Lipopolysaccharide stimulates the expression of pro-opiomelanocortin mRNA in chicken macrophages, as demonstrated with a competitive polymerase chain reaction

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ABSTRACT. The goal of the present study was to develop a competitive PCR assay to measure changes in the expression of pro-opiomelanocortin (POMC) mRNA in myelomonocytic HD11 cells after *Salmonella typhimurium* lipopolysaccharide (LPS) stimulation. Pro-opiomelanocortin mRNA could be detected in HD11 cells by reverse transcription – polymerase chain reaction (RT-PCR). To our knowledge, this is the first observation of a POMC mRNA transcript in avian macrophages. Based on this observation, the effect of stimulation with bacterial LPS on the POMC expression in HD11 cells was investigated by means of a competitive PCR assay. For this purpose, the HD11 cells received a one-hour LPS challenge with LPS concentrations ranging from 10 to 100 ng/ml. A PCR MIMIC (consisting of a heterologous DNA fragment flanked by templates for the gene-specific primers) was used as an internal control in the competitive PCR assay. A ten-fold dilution series of the MIMIC was co-amplified with a constant amount of experimental cDNA. While HD11 POMC mRNA expression showed an increase of one order of magnitude following treatment with 100 ng/ml LPS as compared with untreated controls, no significant differences could be observed after treatment with 50 and 10 ng/ml LPS. Quantitative measurement of POMC mRNA levels is a first step towards a better understanding of the physiological role of non-hypophysial POMC-derived peptides in the response to immune stress in birds.

KEY WORDS: chicken, POMC, HD11 cells, lipopolysaccharide, competitive PCR.

INTRODUCTION

Pro-opiomelanocortin (POMC) is the polypeptide precursor of adrenocorticotropic hormone (ACTH), the opioid hormone β-endorphin (β-END), β-lipotropin (β-LPH) and α-, β- and γ-melanotropin (α-, β-, γ-MSH) (BERTAGNA, 1994). These molecules play a pivotal role in the stress response: ACTH has steroidogenic activity (OTTAVIANI et al., 1999), β-endorphin has analgetic properties (HIRSCH AND MILLINGTON, 1991) and MSH is an interleukin-1 antagonist (STAR et al., 1995; LUGER et al., 1997).

Although pro-opiomelanocortin (POMC) is a well-established hormone precursor in many species, molecular information about chicken POMC has only relatively recently been revealed (BERGHMAN et al., 1998; TAKEUCHI et al.,1999; GERETS et al., 2000). In our laboratory, POMC has been intensively investigated in the pituitary and the brain of the chicken. The chicken POMC coding region appears to consist of 678 base pairs in the pituitary and also in the hypothalamus. The distribution of the POMC mRNA in the pituitary is restricted to the cephalic lobe, whereas in the brain, the signal is limited to the hypothalamic region.

Unlike the situation in mammals, information about peripheral, non-endocrine or neuroendocrine POMC is still relatively scarce in birds. Using the reverse transcription – polymerase chain reaction (RT-PCR) technique, we

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were able to detect a POMC mRNA fragment in non-pituitary tissues including the bursa, thymus, liver, kidney etc. (H. Gerets, manuscript in preparation). The existence of POMC mRNA in different non-pituitary tissues of the chicken has also been demonstrated by Takeuchi et al. (1999). These reports are in line with the earlier work by Ottaviani and co-workers (1992), describing ir-ACTH peptides in chicken phagocytic leucocytes and lymphocytes. To our knowledge, POMC gene expression has not been demonstrated in a permanent avian *in vitro* model, such as HD11 myelomonocytic cells. HD11 cells are chicken macrophages transformed by the *v-myc*-encoding MC29 virus (Beug et al., 1979). This study will focus on the expression and regulation of POMC mRNA in HD11 cells in the presence or absence of *Salmonella typhimurium* lipopolysaccharide (LPS).

