with a sharp decrease in RP areas. This pattern was also in sharp contrast to the unchanged CD14 pattern seen after 12 h LPS treatment (Fig. 3H).

A similar time course analysis was carried out for PC2 in the spleen. In Fig. 4 are shown the representative sections of that study, focusing on the major changes observed at the 12 h time point. As previously mentioned, no PC2 expression could be detected in spleens of control animals (Fig. 4A). Following 12 h of LPS treatment (Fig. 4C), PC2 expression could be detected in the WP areas, suggesting an expression pattern within the GC. This expression pattern was compared to that of CD20, a B cell marker (Fig. 4B and D). As expected, in control sections, CD20 was principally expressed in the white pulp areas, including the GC, but not the PALS. CD20 expression was also observed the MZ (see Table 1). Following LPS treatment, CD20 mRNA was increased in the GC area, but decreased in the MZ area, resulting in the modified pattern observed in Fig. 4D. This expected observation most likely reflects the re-distribution of B cells that responds to T cell-independent II antigen, such as LPS. A strong similarity between PC2 expression and the new CD20 expression pattern was noted (Fig. 4C and D).

The observed distribution of PC1/3, PC2, CD14 and CD20 mRNAs was further examined in emulsion dipped adjacent sections to confirm the regional and cellular expression patterns. In Fig. 5A, PC1/3 mRNA was found mainly in the RP, with few PC1/3 expressing cells in the MZ or the GC. Following a 12 h treatment of LPS (Fig. 5B), PC1/3 expression changed dramatically, with the highest levels being observed in the GC, followed by the MZ. As for PC2, no signal was observed in control sections (Fig. 5C), but a distinct signal was observed in the GC after LPS treatment (Fig. 5D). Spleen sections labeled from control (Fig. 5E) and treated rats (Fig. 5F) showed a re-distribution pattern for CD20 mRNA. Control sections express CD20 in

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Table 1
Summary of observed changes in PC2, PC1/3, CD20 AND CD14 in regional areas of the spleen after LPS treatment

	GC	PALS	MZ	RP
CD20 control	++	–	++++	+
3 h	+++	–	++	+
6 h	++++	–	–	+
9 h	+++++	–	+	+
12 h	+++++	–	+	+
CD 14 control	–	–	++	++
6 h	–	–	+++	+++
12 h	+[a]	+[a]	+++	+++
PC1/3 control	–	–	+	++++
3 h	–	–	+	+++
6 h	–	–	+	++
9 h	+	–	+	++
12 h	+++	+	+	+
PC2 control	–	–	–	–
12 h	++	–	–	–
PENK control	–	–	–	–
12 h	–	–	++[a]	–

[a]Indicates scattered cells.

the MZ and GC, while sections from treated animals express CD20 mRNA mainly in the GC. The GC expression pattern of CD20 (Fig. 5F) corresponded to that observed for PC1/3 (Fig. 5B) and PC2 (Fig. 5D) after 12 h LPS treatment. Sections labeled for CD14 mRNA are also shown in Fig. 5G and H. The CD14 mRNA expression pattern was unchanged after LPS treatment, with a positive signal being observed in the RP and MZ.

3.3. LPS-induction of proenkephalin in the spleen

We then examined the spleen expression patterns of potential PC substrates in the same animal model (Fig. 6). We focused on PENK since previous studies have shown its expression in immune cells, including neutrophils, lymphocytes and macrophages (Rosen et al., 1989; Vindrola et al., 1994; Martin et al., 1987; Tasiemski et al., 2000). Furthermore, recent studies have also demonstrated that antibacterial activities could be derived from the C-terminal processing of PENK (Goumon et al., 1996). Under basal condition, no detectable expression of the PENK mRNA could be observed by in situ hybridization (Fig. 6A). However, PENK expression was strongly upregulated in our model of bacterial infection (Fig. 6B, C and D). After 12 h of LPS treatment, PENK was expressed in scattered cells of a distinct spleen compartment, the marginal zone (MZ) (Fig. 6C and D). Since PENK mRNA has a very restricted expression pattern, this increased expression was easily confirmed by Northern blot analysis (Fig. 6F). As expected, the PENK band was observed at 1.4 kb.

