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Changes in protein expression profiles in bovine endometrial epithelial cells exposed to E. coli LPS challenge

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Abstract

E. coli is one of the most frequently involved bacteria in uterine diseases. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria involved in pathogenic processes leading to post-partum metritis and endometritis in cattle. It also causes inflammation of the endometrium. The increase of cell proliferation by LPS is part of the inflammatory process. The aim of this study was to investigate possible changes in protein expression in relation to the proliferative response of bEECs after challenge with E. coli-LPS. In vitro culture of bEECs was performed from cow genital tracts collected at a slaughterhouse. In passage 5, bEECs from each of 9 cows (3 series of 3 cows) were exposed to 0, 8, and 16 μg ml−1 LPS for 72 h. At time 0 and 72 h later, attached cells/living cells were counted and for each time and

LPS dosage, cells were frozen for proteomic analyses. All samples from the 3 series were analyzed by 2-D gel electrophoresis coupled to MALDI-TOF/TOF mass spectrometry. The samples from the first series were subjected to shotgun nLC-MS/MS analysis. From the whole differential proteomics analysis, 38 proteins were differentially expressed ($p < 0.05$ to $p < 0.001$) following exposure to LPS. Among them, twenty-eight were found to be up-regulated in the LPS groups in comparison to control groups and ten were down-regulated. Differentially expressed proteins were associated with cell proliferation and apoptosis, transcription, destabilization of cell structure, oxidative stress, regulation of histones, allergy and general cell metabolism pathways. The de-regulations induced by LPS were consistent with the proliferative phenotype and indicated strong alterations of several cell functions. In addition, some of the differentially expressed proteins relates to pathways activated at the time of implantation. The specific changes induced through those signals may have negative consequences for the establishment of pregnancy.

Introduction

Due to negative genetic correlations between milk production traits, and reproduction and health traits[1] the genetic selection for high milk production potential has been associated in dairy cows with a reduction in fertility and an increased sensitivity to diseases.[2,3] Modern dairy cows are at high risk of suffering from uterine diseases following calving and the number of exposed cows reached several millions per year in the EU.[2–5] Exposed cows have low fertility, extended unproductive periods with high culling rates[5,6] thus affecting herd economy since rearing replacement animals is one of the main sources of economic losses and welfare due to short life. More costs result from treatment and associated milk withdrawal. The total cost of diagnosed uterine diseases for farmers, dairy and breeding industries has been reported to reach 1.4 billion € per year in the EU.[6] This figure may be underestimated due to undiagnosed forms of uterine dysfunction leading to idiopathic infertility.

Dystocia and retained placenta predispose to uterine diseases due to disruption of physical barriers to infection and perturbation of immune responses that should eliminate pathogens.[7] Metabolic imbalance is also influencing these processes as strong negative energy balance was reported to depress gene expression in relation to immunity in uterine tissue.[8–10]

In most epithelia, specific strains of *Escherichia coli* (*E. coli*) are major sources of infection. For the cow endometrium, *E. coli* is one of the most prevalent bacteria isolated in metritis[6,11,12] and paves the way for infection by other bacteria or viruses.[13,14] A major component explaining the pathogenicity of *E. coli* is related to the interaction of LPS with the host tissue.[12,15]*E. coli* LPS provokes the inflammation of the endometrium through a cascade of events well conserved in different tissues. The presence and the involvement of TLR4 in epithelial and stromal cells of the bovine endometrium has already been demonstrated by Cronin and colleagues.[16] LPS binding to Toll like 4 receptors triggers the production of pro-inflammatory cytokines, the attraction of immune cells in stromal tissue and a shift between PGF2α and PGE2 production by the endometrium. The deregulation of cytokines, chemokines, growth factors and major histocompatibility complexes (MHCs) is a part of the inflammatory processes affecting epithelial barriers. Diagnosed clinical symptoms are the basis for treatment. However, acute uterine infections are often followed by an asymptomatic persistent inflammation which remains untreated. For instance in the cow, it has been shown that the undiagnosed persistence of inflammation of the endometrium following infection contracted at parturition[7,17] perturbs later the embryo–maternal interactions necessary to establish successful implantation thus impairing fertility.[5,6] However, the role of endometrial cells and more especially epithelial cells in the persistence of inflammation and mechanisms involved in infertility at this stage of pregnancy still have to be elucidated.

Successful implantation requires a balanced and accurate molecular communication between conceptus and maternal endometrium. Even a small imbalance due to former bacterial infection/LPS stimulation could affect negatively the dialogue between the mother and the embryo necessary for the establishment of pregnancy.[18] The increased activation of NFκB and the secretion of pro-inflammatory and chemotactic cytokines that proved the activation of the CD14 receptor was demonstrated using bronchial epithelial cell lines.[19] The occurrence of endometritis linked to the production of cytokines has already been investigated through the analysis of mRNA expression 20. The overexpression of IL-1alpha and IL-1-RN mRNA and the down-regulation of cPGES mRNA have been reported in cows with subclinical endometritis when compared to healthy cows. The authors found as well that the expression of CXCL5, IL1B, IL8 and TNF mRNA was significantly higher in cows with subclinical or clinical endometritis. This result came from the analysis of

mRNA expression of the transcripts involved in prostaglandin synthesis in the bovine endometrium.[20,21] The deregulation of these pathways induced by LPS in endometrial cells was further illustrated by Oguejiofor *et al.*, 2015, while using a wider transcriptomic approach.

The differential protein expression in caruncular and intercaruncular areas during the peri-implantation period has been described in ewes.[18] The authors used LC-MS/MS technique and highlighted the important role of structural proteins such as actin in the implantation process.

The differential proteomics profiling of cows with endometritis has already been performed by using 2D electrophoresis on endometrial tissue.[22] Among differentially expressed proteins between healthy cows and endometritis cows, proteins such as peroxiredoxin and heat shock proteins were over-expressed.[22]

As described above, the response of endometrial tissue in terms of pro-inflammatory factors has already been documented from *in vivo* materials generally combining different types of cells resulting in some heterogeneity of the analyzed tissue. Other limitations may have resulted from former proteomic approaches. Due to this, in the present study we investigated the changes induced by LPS at different concentrations on a homogenous population of post-primary bovine endometrial epithelial cells (bEECs) and by using two complementary proteomics approaches (2D electrophoresis and shotgun MS analysis). By using this combination, it was expected to find protein patterns that may reveal the consequences of previous infection in the endometrial epithelium.