MATERIAL AND METHODS

Cell culture

The MC29 virus-transformed chicken macrophage cell line HD11 (Beug et al., 1979), was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL Life Technologies; Merelbeke, Belgium) supplemented with 5% (w/v) fetal bovine serum (FBS) (Gibco BRL Life Technologies), 2 mM L-glutamine (Sigma; Bornem Belgium), 1 mM sodium pyruvate (Sigma) and 50 ng/ml gentamycine (Gibco BRL Life Technologies). HD11 cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

LPS stimulation and total RNA extraction

Cells were maintained in culture and plated at a density of 1.4 10^6 cells per well in 24-well plates (Elscolab; Kruibeke, Belgium). They were treated with different concentrations of lipopolysaccharide (LPS) (Sigma) at 100, 50, 10 and 0 ng/ml, respectively, for one hour. After stimulation, total RNA was extracted from the cells with the RNeasy Mini Kit (Westburg; Leusden, The Netherlands) according to the instructions of the kit.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Reverse transcription – polymerase chain reaction (RT-PCR) experiments were carried out to determine whether HD11 cells express the POMC gene. For this purpose, a RT-PCR reaction was carried out according to the instructions of the GeneAmp RNA PCR kit (Perkin Elmer; Nieuwerkerk a/d IJssel, The Netherlands). To perform the reverse transcription of total RNA into cDNA, an oligo-dT primer (Perkin Elmer) was used. The sequences of the upstream and downstream primers (supplied by Gibco BRL Life Technologies) used in the PCR reactions, were based on the POMC cDNA sequence as determined previously

(Berghman et al., 1998; Gerets et al., 2000). The PCR-primers were designed as follows:

→ 5'-AAGCGCTCCTACTCCATGGAGCATTTCC-3' and

← 5'-GGCGTTTTTGAACAGAGTCATCAGCGGGGTCTG GCTGAGCTCC-3';

→ 5'-AGCGGCCCATGCTGGGAGAAC-3' and

→ 5'-CTGACCCTTCTTGTAGGCGC-3';

→ 5'-CTCTCGGAGAGCATCCGCAAG-3' and

← 5'-GGCGTTTTTGAACAGAGTCATCAGCGGGGT CTGGCTGAGCTCC-3'

amplifying a fragment of 330, 678 and 500 bp, respectively. The PCR cycles consisted of a denaturation (95°C, 60s), an annealing (65°C/50°C/50°C, 60s) and an elongation (72°C, 60s) step. After 36 cycles, the fragments were analyzed by horizontal agarose electrophoresis and visualized by means of ethidium bromide fluorescence. Control experiments involved omission of the reverse transcriptase enzyme and of total RNA, respectively, in order to prove that no genomic DNA contamination accounted for the observed signal.

Construction of the MIMIC

For quantification of POMC mRNA levels, an internal standard (MIMIC) with competing flanking sequences was constructed, using the PCR MIMIC Construction Kit (Clontech – Westburg; Leusden, The Netherlands) with minor modifications from the manufacturer's instructions. In the primary reaction, the cDNA was transcribed using two composite primers (5'-AAGCGCTCCTACTCCATG GAGCATTTCCCAAGTTTCGTGAGCTGATTG-3' as the sense primer and 5'-GGCGTTTTTGAACAGAG TCATCAGCGGGGTCTGGCTGAGCTCCGGGACAA GATACTCATCTGC-3' as the antisense primer). These composite primers were composed of two sections: the 3' portion annealing to the heterologous DNA fragment (supplemented with the kit) and the 5' portion annealing to the specific target gene. In the second PCR reaction only the gene-specific primers (underlined above) were used. This yielded a PCR MIMIC of 251 nucleotides (nt) consisting of a heterologous DNA fragment with templates that were recognized by a pair of gene-specific primers. The yield of the PCR MIMIC was determined by measuring the intensity of the electrophoretic bands generated by PCR MIMICs against those generated by various dilutions of known quantities of a molecular weight marker (φX174/*Hae* III digest) on a horizontal agarose electrophoresis. The intensities of the bands were measured after ethidium bromide staining with the Image Master (Image Master VDS; Amersham Pharmacia Biotech, The Netherlands). Subsequently, the MIMIC was diluted to a concentration of 100 attomoles/µl and used as such in the competitive PCR experiments. MIMIC stocks were stored at –20°C until use.