3.4. Proenkephalin processing in the spleen

We then examined PENK derived peptides in the spleen of control and LPS treated rats, in order to establish whether antibacterial forms previously reported(Tasiemski et al., 2000; Goumon et al., 1996, 1998; Metz-Boutigue et al., 1998; Salzet and Tasiemski, 2001) could be observed. Peptide extracts were prepared from control spleens and spleens from LPS treated rats. The extracts were pre-purified by Sep-Pak and HPLC separation on a C18 column. The obtained fractions were subjected to a rigorous analysis that included three steps: 1) antimicrobial test, 2) DIA tests with PENK antibodies and 3) MALDI analysis of the positive fractions of step 1 and 2. Using antibodies directed against Met–Enk RGL and Leu–Enk, immunoreactivity was observed in fractions eluting from the column at 29% acetonitrile of control and LPS samples. Additionally, immunoreactivity was also observed in fractions eluting at 34% acetonitrile of LPS samples and presented a high antibacterial activity compared to control Table 2. The MALDI-MS analyses of these fractions between both conditions are shown in Figs. 7 and 8.

In the 29% fraction, a mass at a m/z 1214.67 was detected in control (Fig. 7A) and stimulated animals (Fig. 7B) that corresponds to oxidized YGGFM(O)RRVGR peptide, whereas its non oxidized form YGGFMRRVGR (m/z 1198.54) was only detected in injected animals. Masses of m/z of 840.39 and 1154.93 were found in both control (Fig. 7A) and injected (Fig. 7B) animals and corresponded to YGGFLKR peptide and YGGFMRRVG, respectively (Table 1). We also included an inset (Fig. 7C and D) to show average mass of the peptides corresponding to YGGFMRRVG.

The 34% fraction, shown in Fig. 8A and B, was immunoreactive with the anti-Met–EnkRF and anti-Met–Enk antibodies. This fraction had only weak antibacterial activity in the controls (Fig. 8A) and a high activity in challenged animals (Fig. 8B). Analysis of these fractions showed masses at 3346.1, 3384.0, 3491.6 corresponded to MH^+ oxidized enkelytin, MK^+ oxidized enkelytin, and MH^+ diphosphorylated enkelytin. An enlargement of this group of mass is shown in Fig. 8C and D. From the control animals,

Table 2
PENK derived peptides detected in control and in injected animals from fractions 29% and 34% CAN

Peptide	Fractions 29%		Fractions 34%	
	Control	Injected	Control	Injected
YGGFLMR	+	+		
YGGFMRRVG	+	+		
YGGFM(O)RRVGR	+	+		
YGGFMRRVGR		+		
Enkelytin			+	+
(O)Enkelytin			+	+
(O) Na+ Enkelytin			+	+
(O) K+ Enkelytin			+	+
Di(O)monophospho Enkelytin				+
Diphospho Enk				+

Phospho: Phosphorylated, Na+ and K+ are added ions, O: oxidized). A (+) indicates detection of the peptide form, without regard to the levels of that peptide.

