Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

SHORT COMMUNICATION

Expression of Clusterin Isoforms in LPS Treated Porcine Aortic Endothelial Cells

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ARTICLE HISTORY (14-187)

Received: April 15, 2014
Revised: July 03, 2014
Accepted: December 23, 2014

Key words:
Clusterin
LPS
pAEC
Small heat shock proteins

ABSTRACT

Clusterin (CLU) is a multifunctional protein whose expression has been related to many physiological and pathological processes. The most abundant gene product is the secreted form of CLU (sCLU) described as a prosurvival and cytoprotective protein. A nuclear CLU (nCLU) isoform involved in death and inhibition of cell growth has also been reported. The aims of the present work were to verify the differential presence of sCLU and nCLU in a primary culture of porcine aortic endothelial cells (pAEC) and to evaluate whether lipopolysaccharide (LPS) can influence their expression. LPS-treated pAEC expressed both mRNA isoforms but only sCLU protein was detected. LPS exposure induced transient mRNA and protein increases of sCLU at 1 h of treatment. In conclusion our data showed that in LPS-treated pAEC sCLU is the most abundant form of clusterin and nCLU can be considered irrelevant and is rarely translated.

To Cite This Article: Zannoni A, A Zaniboni, C Bernardini, E Seren, ML Bacci and M Forni, 2015. Expression of clusterin isoforms in LPS treated porcine aortic endothelial cells. Pak Vet J, 35(2): 236-238.

INTRODUCTION

The clusterin (CLU) protein plays several roles in biological processes such as cell maturation, lipid transportation, complement inhibition, tissue remodelling, cell-cell interactions, proliferation, cell survival and death (Wyatt *et al*., 2009).

The main transcript of CLU produces an unglycosylated precursor protein (pre-sCLU or p-CLU) that is further glycosylated and proteolytically cleaved into two (α and β) subunits, producing the secretory, mature and most abundant form of CLU (secreted CLU - sCLU, 75-80kDa). sCLU is described as an extracellular chaperone and it is considered a prosurvival/ cyto-protective protein (Wilson and Easterbrook-Smith, 2000).

Alternative splicing variants of the CLU mRNA, that lacks exon 2, were reported in MCF 7 cell lines after X-ray treatment (Leskov *et al*., 2003) and in other cancer and non-cancer cells types (Prochnow *et al*., 2013). The protein product is detected as a precursor in the cytosol (pn-CLU or c-CLU, ~49kDa) and as a glycosylated protein in the nucleus (nuclear CLU- nCLU 55kDa). The latter is considered as an inducer of cell death and a cells growth and survival inhibitor (Leskov *et al*., 2011). LPS induces apoptosis in *in vitro* endothelial cells cultures of different animal species and in *in vivo* models. Our previous research on primary culture of porcine aortic endothelial cells (pAEC) indicated that LPS is able to induce the so-called "heat shock response" consisting in an increase of non specific protective molecules, such as Hsp70 and small HSPs as HO-1 (Bernardini *et al*., 2010; Bernardini *et al*., 2012). Considering the different roles of CLU, as pro-survival (sCLU) and/or proapoptotic (n-CLU), the aim of the present study was to investigate, in a well characterized model of LPS-induced apoptosis in pAEC, the presence of sCLU and nCLU.

MATERIALS AND METHODS

Clusterin PCR and mRNA Exon 1 sequencing: In order to sequencing the initial portion of the gene not available in NCBI database for pig a well described model of apoptosis was used. Ovaries from non-pregnant sows were collected at a local slaughterhouse and granulosa cells were recovered from atretic follicles. A forward primer (5'-GCGTGAGTCATGCA GGTTTG-3') was designed on exon 1 (position 1-21) on the alignment among the bovine (BC_149632) and the human (NM_001831) sequences; reverse primer (5'-GCTCTTCATTTGATTGTTCTATTAGG-3') (position 365-348) was designed on the basis of porcine sequence (AN: NM_213971.1). The PCR products were purified and sequenced (BMR, Padova, Italy).

Porcine aortic endothelial cells (pAEC) culture and LPS treatment: Porcine Aortic Endothelial Cells

(pAECs) were isolated and cultured as previously described (Bernardini *et al*., 2010; Bernardini *et al*., 2012). LPS (E. coli 055:B5, Sigma-Aldrich Co, St Louis, MO, USA) (10 µg/ml) was added to the culture medium for different time (1, 7, 15 and 24 hours).