The information obtained here showing that a multiplicity of pathways are deregulated by LPS provides new insights on the mechanisms involved in persistent inflammation following bacterial infection and suggests new perspectives to limit the impact of inflammation on the endometrial epithelium.

Materials and methods

Sample selection and endometrial epithelial cell culture

Bovine uteri without any morphological signs of inflammation were collected from a slaughterhouse and brought back on ice to laboratory within 1 hour of collection. The stage of the estrus cycle was determined at first from the ovarian morphology and the presence or absence of mucus in the uterine body. Genital tracts from 9 cows, showing an orange CL >15 mm diameter and without abundant mucus, were estimated

to be associated with luteal phase 23 and subsequently used for cell culture. The stage of the cycle was further confirmed by histology from measurement of the number of cross-sections of the glands in full sections of the endometrium taken 5 cm from the tip of the horn 24. The left uterine horns were dissected and cut into 5–6 cm long and 4–5 mm deep pieces. Uterine tissue was digested with collagenase IV (C5138, Sigma) and hyaluronidase (250 U $m1^{-1}$; H3506, Sigma) diluted in PBS (phosphate-buffered saline) plus 2% BSA while stirring for 2 hours at 39 °C. The suspension was then filtered through a 250 µm gauze to remove residual mucus and undigested tissue. The filtrate was then passed through a 40 µm nylon sieve, which allowed the fibroblast and blood cells to pass through while epithelial cells were retained. Epithelial cells were collected from the filter by backwashing with 30 ml PBS. Cells were centrifuged at 170*g* for 6 minutes and the pellet was re-suspended in 3 ml of PBS. To disperse the pellet into a single cell suspension, cells were disrupted by passing through a fine gauge needle. Then, cells were cultured in F-12 medium (Dulbecco's modified eagle's medium, Sigma D6434) containing 10% Fetal Bovine Serum (FBS), 50 U ml^{-1} of penicillin/streptomycin, 2 mM l-glutamine, 0.5 × Liquid Media Supplement (ITS, I3146, Sigma), 10 µg ml^{-1} gentamycin and 100 U ml^{-1} nystatin. Cells were seeded into a 25 cm^2 ventilation flask and cell cultures were kept in a water-jacked incubator with 5% CO_2 at 39 °C. The medium was changed every 1–2 days. Sub-cultivations were performed at 5–6 days when epithelial cells attained 80 to 90% confluence. The process was renewed until passage 4 and the cells were exposed to LPS challenge. LPS powder was dissolved in 5 mg ml^{-1} water as a stock. Before challenge, the adjusted concentrations of LPS and media were completely mixed. At 72 hours following LPS challenge, the supernatant was removed and non-adherent (floating) cells in media were counted under a microscope by using a burker neubauer chamber (hemocytometer, 40443001, Hecht Assistent®, Rhon, Germany). The attached cells were then detached with trypsin (TrypleTMExpress, gibco®, Waltham, USA). The cells were exposed systematically two times to 4 ml trypsin at 39 °C for 4 minutes. Flasks were then checked for the remaining cells. This protocol was applied again when some cells remained attached. After trypsinization, all cells were pipetted from flasks and then transferred into 15 mL falcon tubes. The solution was gently mixed for 2–3 seconds and 70 µl was taken and mixed with the same volume of trypan blue solution (T8154 trypan blue solution 0.4%, Sigma®, St Louis, MO, USA) in Eppendorf tubes. Then, 10 µl of the mixed solution was immediately transferred to the counting

chamber (same as above) following manufacturer's instructions. The living cells were detached with trypsin and pellets of 2 to 3 million post-primary cells per group were collected and immediately deep frozen (−80 °C) until subsequent proteomic analyses.[23]

LPS challenges

In preliminary experiments, epithelial endometrial cells were exposed to a wide range of concentrations of LPS reflecting concentrations found in uterine fluid in the case of clinical endometritis[24] and cell survival was estimated.[25] The dosages of LPS used in the present experiment (Sigma L2630 *E. coli* O111:B4, 0 as controls, 8 and 16 μg ml^{-1}) were chosen according to epithelial cell survival at 72 hours which was shown to be maximal with 8 μg ml^{-1} LPS (+30 to +40% living cells when compared to controls). This increase in cell survival progressively vanished with increasing dosage, cell survival being not different from controls following addition of 16 μg ml^{-1} and being lower than controls at higher dosages.

LPS challenges (0, 8 and 16 μg ml^{-1} LPS) were applied on cells issued from 9 different cows (in 3 series of 3 cows). Living cells were counted by trypan blue staining and cell survival profiles at 72 hours obtained from this precise subset of cows, measured as (number of cells “LPS treated” – number of cells “controls”/number of cells “controls”) were analyzed by ANOVA (SAS ver 9.2, proc GLM). Effects of the cow, series of experiments and LPS dosage on cell survival were analyzed by ANOVA (SAS Ver 9.2, proc GLM). The cow ID (9 levels; 3 different cows in each of 3 series), the series No (3 levels) and LPS dosage (2 levels) were included in the model as well as LPS × cow and LPS × series interactions. The tests were made while comparing the LS mean ratio, observed for a given factor (or second order interactions) to 0, which should be the mean value observed if these ratios were distributed at random. LS mean ratios (either negative or positive) corresponding to the effect of LPS differing from 0 at $p < 0.05$ were considered as significant.

Proteomics analyses

Proteomics analyses have been performed according to Fig. 1. All 9 biological replicates have been analyzed in groups of three through 2D electrophoresis followed by MALDI TOF MS for protein identification. Moreover, the first three biological replicates have been analyzed through label-free nanoflow liquid chromatography mass spectrometry (nLC-MS^E) analysis and each run was performed in triplicate.