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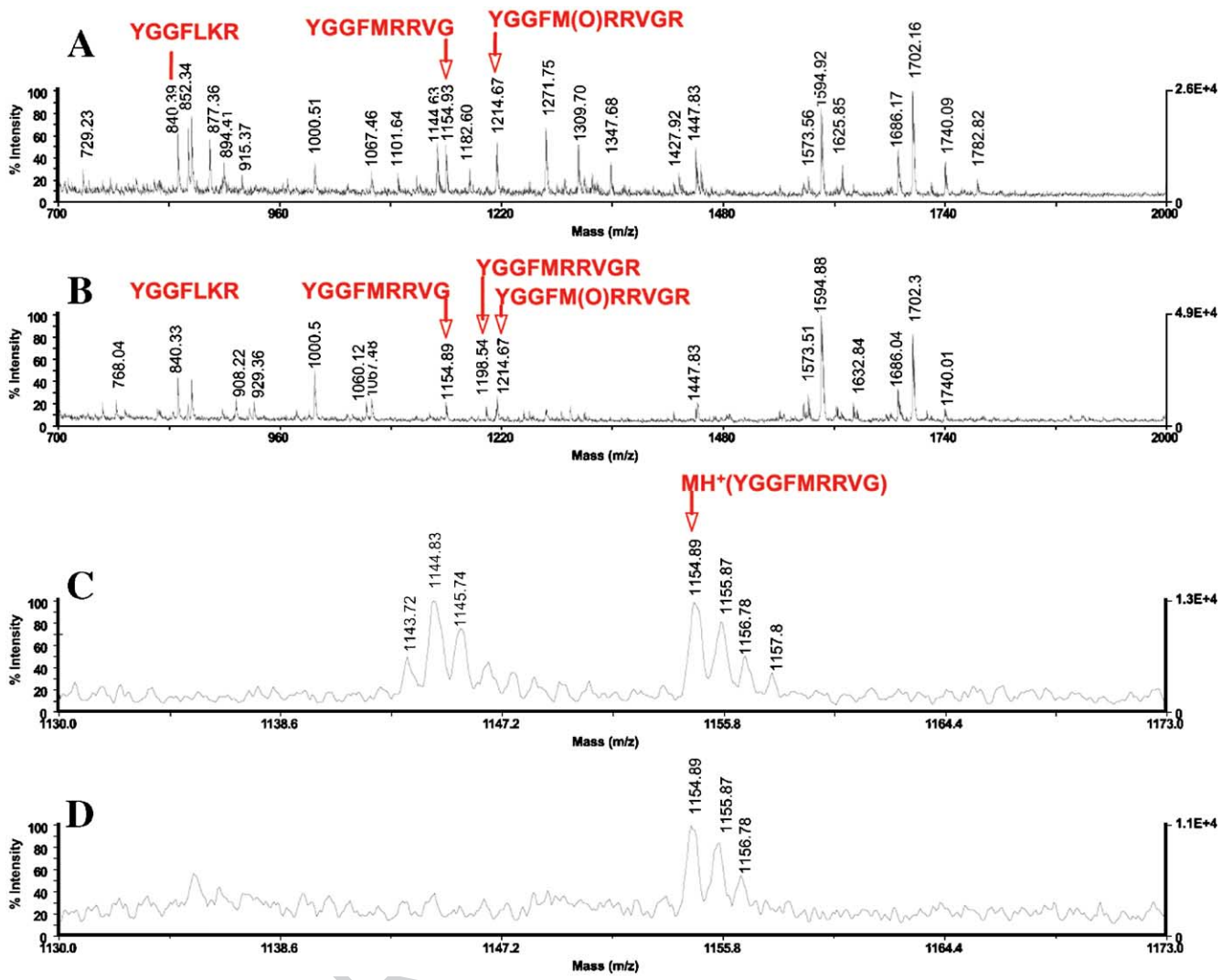

Fig. 7. MALDI-time of flight mass spectra of HPLC fractions eluted at 29% ACN. (A) Control (saline solution injection) and (B) challenged animals (LPS injection) were analyzed. m/z at 840.39, 1154.93, and 1214.67 correspond to YGGFLKR, YGGFMRRVG, oxidized YGGFM(O)RRVGR, respectively were observed in controls. However, in the challenged animals (B) an additional peptide with m/z at 1198.54 corresponding to YGGFMRRVGR was observed, the non-oxidized form of the latter peptide. (C) and (D) are enlargements of the YGGFMRRVG peak in (A) control and (B) in LPS challenged animals, respectively. In (C) an unidentified group of masses of 1143.72 1144.83 and 1145.74 are absent in injected rats. However, the peaks at 1154.89, 1155.87 and 1156.78 are present in the controls and correspond to average mass of the peptide YGGFMRRVG.

(Fig. 8C) masses at 3331.6 and 3368.02 corresponding to MH^+ enkelytin and MNa^+ oxidized enkelytin at lower levels. These observed masses are in agreement with a previous analysis carried out to identify the different forms of the C-terminal proenkephalin derived peptides (Goumon et al., 1996, 1998). Following LPS injection (Fig. 8D), the enkelytin levels of diphosphorylated forms greatly increased whereas the oxidized forms decreased. We observed masses at 3331.6, 3346.1, 3442.59, 3491.6 corresponding to MH^+ enkelytin, MH^+ oxidized enkelytin, MH^+ dioxidized monophosphorylated enkelytin and MH^+ diphosphorylated enkelytin. Furthermore, the mass at 3342.59 corresponding to MH+ deoxidized monophosphorylated was observed only in the challenged animals. It is noted that MH^+ enkelytin greatly increased in LPS animals as well as MH^+ diphosphorylated enkelytin. These results are in agreement with the high antibacterial activity found in the 34% fraction of LPS injected animals. The diphosphorylated enkelytin forms have previously been shown to have highly antibacterial activity (Goumon et al., 1998) (Table 1). These data support previous studies in chromaffin granules, bovine infectious fluids and human plasma after coronary cardiology bypass, where all the different forms of enkelytin were detected (Tasiemski et al., 2000; Goumon et al., 1996, 1998).