Competitive PCR assay

The entire pool of total RNA from one condition was simultaneously reverse transcribed to cDNA, with the use of the reverse transcriptase enzyme and the oligo(dT) primer. In the competitive PCR (cPCR) reaction, a ten-fold dilution series of the MIMIC was amplified together with a constant amount of experimental cDNA. The cPCR reactions were performed as described above. The resulting fragments were separated by horizontal agarose gel electrophoresis, stained with ethidium bromide and analyzed with the Image Master. Variations in fluorescence due to the molecular weight differences between target and MIMIC were corrected using the following formula: (Target / MIMIC fluorescence) x (MIMIC / target size (bp)) = corrected fluorescence ratio. For each individual PCR reaction the corrected fluorescence ratio (y-axis) was plotted against the initial MIMIC concentration on logarithmic scales. A line was drawn from a linear regression analysis of the data points. The amount of target POMC molecules was calculated by determining the x-intercept for the points of the curve where the ratio target / MIMIC equals 1 (when y = 0).

Statistical Analysis

Data are represented as means ± SE (n = 8). Results from the four groups of HD11 cells (controls, 10, 50 and 100 ng/ml LPS-stimulated) were compared using the non-parametric Wilcoxon test found on the website *http://fonsg3.let.uva.nl :8001/Service/Statistics/ Signed_Rank_Test.html*. Differences with a *P* < 0.05 were considered significant.

RESULTS

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions showed that HD11 cells indeed express POMC mRNA: fragments of 330 nt (Fig. 1A) and 500 nt (Fig. 1C) were detected. Also the entire coding region of POMC (678 bp) was amplified in these cells (Fig. 1B), although seemingly in a very low concentration when compared with the 330 nt fragment. When this fragment was amplified, two other bands of approximately 350 and 400 bp also appeared. Control experiments, which did not receive any total RNA input in the reverse transcription reaction, were all negative (data not shown).

Competitive PCR assay

As an internal standard, a PCR MIMIC consisting of a heterologous DNA fragment flanked with templates for the gene-specific primers was used. Due to the different sizes of the MIMIC and the target DNA (330 nt *vs*. 251 nt), these can be distinguished by horizontal agarose gel electrophoresis. Fig. 2 (p. 122) shows the competitive PCR reaction on
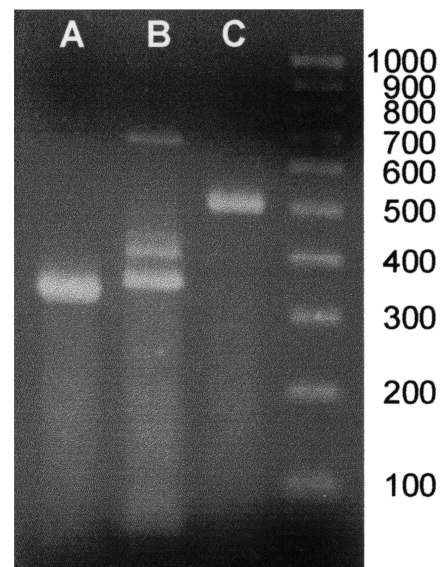

Fig. 1. – Polymerase chain reaction amplification of pro-opiomelanocortin cDNA of HD11 cells. Three different primer sets were tested to determine the length of the POMC transcript in HD11 cells. **A**. A 330 bp fragment of POMC used as the target in the competitive PCR reaction. **B**. The entire POMC coding region (~ 678 bp). **C**. An intermediate 500 bp fragment. These primer combinations indicate that the entire coding region of the POMC transcript is being transcribed in HD11 cells.

mRNA from HD11 cells stimulated with different concentrations of LPS (100 - 50 - 10 and 0 ng/ml). POMC mRNA expression in cells stimulated with 100 ng/ml LPS increased with as much as an order of magnitude as compared with untreated controls. On the other hand, no statistically significant differences could be observed between the 50 and 10 ng/ml LPS-stimulated cells as compared with untreated controls (Fig. 3). Densitometric analysis of the ethidium bromide stained gels is demonstrated in Fig. 2.
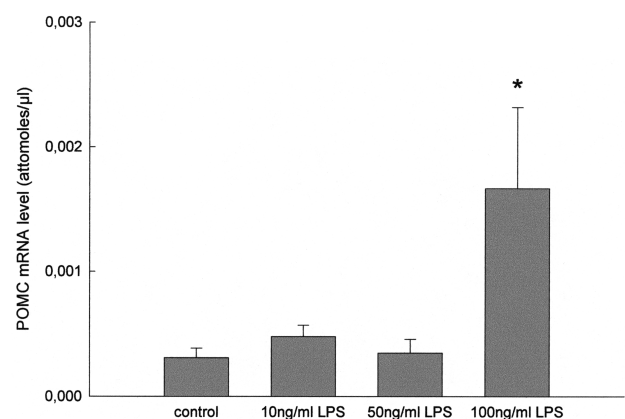