Extraction of cell samples

Cellular pellets (cell amounts per pellet varied from 1.6×10^6 to 3.3×10^6) have been solubilized in a buffer containing 7 M urea, 2 M thiourea and 2% chaps with protease inhibitors. Briefly, the frozen pellet has been defrosted in ice and centrifuged for 10 minutes at 14 000 rpm for 10 minutes. The supernatant was carefully discarded and remaining pellets then solubilized with 20 µl of the above described buffer (7 M urea, 2 M thiourea and 2% CHAPS). The samples were solubilized with 2 cycles of 1 h under magnetic gentle stirring interspersed by 1 h at room temperature. The samples have been subsequently sonicated for 20 minutes and then centrifuged for 10 minutes at 10 000 rpm at room temperature. The pellet has been discarded and the supernatant with the extracted proteins frozen at −20 °C until use. Protein quantification has been performed using a BioRad Protein Assay quantification kit.

2D electrophoresis and image analysis

From each sample, 100 micrograms of protein have been loaded on a 7 cm strip through active rehydration performed overnight at 50 V in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% Ampholytes 3–10 Amersham, and 26 mM DTT. Isoelectric focusing (IEF) was performed on a protean IEF platform using the following protocol: 100 V/1 h linear, 250 V/2 h linear, 4000 V/5 h linear, 4000 V step/50 000 total volt-hours (VhT). When the final amount of VhT was reached, immobilized pH gradient (IPG) strips were frozen up to the next step or directly equilibrated in two steps of 15 minutes under gentle stirring. The first one was performed in equilibration buffer (6 M UREA, 2% SDS, 0.05 M Tris-HCl pH 8.8, 20% glycerol) supplemented with 1% DTT w/v and the second one with the addition of 2.5% w/v iodoacetamide. After this equilibration step, the IPG strips were loaded in a 12% home made acrylamide gel and IEF run under constant amperage of 15 mA per gel until the Bromophenol Blue (BFB) reached the front.

The gels were then removed from the plates, washed three times with double-distilled water and stained overnight (ON) with Coomassie Brilliant Blue.

Gels were digitized using an Imagescanner III (GE Healthcare) and image analysis was performed using SameSpots software (Version 4.5, Nonlinear Dynamics U.K.). Spots with a *p* value lower than 0.05 were manually excised and subjected to mass spectrometry (MS) analysis and protein identification. The protein

identification was performed according to the methodological protocol previously described.[26–29] Briefly, after steps of dehydration, reduction and alkylation, single spots were digested with a solution of 0.01 µg µl^{-1} of porcine trypsin (Promega, Madison, WI) at 37 °C for 16 h. Peptides were concentrated using C18 ZipTip (Millipore) and then were spotted on a Ground Steel plate (Bruker-Daltonics, Bremen, Germany).

MALDI TOF TOF MS analysis

The peptide mass fingerprinting analysis was performed according to Piras *et al.*[30]

Briefly, the MS analysis was performed on an Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics) in positive reflectron mode and MS spectra were analyzed by FlexAnalysis 3.3 software (Bruker-Daltonics) to select monoisotopic peptide masses. The external calibration was done by the standard peptide mixture calibration (Bruker-Daltonics: *m*/*z*: 1046.5418, 1296.6848, 1347.7354, 1619.8223, 2093.0862, 2465.1983, 3147.4710).

After an internal calibration (known autolysis peaks of trypsin, *m*/*z*: 842.509 and 2211.104) and exclusion of contaminant ions (known matrix and human keratin peaks), the created peak lists were analyzed by MASCOT v.2.4.1 algorithm (www.matrixscience.com) against the SwissProt database released 2013_12 (25 245 entries) restricted to *Bos taurus* taxonomy. Database search was performed according to these parameters: carbamidomethylation of cysteines as fixed modification; oxidation on methionines as variable modification; one missed cleavage site set for trypsin; and maximal tolerance was established at 70 ppm. For protein identification assignment, only Mascot scores higher than 56 were considered as significant ($p < 0.05$).

To confirm the PMF identifications, the instrument was switched in LIFT mode with 4–8 × 10^3 laser shots using the instrument calibration file. For the fragmentation, precursor ions were manually selected and the precursor mass window was automatically set. Each MS/MS spectrum acquired was processed by spectral baseline subtraction, smoothing (Savitsky–Golay) and centroiding using Flex-Analysis 3.3 software. For search analysis, the following parameters were set: carbamidomethylation of cysteines and oxidation on methionine, respectively, among fixed and variable modifications; maximum of one missed cleavage; the mass tolerance to 50 ppm for precursor ions and to a maximum of 0.5 Da for fragments. The taxonomy was restricted to Bos taurus. The confidence interval for protein identification was set to 95% ($p < 0.05$) and only

peptides with an individual ion score above the identity threshold were considered as correctly identified.

Statistical analysis for validation of 2-DE datasets

For a subset of 5 proteins, the repeatability of the results over the 3 series of analyses has been studied. From mean MS results from the 3 technical replicates of each sample the ratio of expression when compared to the control was calculated and the results were analyzed by ANOVA (SAS Ver 9.2, proc GLM). The cow ID (9 levels; 3 different cows in each of 3 series), the series No (3 levels) and LPS dosage (2 levels) were included in the model as well as LPS × cow and LPS × series interactions. The tests were conducted while comparing the LS mean ratio, observed for a given factor (or second order interactions) to 0, which should be the mean value observed if these ratios were distributed at random. For a given protein, ratios of expression (either negative or positive) corresponding to the effect of LPS differing from 0 at $p < 0.05$ were considered as significant through the 3 series of experiments.