4. Discussion

The neuroendocrine cells secrete their products in a regulated manner, in response to a specific stimulus. The expression of markers such as neuropeptides, chromogra-

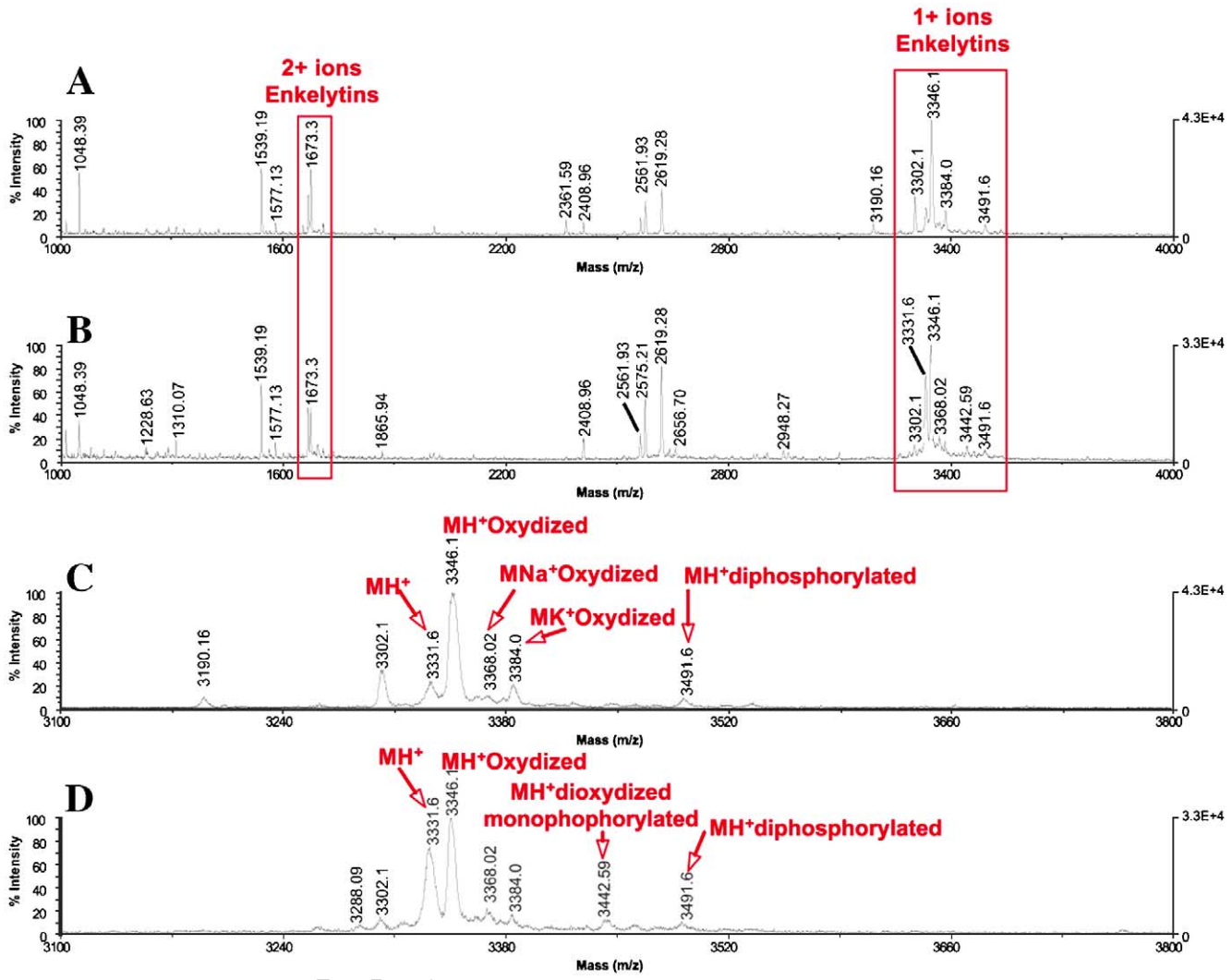

Fig. 8. MALDI-time of flight mass spectra of HPLC fractions eluted at 34% ACN. (A) Control animals and (B) LPS injected animals. In (A) masses at 3346.1, 3384.0 and 3491.6 correspond to respectively MH^+ oxidized enkelytin. MK^+ oxidized enkelytin and the MH^+ diphosphorylated enkelytin. In (B) m/z 3331.6, 3346.1 3442.59, 3491.6 correspond to respectively MH^+ enkelytin, MH^+ oxidized enkelytin, MH^+ dioxidized monophosphorylated enkelytin, MH^+ diphosphorylated enkelytin. In (A) and (B) the group of masses at 1673.3 correspond to MH^{2+} oxidized enkelytin. (C) and (D) are the enlargements of the group of peaks at 3346.1 scanned between 3100 and 3800.