Fig. 3. – Histogram of untreated cells (controls) vs. 10 – 50 – 100 ng/ml LPS-stimulated HD11 cells. The bars represent the mean ± SE of the POMC RNA levels. The asterisk indicates a statistically significant difference between control cells and 100 ng/ml LPS stimulated cells in the Wilcoxon test (*: *P*<0.05); control and 100 ng/ml LPS (n=8); 50 and 10 ng/ml LPS (n=7) (n = number of independent experiments).
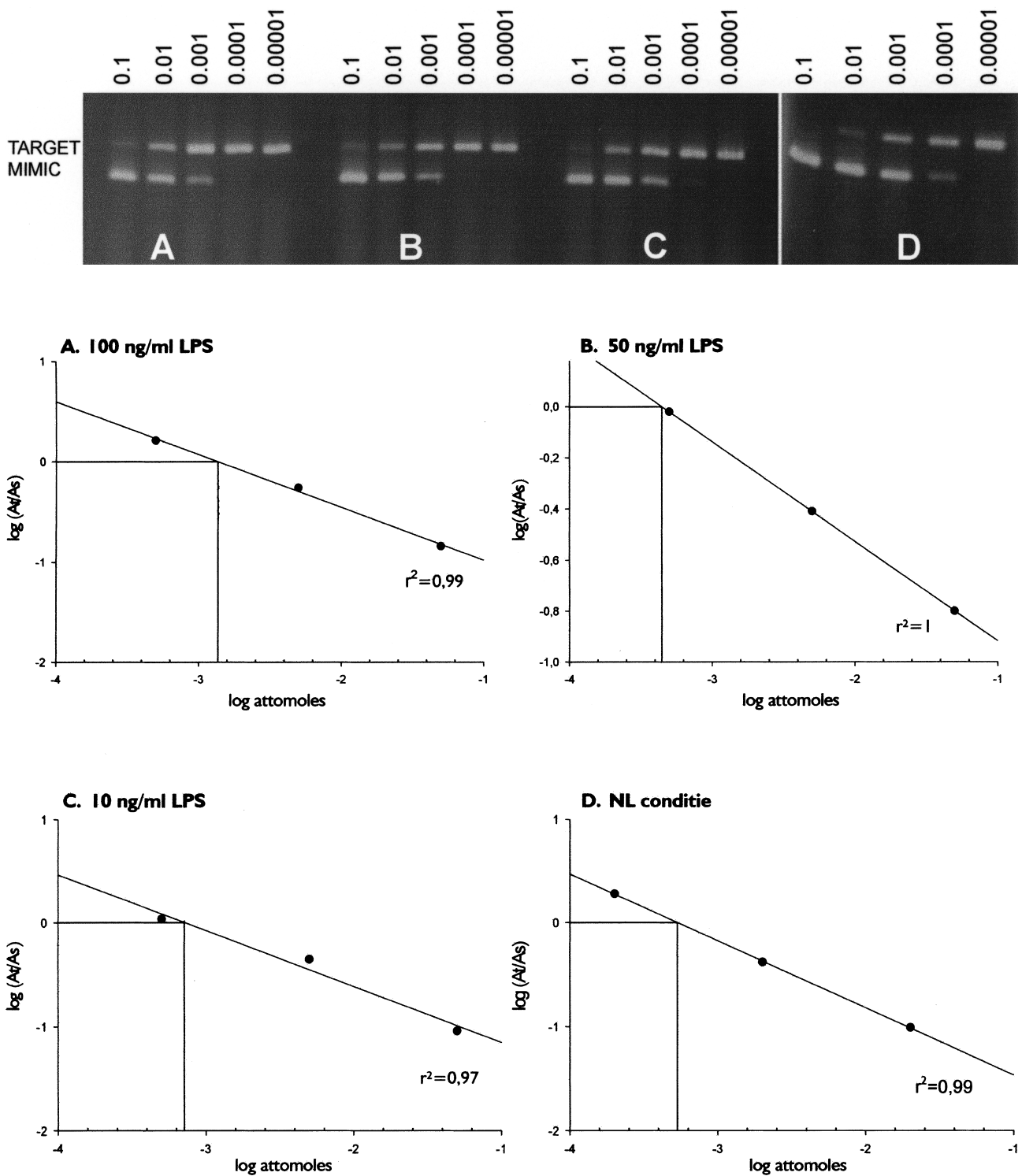
Fig. 2. – (Upper part) Competitive PCR analysis of the POMC mRNA expression in HD11 cells upon treatment with LPS. In this cPCR reaction, a ten-fold dilution series of the PCR MIMIC was co-amplified with a constant amount of experimental cDNA. The size of the target is 330 nucleotides (nt) and that of the PCR MIMIC is 251 nt.