Expression analysis by nLC-MS^E

Label-free nanoflow liquid chromatography mass spectrometry (nLC-MS^E) was performed as previously described in ref. 30–32. Briefly, the protein extracts of nine biological samples (3 dosages LPS from cell culture from 3 cows) were precipitated with a cold mix of ethanol, methanol, and acetone (ratio 2 : 1 : 1, v/v), then dissolved in 6 M urea, 100 mM Tris pH 7.5 and digested 50 : 1 (w/w) with sequence grade trypsin (Promega, Madison, WI, USA) at 37 °C overnight after reduction with 10 mM DTT and alkylation with 20 mM IAA. The reaction was stopped by adding a final concentration of 0.1% TFA. Separation of tryptic peptides and subsequent qualitative and quantitative nLC-MS^E analysis were performed using a nanoACQUITY UPLC System (Waters Corp., Milford, MA) coupled to a Q-Tof Premier mass spectrometer (Waters Corp., Manchester, U.K.). An amount of 200 fmol μl^{-1} of digestion of Enolase from *Saccharomyces cerevisiae* was added to each sample as an internal standard, then a final concentration of 0.6 µg of protein digestion was loaded onto a column for peptide separation. Peptides were loaded onto a Symmetry C18 5 µm, 180 µm × 20 mm precolumn (Waters Corp.) and subsequently separated by a 170 min reversed phase gradient at 250 nL min^{-1} (3–40% CH_3CN over 145 min) using a NanoEase BEH C18 1.7 µm, 75 µm × 25 cm nanoscale LC column

(Waters Corp.) maintained at 35 °C. The Q-Tof Premier mass spectrometer was directly coupled to the chromatographic system operated in "Expression Mode" switching between low (4 eV) and high (15–40 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over 50–1990 *m*/*z*. The processing of low and elevated energy, added to the data of the reference lock mass, provides a time-aligned inventory of accurate mass-retention time components for both the low and elevated-energy (EMRT, exact mass retention time).

Each sample was run in three technical replicates. For qualitative and quantitative analysis, LC-MS data from three replicate experiments for each nine samples were processed using ProteinLynx GlobalServer v.3.0.2 (PLGS, Waters Corporation). Protein identifications were obtained with the embedded ion accounting algorithm of the software searching into the UniProtKB/Swiss-Prot Bos taurus database release 2013_12 (25 245 entries) to which the sequence of enolase (UniProtKB/Swiss-Prot AC: P00924) was appended. The search parameters were set at: automatic tolerance for precursor ions and for product ions, minimum 3 fragment ions matched per peptide, minimum 7 fragment ions matched per protein, minimum 2 peptide matched per protein, 1 missed cleavage, carbamydomethylation of cysteines and oxidation of methionines as fixed and variable modifications, false positive rate (FPR) of the identification algorithm under 1% and 200 fmol of the enolase internal standard set as calibration protein concentration. The most reproducible proteotypic peptides for retention time and intensity of enolase digestion (*m*/*z* 814.49; *m*/*z* 1159.59; *m*/*z* 1288.70; *m*/*z* 1755.95; *m*/*z* 1840.91, *m*/*z* 2441.12) were used to normalize the EMRT table. The expression analysis was performed considering 3 technical replicates available for each experimental condition (*i.e.*, one experimental condition, control, LPS 8 and LPS 16 groups, × three biological replicates × three technical replicates). The list of normalized proteins was screened according to the following criteria: protein identified in at least 2 out of 3 runs of the same sample with a fold change of regulation higher than ±20%; we considered significant only differentially expressed proteins with a *p* value < 0.05. Finally, the GO molecular function classification from the Panther Classification System[33] was used to allocate the differentially expressed proteins into functional groups according to the best fitness in the biological system analyzed.

Results

Cell survival profiles following LPS challenges

The profile of cell survival following LPS challenges made in the subset of cows used in this study was very similar to those observed in former experiments. A strong increase in cell survival was observed with the 8 µg ml^{-1} LPS dosage (+24%, $p < 0.0001$) whereas results were not different from controls for the 16 µg ml^{-1} LPS group (0.4%, NS).

Proteomic profiling from 2D electrophoresis coupled with MALDI TOF TOF

From 2D electrophoresis and imaging analyses a total of 1096 different spots were visualized (Fig. 2) from pellets of endometrial epithelial cells and proteins subsequently identified from MALDI TOF TOF MS analysis. From those, a total of 7 proteins were found to be differentially expressed between controls and LPS treated samples (Table 1).

The results obtained by 2D electrophoresis coupled with MALDI TOF identification revealed that response to LPS was similar over the 3 groups of experiments for 5 proteins especially for the 8 µg ml^{-1} dosage whereas response was more variable for the 16 µg ml^{-1} LPS dosage. For all these proteins no significant interaction between LPS dosage and series was found. Annexin 2 was significantly under-expressed for the 8 µg ml^{-1} dosage ($p < 0.02$) but only a similar trend was observed for the 16 µg ml^{-1} LPS dosage (Fig. 4). In contrast, Eukaryotic Initiation Factor 4A1 (EIF 4A1), Protein Disulfide isomerase (PDIA3), Superoxide Dismutase and Transketolase were significantly over-expressed following the 8 µg ml^{-1} LPS challenge ($p < 0.001$ to $p < 0.05$). Similar effects were seen with 16 µg ml^{-1} but only a trend was seen for EIF 4A1. For all proteins, differences between 8 and 16 µg ml^{-1} LPS are non-significant.

Proteomic profiling from shotgun MS and analysis of differentially expressed pathways

Shotgun MS analysis qualitatively identified a total of 226145 EMRTs and 160 proteins across all conditions. Quantification was performed following analysis of EMRTs and protein normalization.[34,35] The overall statistical analysis from shotgun MS highlighted 35 differentially

296 expressed proteins between the three different experimental groups (Table 1 and Fig. 5). These
297 divide into 25 up-regulated and 10 down-regulated for at least one of the LPS dosages when
298 compared to controls ($p < 0.05$).

299 From the Panther Classification System and using GO Molecular function classification the
300 differentially expressed proteins clustered well in structural proteins, metabolism proteins
301 (energy metabolism), transcription and protein synthesis, oxidative stress, cell
302 growth/apoptosis, immune response, and chromatin DNA binding pathways.

303 **Structural/Cytoskeleton, calcium metabolism and membrane properties**

304

305 Structural proteins such as actin, peripherin and related proteins that contribute to actin
306 stabilization and anchorage on the plasma membrane such as radixin and tropomyosin alpha 1
307 chain are strongly down-regulated in both LPS groups in comparison to the control group. In
308 contrast, Tropomyosin alpha 3 chain is up-regulated with both LPS dosages. Proteins involved
309 in cell membrane function and calcium metabolism are either down- (annexin A1 and annexin
310 A2, both LPS dosages) or up-regulated (calreticulin both LPS dosages).
311 Protein disulfide isomerase (P4HB) and protein disulfide isomerase A3 (PDIA3) are both up-
312 regulated. In addition, a strong down-regulation of a protein with proteolytic activity
313 (Cathepsin) and an up-regulation of a strong inhibitor of endopeptidases (Cystatin-B) in the
314 LPS8 group *versus* control group were observed.