Real-time PCR quantification (qPCR) of CLU isoforms: Total RNA extraction from pAEC, cDNA synthesis and qPCR were performed according our previous studies (Bernardini *et al*., 2012). Forward (5'-CTGCTGACCTGGGAGAATGG-3') and reverse (5'-GCTCTTCATTTGATTGTTCTATTAGGG-3') primers for porcine Long Clusterin (L-CLU) and forward (5'-GGGCACCGCTGACTGAAATG-3') and reverse (5'-CGCTCTTCATTTGATTGTTCTATTAG GG-3') primers for porcine Short Clusterin, (S-CLU) were designed on the determined sequences (JQ840388 and JQ840389).

Western Blot analysis for intra-cellular CLU: After LPS treatment (1, 7, 24 hours), pAEC were harvested and lysed in SDS solution (Tris-HCl 50mM pH 6.8; SDS 2%; glycerol 5%). The immunodetection of clusterin was performed using an anti-human clusterin antibody (05-354 Upstate Biotech; 1:1000 dilution, overnight at 4°C). The relative protein content (protein of interest/β Tubulin) was expressed as arbitrary units (AU).

Western Blot analysis for secreted CLU in culture medium: Collected culture media (210 µl) were placed into Microcon YM-100 column (Amicon, Millipore, Billerica MA, USA) and centrifuged at 12000xg for 1 min. The filtrated medium was freeze dried under vacuum centrifuge and resuspended in 15 µl of water. Five µl of resuspended medium was used in western blot analysis.

Statistical Analysis: Each treatment was replicated three times. Data were analyzed by a one-way analysis of variance (ANOVA); the significant differences ($P<0.05$) were analyzed by the Duncan's test (SPSS program version 13.0; SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

Our data showed for the first time that, in granulosa cells of atretic follicles, a well described model of apoptotic cell death (Zwain and Amato, 2000), the primers used were able to amplify two Clusterin mRNA alternative sequences: the Long-CLU (365bp) and the Short-CLU (230bp) generated by alternative splicing of exon 2 (Fig. 1). The two cDNA sequences were published in the NCBI database (http://www.ncbi.nlm.nih.go) with Accession Numbers JQ840388 and JQ840389. The Long-CLU sequence aligns with the human mRNA and is consistent with the mature, secreted, prosurvival and cytoprotective sCLU. The sequence of the Short-CLU transcript, herein described, showed that exon 1 is directly linked to exon 3, and is consistent with the non-secreted isoform nCLU described by Leskov *et al*. (2003).

In pAEC the mRNA expression level of Long-CLU was definitely higher than Short-CLU and LPS induced a transient increase of about 2.5 times of the Long-CLU mRNA whereas Short-CLU mRNA was not affected (Fig.

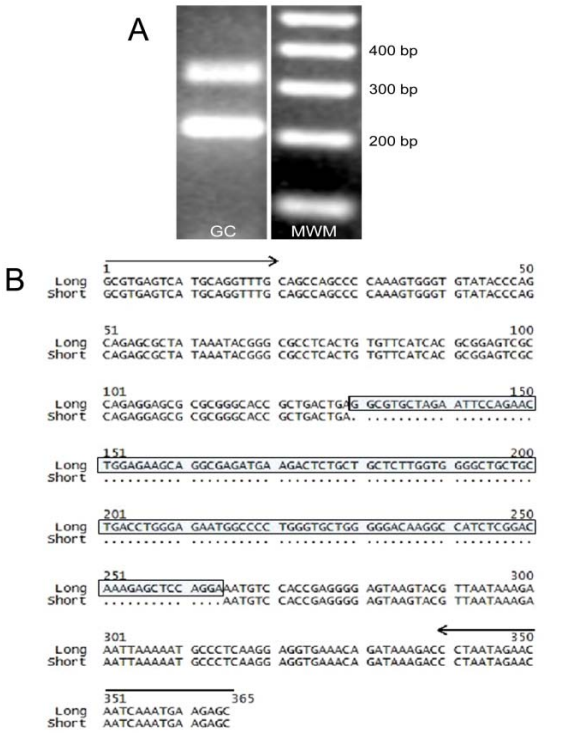

Fig. 1: Clusterin isoforms and sequence of PCR products. Representative gel electrophoresis (2%) of two alternative amplicons (365bp and 230bp) from cDNA of granulosa cells (GC) of porcine atretic follicles. MWM= molecular weight marker. (A). Sequence of PCR products obtained. The rectangular box indicates the spliced exon II sequence (B).