(Lower part) Representative plot of the ratio of target DNA intensity (At) to MIMIC intensity (As) plotted against the initial concentration of MIMIC DNA in attomoles on logarithmic scales. A line was drawn from a linear regression analysis of the data points, and the amount of target POMC was calculated by determining the x-intercept for the point of the curve where the ratio target to MIMIC equals 1 (when y = 0). **A.** HD11 cells stimulated with 100 ng/ml LPS. **B.** HD11 cells stimulated with 50 ng/ml LPS. **C.** HD11 cells stimulated with 10 ng/ml LPS. **D.** Untreated HD11 cells (controls). The ten-fold dilution series of the MIMIC starts at 0.1 attomoles/μl and goes down to 0.0001 attomoles/μl indicated above the gel.

DISCUSSION

So far, information about non-pituitary pro-opiome-lanocortin (POMC) in the chicken has been relatively scarce. Using the RT-PCR technique, we were able to detect a POMC fragment in the bursa, thymus, liver, kidney, etc. (H. GERETS, manuscript in preparation). The expression of the chicken POMC gene in extra-hypophysial tissues, including adrenal gland, gonads, kidney and a host of other tissues has also been reported by TAKEUCHI et al. (1999). These observations were among the first to demonstrate that POMC mRNA is indeed present outside the pituitary and the brain of the chicken. In mammals, by contrast, non-pituitary POMC gene expression has already been described in detail a number of years ago (DEBOLD et al., 1988a,b).

Using the RT-PCR technique on HD11 cells, we presently report the expression of the POMC gene in a myelomonocytic cell line. Elaborating on these results, the regulation of these cells by lipopolysaccharide (LPS) was investigated. For this purpose, a competitive PCR assay was developed. Lipopolysaccharide, an integral component of the outer membrane of gram negative bacteria, activates important cellular mechanisms in the acute phase of a bacterial infection (GOETHE et al., 1998).

HD11 cells belong to the myelomonocytic lineage and our present results show a low level of POMC mRNA expression under normal conditions. Upon treatment with LPS, increased levels of POMC mRNA in HD11 cells were rapidly induced, with a minimal effective dose of 100 ng/ml. Lower doses of 10 and 50 ng/ml LPS had no effect compared with control cells; possibly these concentrations are too low to be effective. GOETHE & PHI-VAN (1998) used concentrations up to 5 μg/ml LPS to stimulate HD11 cells.

An important conclusion from these findings is that the non-pituitary POMC transcript in chicken macrophages seems to be subject to physiological regulation at the transcriptional level. This corroborates the hypothesis that also in birds POMC-derived peptides of non-hypophysial origin play a biological role in the immune response, similar to what has been shown before in mammals (OTTAVIANI et al., 1999). This is perfectly in line with experiments done by HENDRICKS & MASHALY (1998) and by HENDRICKS et al. (1995) showing that only a particular subset of avian splenic leucocytes, the macrophages, produce ACTH in response to corticotropin releasing hormone (CRH) stimulation and that this effect is inhibited by corticosterone.