315 **Energy metabolism**

316

All proteins clustering in this category are strongly up-regulated for both LPS dosages. This includes phosphoglycerate mutase, glyceraldehyde-3-phosphate deshydrogenase, triosephosphate isomerase and β enolase, all of them being part of the glycolytic process.

Oxidative stress

All proteins involved in oxidative stress response and protein folding and refolding have been found to be over-expressed following LPS treatment. Among them peroxiredoxin, protein disulfide-isomerase, protein disulfide-isomerase A3, endoplasmin, SH3 domain binding glutamic acid rich like protein 3 and heat shock related 70 kDa protein-2. For most of them over-expression is a little bit higher with the 16 than for the 8 μg ml^{-1} LPS dosage, but the response is already very significant with 8 μg ml^{-1}.

Transcription processes

Proteins such as transcription, initiation and elongation factors and ribonucleoproteins involved at different steps of the transcription process have been also found to be over-expressed in the groups of cells challenged with LPS. This includes elongation factor 1-alpha, elongation factor 1-delta, elongation factor 2, eukariotic initiation factor 4A-1 (EIF4A-1), heterogeneous nuclear ribonucleoprotein A1 and 60S acidic ribosomal protein P2. For all of them except EIF4A-1 changes in expression are more pronounced for the 16 than for the 8 μg ml^{-1} LPS dosage.

Cell growth/cell cycle/proliferation/apoptosis

Differentially expressed proteins involved in these pathways dispatch in up- (78 kDa glucose-regulated protein, phosphoglycerate kinase 1) and down-regulated ones (three different types of metallothioneins (-1, -1A, -2) and galectin-1). Galectin-1 (regulating apoptosis) is down-regulated for both LPS dosages whereas the 3 metallothioneins (involved in negative regulation of growth/proliferation) are only up-regulated significantly for the 8 µg ml^{-1} LPS dosage.

Immune response

Histone H2B type 1 (involved in innate immune response in mucosa as a defense mechanism against bacteria) and 14-3-3 protein zeta delta (involved in response proteins from bacterial origin) are strongly up-regulated with the 16 µg ml^{-1} LPS dosage (no change with 8) whereas alpha enolase (stimulating IgG production, pro-inflammatory and involved in allergy mechanisms) is strongly up-regulated with both LPS dosages.

Chromatin/DNA binding proteins

Histones H2A type 1, H2A.J, and H2B type 1-K are more strongly down-regulated with the 16 than with 8 µg ml^{-1} LPS dosage whereas Histone H4 is more de-regulated with 8. In contrast, Histone H2B type 1-N has been found to be up-regulated in the LPS16 group.

The whole panel of differentially expressed proteins, their relationships and sense of deregulation have been represented through an interactomic analysis performed with STRING 10[36] (Fig. 6).

Discussion

LPS used at concentrations in the range of those found from *in vivo* studies at 72 hours after challenge of bEECs induce either a strong increase in cell number (8 μg ml^{-1} dosage) or no change in epithelial cell survival (16 μg ml^{-1} dosage).[25] These results are in agreement with studies reporting that LPS induced an increase in epithelial cell proliferation from different tissues.[37–39] Differences with results showing an inhibition of epithelial cell number[40,41] could be partly due to the dosage of LPS that induced an inhibition with the highest LPS dose (16 μg ml^{-1} dosage). However, despite this increase in the number of living cells with a normal morphological appearance, the results of the present study reveal that many changes in protein expression occur following exposure of bEECs to *E. coli* LPS showing that several functions of these cells were highly de-regulated. The consequences of observed changes in protein expression for cell function and possible implications for implantation will be discussed according to the molecular function retrieved from GO annotations.

Structural/cytoskeleton, calcium metabolism and membrane properties

Actin and peripherin have been found to be down-regulated with both LPS dosages. These proteins play a key role as structural constituents of cytoskeleton. Such changes in actin remodeling after LPS stimulation have already been described in macrophages and changes in cell mobility have been reported from the same study.[42] The down-regulation of actin 2 in this model could also be linked to a rearrangement of other structural proteins following LPS stimuli. This is supported by changes in the regulation of PHB4 and PDIA3 which control the cleavage and rearrangement of disulfite bonds both inside and outside the cell, modifying proteins attached to cell or nascent proteins.

Annexin A1 and annexin A2 were down-regulated by LPS. They are both involved in calcium metabolism. Annexins are considered as scaffolding proteins which participate in membrane dynamics. In particular, annexin A2, that has structural similarities to annexin A1, has been

shown to exhibit anti-inflammatory activities in several animal models of inflammation.[43–47] This protein has been included here in the calcium metabolism proteins because of its calcium binding properties and dependent activity. However, beside these properties in relation to GO annotations, structural activities linked to lipid bilayer and lipid rafts and to actin binding have been reported as well.[48] The regulation of annexins and actin are following the same trend. The anti-inflammatory properties of annexins and the down-regulation observed here are in agreement with the pro-inflammatory role of LPS.

In contrast, we observed an over-expression of calreticulin which is mainly involved in calcium metabolism and in protein folding (Fig. 6). As a chaperone protein, calreticulin is also linked with HSP90. This protein is expressed on the cellular surface and its putative role in cell adhesion, migration or apoptosis has been documented.[49] In addition, calreticulin modulates integrin-dependent Ca^{2+} signaling [Michalak *et al.*, 1999] and different patterns of expression have been reported during implantation in mice [Cheng *et al.*, 2009]. This protein is expressed on the cellular surface and its putative role in cell adhesion, migration or apoptosis has been documented.[49] In addition, calreticulin modulates integrin-dependent Ca^{2+} signaling[50] and different patterns of expression have been reported during implantation in mice.[51] The significance of its over-expression in our model in relation to the above still has to be elucidated.