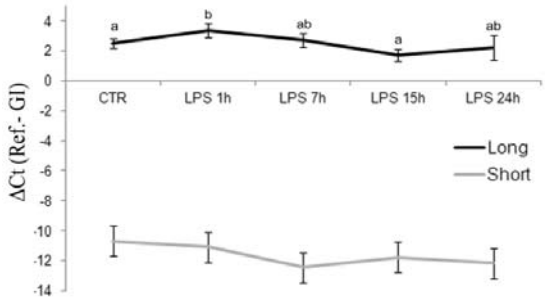

Fig. 2: Expression levels of different CLU isoforms in LPS-treated pAEC (10 µg/ml). mRNA expression level of Long-and Short-CLU in pAEC during LPS treatments and in standard culture condition (CTR). Relative mRNA data are shown as ΔCt (Ref-GI) which is directly correlated with expression level (Ref. = Reference gene; GI = Gene of Interest).

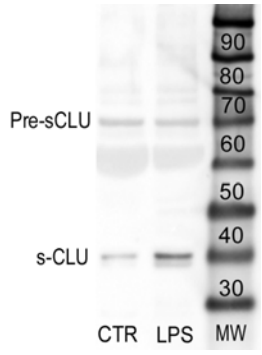

Fig. 3: Western blot of clusterin in pAEC. Representative image of a western blot of clusterin in control (CTR) and LPS-treated pAEC.

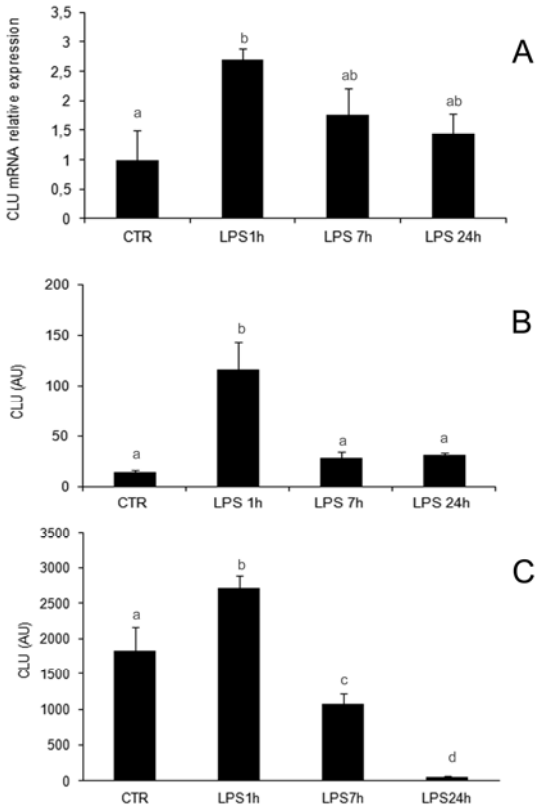

Fig. 4: Expression of secreted clusterin in LPS-treated pAEC (10 μg/ml). Long CLU mRNA expression ($2^{\Delta\Delta CT}$ method) in relation to the standard culture condition (CTR) (A). sCLU protein expression (B). sCLU protein level in culture media (70 μl) (C).

2). At protein level, different clusterin isoforms (pre-sCLU, nCLU, sCLU) were well described only in human colon tumorigenesis (Pucci *et al.,* 2004), our data showed the presence of clusterin protein of about 40kDa corresponding to the glycosilated subunit of mature secreted isoform (sCLU) (Fig. 3). Further bands (~70-60kDa), ascribable to the uncleaved precursor holoprotein forms (pre-sCLU), were also evidenced (Fig. 3). No bands ascribable to nuclear form (nCLU, 55kDa) were detected in control nor in LPS treated cells. In parallel with the Long-CLU mRNA variation (Fig. 4A), LPS induced a transient significant increase of sCLU protein in cells (Fig. 4B) and in the culture media (Fig. 4C).

According to previous study on LPS-treated microglial cells (Saura *et al.,* 2003), our data showed that LPS influences sCLU expression in pAEC inducing both mRNA and protein upregulation. The precocious induction of sCLU (1 h of LPS) is consistent with the well-known function of small HSP that, together with Hsp70, protect the stressed cells by stabilizing proteins, and preventing stressed protein precipitation (Wyatt *et al*., 2009). In conclusion, our data confirm that the sCLU is the most abundant form of clusterin also in pAEC and that Short-CLU can be considered irrelevant and rarely translated also in LPS-induced damage of endothelial cells as in other cell models (Prochnow *et al*., 2013).

Acknowledgment: This work was supported by RFO and Fondazione del Monte Bologna-Ravenna.

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