Earlier experiments in mice also demonstrated that macrophage-like cells and other immune cells produce ACTH and β-END when stimulated with different stimuli such as LPS, Newcastle Disease Virus (NDV) and CRH (HARBOUR et al., 1991). Interestingly, different stimuli seem to result in differential processing of the POMC-derived peptides: Newcastle Disease Virus and CRH induce the production of POMC peptides with the molecular weight of $ACTH_{1-39}$ and β-END, whereas LPS leads to the production of a truncated form of ACTH ($ACTH_{1-24\ to\ 26}$) and β-END (α- or γ-END) (HARBOUR-MCMENAMIN et al., 1985; HARBOUR et al., 1987). These data seem to point towards the existence of distinct regulation mechanisms for the respective processing enzymes involved in the post-translational processing of the POMC precursor protein (HARBOUR et al., 1987).

Another important question that remains to be answered in the presently described avian in vitro model, is whether the observed POMC message will be translated and if the precursor protein will be properly processed into biologically active, secreted peptides. RT-PCR experiments using the primer set that spans the entire POMC coding region yielded a fragment of 678 bp, corresponding to the size of the pituitary POMC transcript as determined previously (GERETS et al., 2000), but since none of the primer sets used in this study included the message for the signal peptide, our present data do not allow us to answer the question.

However, since the entire coding region of POMC is synthesized in the HD11 cells, it is theoretically possible that the POMC-derived peptides are being translated and have a biological function in the cells of the macrophage lineage. It has been shown that POMC-derived peptides are important mediators in the overall response to endotoxin in immune cells (HARBOUR et al., 1991). MECHANICK et al. (1992) demonstrated that both POMC mRNA and ir-β-END exist in rat spleen and lung macrophages. Because immune cells possess receptors for POMC-derived peptides, it is possible that these peptides have autocrine and/or paracrine roles within these immune tissues (MECHANICK et al., 1992). β-Endorphin for example has been found in macrophages where it acts on opioid receptors to lessen pain (BLALOCK, 1994). GALIN et al. (1991) did not detect a full length POMC transcript in murine lymphocytes upon CRH treatment despite the inherent sensitivity of the PCR technique. Instead, two truncated POMC transcripts were observed that were quite different in structure in that one lacked the extreme 5' end of exon 3 while the other contained all of exon 3.

Finally, proteolytic cleavage of the POMC precursor is another requirement for the production of biologically active, secreted peptides. This implies expression of the prohormone convertases enzymes PC1 and/or PC2, which have been shown to be indispensable for cleavage of the POMC precursor (SEIDAH & CHRÉTIEN, 1992). The particular enzyme profile present within the cell indeed determines the nature of the peptides synthesized and secreted. Therefore, future studies will focus on the question whether the chicken equivalents of PC1 and/or PC2 are being expressed in chicken HD11 cells, in particular, and in avian immune tissues, in general.

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REFERENCES

BERGHMAN, L.R., B. DEVREESE, P. VERHAERT, H. GERETS, L. ARCKENS, J. VANDEN BROECK, J. VAN BEEUMEN, H. VAUDRY, & F. VANDESANDE (1998). The molecular characterization of chicken pituitary N-terminal pro-opiomelanocortin (POMC). *Mol. Cell. Endocrinol.,* 142 : 119-130.

BERTAGNA, X. (1994). Pro-opiomelanocortin-derived peptides. *Endocrinol. Metab. Clin. North. Am.,* 23 : 467-485.

BEUG, H., A. VON KIRCHBACH, G. DODERLEIN, J.-F. CONSCIENCE, & T. GRAF (1979). Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell*, 18 : 375-390.

BLALOCK, J.E. (1994). The syntax of immune-neuroendocrine communication. *Immunology Today*, 15 : 504-526.

DE BOLD, C.R., J.K. MENEFEE, W.E. NICHOLSON, & D.N. ORTH (1988a). Pro-opiomelanocortin gene is expressed in many normal human tissues and in tumors not associated with ectopic adrenocorticotropin syndrome. *Mol. Endocrinol.*, 2 : 862-869.

DE BOLD, C.R., W.E. NICHOLSON, & D.N. ORTH (1988b). Immunoreactive pro-opiomelanocortin peptides and POMC-like messenger ribonucleic acid are present in many rat non-pituitary tissues. *Endocrinology*, 66 : 2648-2657.