nins and neuropeptide processing enzymes PC2 and PC1/3 was thought to be restricted to the neuroendocrine system. However, neuropeptide expression has been found elsewhere than in neurons or endocrine cells such as in tumor cells (Moody et al., 2003), cardiomyocytes (Springhorn and Claycomb, 1989), and immune cells (Weigent and Blalock, 1997; Blalock, 1985; Mousa et al., 2004; Przewlocki et al., 1992; Behar et al., 1994). The unexpected distribution of PENK seen in this study, as well as the presence in immune cells of several so-called markers of neuroendocrine system such as chromogranins (Tasiemski et al., 2002) and opioids peptides derived from PENK and POMC (Weigent and Blalock, 1997; Blalock, 1985; Stefano et al., 1996; Mousa et al., 2004) lead us to suggest that immune cells are capable of cellular plasticity such that they can potentially express a neuroendocrine phenotype. This also leads us to ask the question if the full complement of processing enzymes are co-expressed with these neuroendocrine precursors in immune cells.

The PC family has been shown to be important in the activation of neuropeptides. The present study demonstrated that PCs with ubiquitous or widespread distribution such as furin, PC5/6 and PC7 are present in various cells of the immune system. While any PC could potentially generate neuropeptides, most of our attention has been focused on PC1/3 and PC2. These two enzymes are considered excellent markers of neuroendocrine cells (Bergeron et al., 2000). Surprisingly, we observed in the spleen PC1/3 mRNA under basal condition and up-regulation of both PC1/3 and PC2 mRNA following a pathogen-like infection. Regulation of PC1/3 and PC2 mRNAs levels are not surprising in endocrine systems, as they have been shown to be modulated under a variety of conditions, such as glucocorticoids, dopaminergic or thyroid hormone manipu-

lations (Day et al., 1992). These effects are all mediated through appropriate receptor-signaling pathways of the target endocrine cells. In contrast, modulations of PC1/3 and PC2 expressions are not well understood in immune cells. LPS mimics pathogen-like infection through its interaction with TLR4. TLRs are a family of receptors that contact the environment and provide specific sensing for an important component of the innate immunity (Pasare and Medzhitov, 2005). TLRs are found on immune cells and recognize specific microbial molecular patterns. The activation of these sentinel molecules leads to a process of intracellular signaling through to transcriptional control that culminates with an array of host immune responses. Therefore, TLRs play an important part in the innate immune recognition of invading pathogens, initiating immune responses characterized by the release of pro-inflammatory cytokines and interferons via transcription factors such as NFkB and IRF3. From our data, we can surmise that these signaling pathways/transcriptional factors also modulate/induce PC1/3 and PC2 in immune cells as well as one of their target substrates, namely PENK. One potential function of this powerful modulatory process is to produce antibacterial peptides such as enkelytin. Since PC1/3 and PC2 are processing enzymes for many precursors, a number of other important functions could also be uncovered, directly related to innate and even acquired immunity.

The induction of PENK gene expression within the spleen MZ following LPS induction and its processing into antibacterial peptides is not surprising as this compartment is harboring spleen sinuses, weakly assembled lymphocytes, plasmocytes and macrophages. Indeed, terminal branches of the arterial system discharge their content between the cells of the MZ so that it offers a good opportunity for cells to meet systemic antigens arriving in the spleen. In fact, lymphocytes crossing this frontier meet several reticular dendritic cells which are able to present antigens on their surface to the immunocompetent lymphocytes exactly in the same manner that macrophages do during the immune reaction. MZ immunocytes form then a first line of defense against systemic invaders where antibacterial peptides might play a critical role. The secondary response is very fast and the spleen becomes the organ that produces the most important amount of antibodies of the organism.