The full mechanism by which LPS provokes these changes remains to be deciphered. However, our results suggest that LPS destabilizes cell structure and modify cell membrane properties in a way cell adhesion is reduced. To our knowledge, the possible roles of LPS in altering such functions have not been much described before especially in relation to implantation. The changes observed could be of considerable importance while considering the role of adhesion molecules and membrane function in this process.

Another response to LPS in relation to cell structure could be part of a defense mechanism contributing to inhibit protease activity through down-regulation of the protease cathepsin and

up-regulation of the peptidase inhibitor Cystatin-B. As Cystatin-B is an inhibitor of cathepsin protease, the trend of expression of these two proteins is consistent and such changes may protect cells from proteolytic activity. The role of this anti-protease mechanism and its relation with the changes reported above in cell structure and membrane permeability requires further investigation.

Energy metabolism

Differentially expressed proteins related to energy metabolism proteins were all found to be over-expressed in both LPS groups when compared to controls. These proteins are mostly glycolytic proteins such as phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, beta-enolase OS and alpha-enolase. The interactomic analysis (Fig. 6) shows clearly that energy metabolism occupies a very central place in this system. This is consistent with the fact that LPS induced glycolysis which has been constantly reported in the literature from other tissues and other species[52–54] determines other cell responses thus contributing in many ways to the development of pathological processes.

Oxidative stress response

Even if this *in vitro* model is working outside the *in vivo* machinery and based on a single population of cells, many proteins involved in this pathway and in protection from oxidative stress are de-regulated showing the key role of LPS while inducing oxidative stress. They occupy a place close to proteins involved in metabolism (Fig. 6) and most of them may result from LPS induced glycolysis changes. The only protein that is not linked with other proteins, in this interactomic analysis, is the SH3 domain-binding glutamic acid-rich-like protein 3 that, according to GO classification, was related to cell redox homeostasis and is also involved in regulation of actin cytoskeleton organization. Interestingly, our results also confirm the up-

regulation of peroxiredoxin-1, which has been described as a strong promoter of inflammation through stimulation of the synthesis of proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α).[55]

Changes in cytokines were not evidenced from this epithelial cell model. However, the up-regulation of peroxiredoxin-1 and other pro-inflammatory molecules such as enolases may represent an important link between LPS stimulation and the generation of inflammatory cascade in surrounding cells.

Translation process/protein synthesis, regulation of cell cycle and proliferation

As for the energy metabolism, several proteins involved in protein biosynthesis such as initiation and elongation factors have been found to be strongly up-regulated. This demonstrates that the translation machinery is stimulated by LPS. The strong links between these proteins in the interactome and their upregulation is very consistent with the role of this family of factors in the stimulation of cell growth and proliferation[56,57] and the present changes in this phenotype induced by LPS. In addition to the above, other proteins have been reported, from their GO annotated molecular functions, to be related with regulation of cell cycle and cell proliferation. In this case, the interpretation of the interactomic analysis is more complex than for other functions. A strong link was found, with a high significance value, between transketolase, phosphoglycerate kinase 1 and 78 kDa glucose-regulated protein, the latest being linked to the above translation machinery. From GO molecular functions, all these proteins are involved in regulation of cellular growth, epithelial cell differentiation and act as positive regulators of cell migration. As for proteins involved in translation their up-regulation is consistent with the proliferative phenotype observed following LPS challenge.

No links were found between the above and metallothioneins nor galectin-1. Metallothioneins were found here to be up-regulated only in cells exposed to 8 μg ml^{-1} LPS and

galectin-1 is down-regulated for both LPS dosages. These proteins are classified, from GO annotated molecular functions, as involved in the negative regulation of growth. The up-regulation of metallothioneins looks in contrast to the growing phenotype observed in our model. However, their up-regulation could be related to a compensatory mechanism from cells to counteract the proliferative response induced by the activation of metabolism and transcription machinery. This response could be also associated with different functions as these proteins have been reported to be involved in bactericidal activity and nitric oxide production following LPS stimulation.[58]

Immune response; relationships with establishment of pregnancy

Our results showing that 14-3-3 protein zeta/delta is up-regulated in the LPS16 group *versus* control group are consistent with former functions of this protein described in the literature. Its major role in the regulation of corneal epithelial proliferation and differentiation in corneal mice cell culture has been reported.[59] This protein has been shown to be also involved in the regulation of the production of cytokines[60] and, the gamma proteoform, is up-regulated during LPS-induced cardiomyocyte injury.[61] Its major role in Toll-like receptor activation has also been demonstrated and its involvement in the lipopolysaccharide-induced production of tumor necrosis factor by macrophages has been documented.[62] A recent study demonstrated that 14-3-3γ was able to attenuate the LPS-induced inflammatory responses and to induce the proliferation of dairy cow mammary epithelial cells.[63] The up-regulation of this protein we observed may be part of a similar anti-inflammatory mechanism taking place in our model in response to LPS.

One of the most interesting findings in our results in relation to the establishment of pregnancy is the strong down-regulation of galectin-1 induced by LPS. To our knowledge this has not been reported before. Galectin-1 has been mostly studied in human and rodents and

has been shown to be expressed in the bovine endometrium.[64] In humans, this protein is abundantly expressed in the non-immune cells at the fetus–maternal interface, down-regulates the production of pro-inflammatory cytokines and promotes maternal immune tolerance.[65] In the mouse, Yakushina *et al.*, 2015[66] have shown that galectin-1 stimulates the differentiation of $CD4^+$ cells into T-regulatory cells than being one of the key molecules involved in immune-tolerance. Probably as the result of the above, low expression in the endometrium has been associated with an increased frequency of early pregnancy failures and miscarriages.[67,68]

As part of the metabolic changes both α and β-enolase are both up-regulated following challenges with similar responses for both LPS dosages. Among other roles, α-enolase has been reported as an allergenic molecule with immune and strong pro-inflammatory properties.[69–71] In rats, high expression of α-enolase has been associated with increased numbers of $CD4^+$ T cells and immunorejection in an allograft transplantation model.[72]

The impact of immune imbalance induced by deregulations of galectin-1 and α-enolase on the establishment of pregnancy has to be demonstrated in the bovine through functional studies. If existing and persistent, the lack of immunosuppression induced by LPS through down-regulation of galectin-1 and up-regulation of pro-inflammatory processes through enolases could be part of the mechanisms altering implantation success even in the absence of bacterial infection.