GALIN, F.S., R.D. LEBOEUF & J.E. BLALOCK (1991). Corticotropin-releasing factor upregulates expression of two truncated pro-opiomelanocortin transcripts in murine lymphocytes. *J. Neuroimmunol.*, 31 : 51-58.

GERETS, H.H.J., K. PEETERS, L. ARCKENS, F. VANDESANDE, & L.R. BERGHMAN (2000). Sequence and distribution of pro-opiomelanocortin in the pituitary and the brain of the chicken (*Gallus gallus*). *J. Comp. Neurol.*, 417 : 250-265.

GOETHE, R., & L. PHI-VAN (1998). Posttranscriptional lipopolysaccharide regulation of the lysozyme gene at processing of the primary transcript in myelomonocytic HD11 cells. *J. Immunol.*, 160 : 4970-4978.

HARBOUR-MCMENAMIN, D., E.M. SMITH & J.E. BLALOCK (1985). Bacterial lipopolysaccharide induction of leucocyte-derived corticotropin and endorphins. *Infect. Immun.*, 48 : 813-817.

HARBOUR, D.V., E.M. SMITH & J.E. BLALOCK (1987). Novel processing pathway for pro-opiomelanocortin in lymphocytes : endotoxin induction of a new prohormone-cleaving enzyme. *J. Neurosci. Res.*, 18 : 95-101.

HARBOUR, D.V., F.S. GALIN, T.K. HUGHES, E.M. SMITH & J.E. BLALOCK (1991). Role of leukocyte-derived pro-opiomelanocortin peptides in endotoxic shock. *Circ. Shock*, 35 : 181-191.

HENDRICKS G.L., M.M. MASHALY & H.S. SIEGEL (1995). Effects of corticosterone in vivo and in vitro on adrenocorticotropic hormone production by corticotropin releasing factor-stimulated leucocytes. *Proc. Soc. Exp. Biol. Med.*, 209 : 382-386.

HENDRICKS G.L. & M.M. MASHALY (1998). Effects of corticotropin releasing factor on the production of adrenocorticotropic hormone by leukocyte populations. *Br. Poult. Sci.*, 39 : 123-127.

HIRSCH M.D. & W.R. MILLINGTON (1991). Endoproteolytic conversion of beta-endorphin-1-31 to beta-endorphin-1-27 potentiates its central cardioregulatory activity. *Brain Res.*, 550 : 61-68.

LUGER T.A., T. SCHOLZEN & S. GRABBE (1997). The role of alpha-melanocyte-stimulating hormone in cutaneous biology. *J. Investig. Dermatol. Symp. Proc.*, 1 : 87-93.

LYONS, P.D. & J.E. BLALOCK (1995). The kinetics of ACTH expression in rat leukocyte subpopulations. *J. Neuroimmunol.*, 63 : 103-112.

MECHANICK, J.I., N. LEVIN, J.L. ROBERTS & D.J. AUTELITANO (1992). Proopiomelanocortin gene expression in a distinct population of rat spleen and lung leukocytes. *Endocrinology*, 131 : 518-525.

OTTAVIANI, E., A. FRANCHINI, A. COSSARIZZA & C. FRENCESCHI (1992). ACTH-like molecules in lymphocytes. A study in different vertebrate classes. *Neuropeptides*, 23 : 215-219.

OTTAVIANI, E., A. FRANCHINI & S. GENEDANI (1999). ACTH and its role in immune- neuroendocrine functions. A Comparative study. *Curr. Pharm. Des.*, 5 : 673-681.

SEIDAH, N.G. & M. CHRÉTIEN (1992). Proprotein and prohormone convertases of the subtilisin family. Recent developments and future perspectives. *TEM*, 3 : 133-140.

STAR, R.A., N. RAJORA, J. HUANG, R.C. STOCK, A. CATANIA & J.M. LIPTON (1995). Evidence of autocrine modulation of macrophage nitric oxide synthase by α-melanocyte-stimulating hormone. *Proc. Natl. Acad. Sci.*, 92 : 8016-8020.

TAKEUCHI, S., K. TESHIGAWARA & S. TAKAHASHI (1999). Molecular cloning and characterization of the chicken pro-opiomelanocortin (POMC) gene. *Biochim. Biophys. Acta*, 1450 : 452-459.