Our results demonstrate that bacterial-like challenge can result in very impressive and dynamic levels of induction of PCs in the spleen. Such striking modulation supports the notion that the system is mounting a response where PCs play an important role. PC regulation/induction could result in the differential and temporal processing of PENK. As the spleen is intimately implicated in blood filtration and because treatment conditions such as LPS can alter the sub-regional distribution of immune cells, changes in PCs and PENK mRNA expression could partly be explained by redistribution, infiltration or migration of some immune cells. However, the concept of cellular traffic cannot entirely explain the changes seen in mRNA expression following LPS injection for PC2 and PC1/3. In fact, as shown in Fig. 5, and following LPS treatment, PC1/3 and PC2 are strongly expressed in the germinal centers, which are very rich in CD20, a B lymphocyte marker.

The biochemical data confirm the presence of PENK derived peptides in spleen and with a processing similar to what is occurring in the central nervous system. In fact, the four enkephalin peptides (YGGFMRRVGR, YGGFMRRVG, YGGFLKR and enkelytin) found are the ones present at the C-terminal part of the rat PENK. Moreover, these four peptides must derive from PCs action and most likely PC2 and/or PC1/3. For the peptide YGGFMRRVGR, only PC2 can act due to the sequence RXXR↓P where the P1′ is a Pro, as previously shown in dynorphin 1–17 to yield dynorphin 1–8 (Day et al., 1998). Furthermore, after LPS treatment, this peptide is overexpressed like PC2 and the antibacterial peptide enkelytin. This is in line with the data we previously demonstrated in monocytes during interleukin-6 stimulation (Tasiemski et al., 2002). PENK processing after immune response stimulation allows the production of the antibacterial peptide enkelytin which is found in phagolysosomes (Tasiemski et al., 2002) whereas Met–Enk, Met–Enk RF are released in order to stimulate chemotaxis (Tasiemski et al., 2000) and act as cytokines (Plotnikoff et al., 1997). It is also interesting to note that in non-stimulated animals, the oxidized forms of enkephalin derived peptides are high compared to injected animals. This indicates that oxidation could be an inactivating process for enkephalins as previously shown for FMRFamide peptides family (Salzet et al., 1993).

As in the neuroendocrine system, processing might be essential in immune cells to generate different biologically active neuropeptides depending on the function required. For example, PENK could be cleaved by a combination of PCs shortly after LPS induction resulting in the production of short peptides such as Met–Enk–Arg–Phe and the antibacterial peptide enkelytin as observed in our study. Therefore, differential PCs expression in immune cells might be an important mechanism by which defense activities are modulated. However, the fact that PENK derived peptides such as YGGFMRRVG, YGGFLKR and YGGFM(O)RRVGR are detected in the spleen of non-infected animals could also reflect a bifunctional role of these peptides independently of the infection and thus could have a role in cell to cell communication/modulation.

Differential processing of PENK by the PCs appears to generate multiple biological activities in immune cells. The opioid Met–Enk and its analogs, through their delta opioid receptor activities can enhance the immune reaction by stimulating immunocyte chemotaxis, phagocytosis and secretion of immunostimulatory factors, but would also mediate an analgesic effect in peripheral inflamed tissues (Stefano et al., 1996; Plotnikoff et al., 1997; Kamphuis et

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al., 1998; Stein et al., 1993). Furthermore, the release of enkelytin and Met–Enk peptides is increased in different cases such as LPS injection, surgical trauma or bacterial exposure (Tasiemski et al., 2000; Goumon et al., 1998). These data suggest that the co-processing and co-liberation of enkelytin and Met–Enk peptides could represent a unified neuroimmune protective response to an immediate threat to the organism, regardless of the specific stimulus. According to this hypothesis, enkelytin would be released to deal immediately with the microbial threat, thus allowing time for the opioid peptides simultaneously liberated from PENK to stimulate, activate or recruit immunocytes. This hypothesis is further supported by the presence of specific opioid receptors on immunocytes (Bidlack, 2000). This unified mechanism could represent an important survival strategy.

PCs most likely have numerous and uncharacterized functions in the immune system, as they could play a role in protein processing necessary for classically defined immune cell functions such as immunoglobulins, cytokines and chemokines production. Furthermore, the important spatial and temporal changes observed in PC expression following pathogen-like challenge provide strong evidence that they are implicated not only in the innate immune response but also in the initiation of the adaptive immune response. The strong upregulation of PC2 and PC1/3 in the white pulp is of particular interest, as this could mean that other proneuropeptides might be expressed and processed within lymphocytes. Taken together, these results, in agreement with previous reports (Mousa et al., 2004; Salzet et al., 2000; Day and Salzet, 2002; Salzet, 2002), allow us to add PCs to the growing numbers of endocrine markers expressed by immune cells and point to a significant role in the immune response mechanisms.

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