Chromatin and DNA binding

A differential expression of several DNA binding proteins such as histones has been found. As said before for other processes, the interactome picture is quite complex here with 4 of them under-expressed and 2 over-expressed following LPS challenge and more specific work is needed to decipher the mechanisms explaining the over-expression of some specific histones and the down-regulation of some other isoforms. However, from the 4 under-expressed

histones we found 2 (histone H2A type1 and H2AJ), which were associated with chromatin silencing consistent with the over-expression of many pathways. The over-expression of histones H2B type1 and H2B type1N following exposure to the 16 μg ml^{-1} dosage is also consistent with their roles in innate immune response in mucosa and DNA protein binding. Effectively, some histones, such as H2B type1, could represent LPS binding proteins[73] and their differential expression could contribute to the formation of an antimicrobial and of an endotoxin-neutralizing barrier against microorganisms.[74]

Conclusion

This study shows that many pathways involved in a wide range of functions are affected by LPS and we addressed the corresponding changes in mRNAs and proteins expression. However, even for these pathways which have been studied intensively and largely documented, such as immune response, this proteomic approach reveals that deregulation occurs for specific molecules which have not been described in former studies and/or not related to the role of LPS and its possible impacts on the establishment of pregnancy. Taken together, the results from this cow model based on a pure population of epithelial cells provide evidence that LPS induces the activation of pro-inflammatory mechanisms and at the same time down-regulates signals reported to be involved in immune-tolerance in other species (such as galectin-1). The persistence of changes possibly induced by LPS due to exposure to pathogens during the post-partum period and their subsequent implication in fertility failures at the time of establishment of pregnancy deserves further investigation.

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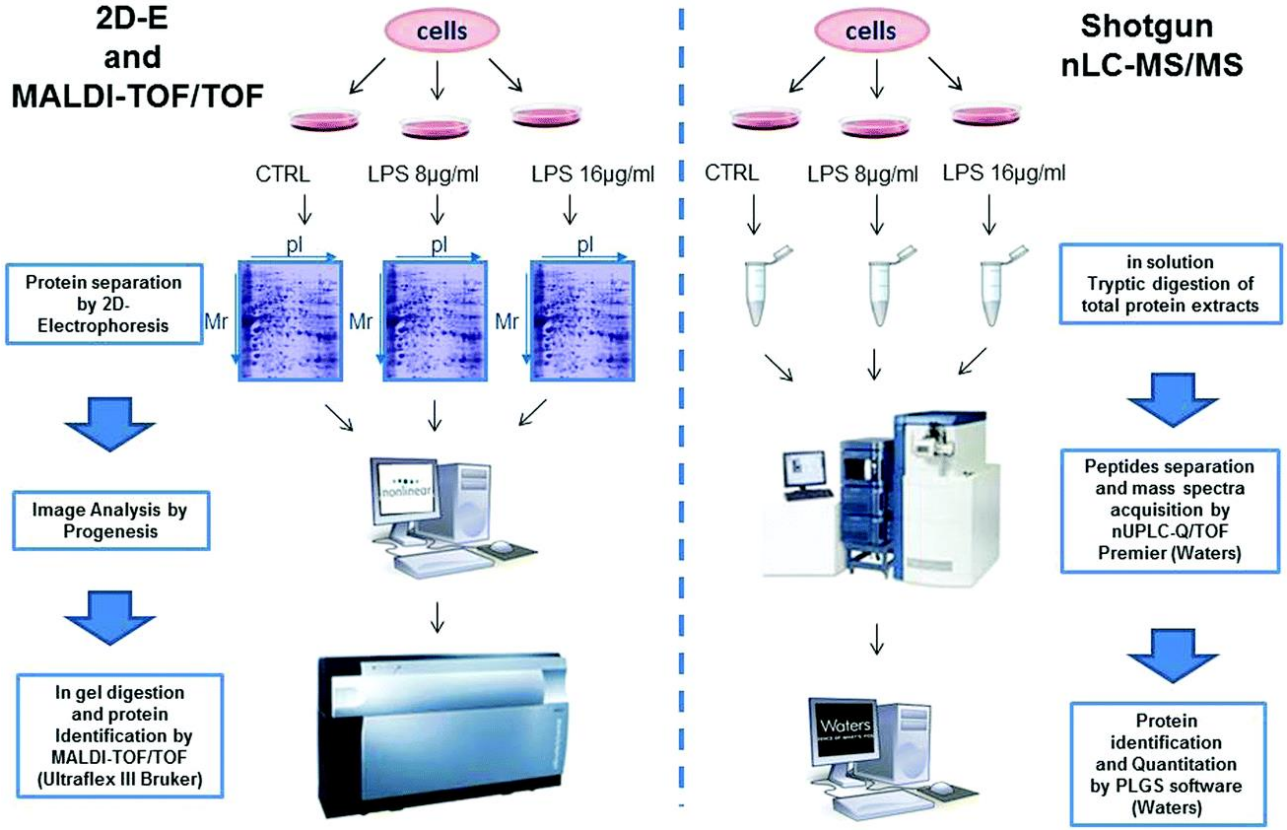
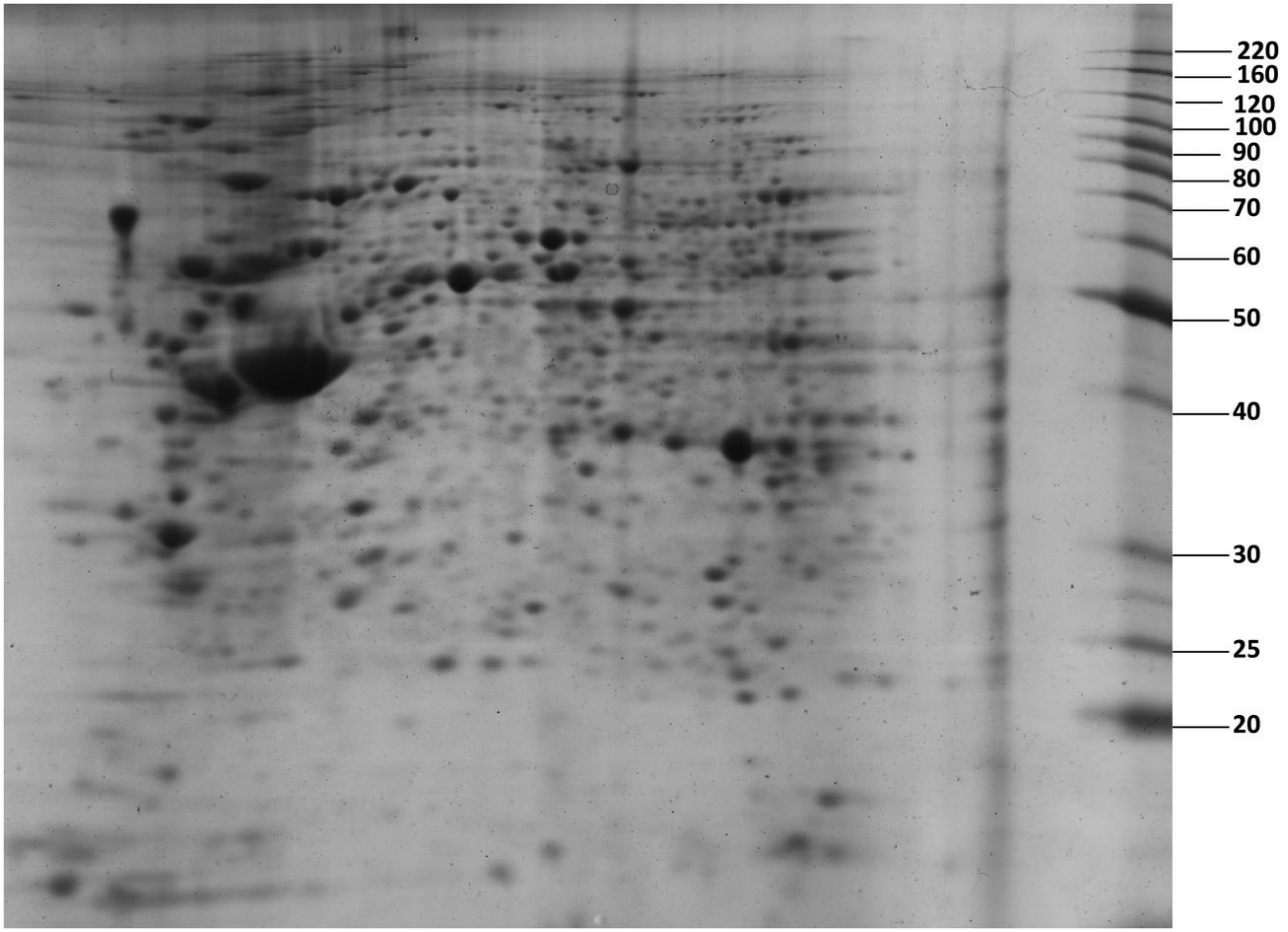


Fig. 1 Strategy for the proteomic analyses. Left part of graph: 2D electrophoresis and MALDI TOF TOF. All cell pellets from 9 cows (3 samples per cow, Control CTRL and LPS treated cells; LPS 8 µg ml−1, LPS 16 µg ml−1) were analyzed following separation of protein spots by 2D gel electrophoresis and identification of spots by image analysis (Progenesis), gel spots were digested and then differentially expressed (DE) proteins identified by MALDI TOF-TOF MS (Ultraflex III, Bruker) and quantified. Right part: nLC-MS/MS analysis was performed in the first series of three cows (3 samples per cow as above) and peptide separation and identification was done following tryptic digestion of total protein extracts. Assays were run in triplicate for each sample in each type of analysis.



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683 Fig. 2 Representative image of 2D map of one biological replicate.

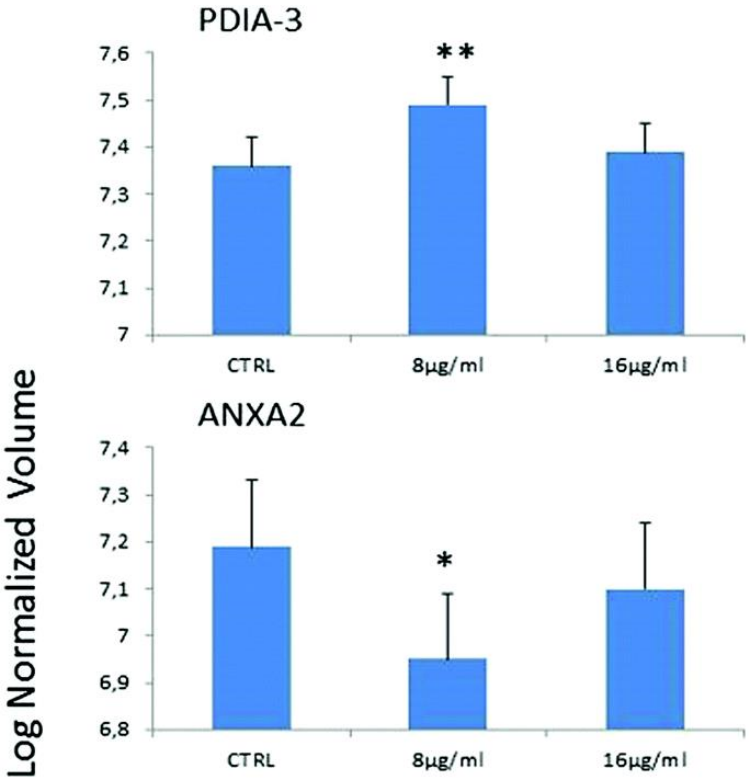

Fig. 3 Differentially expressed proteins observed from 2D electrophoresis followed by MALDI TOF TOF MS analysis in response to LPS (CTRL Control, LPS 8 µg ml−1, LPS 16 µg ml−1). For each bar is reported the mean ± SD value of nine biological replicates (n = 9). Proteins follow the survival profile of epithelial cells (PDIA3 Protein Disulfide Isomerase) or is inverse (ANXA2 Annexin 2). Significance (* $p < 0.05$, ** $p < 0.01$) indicates differential expression between treated samples and controls.

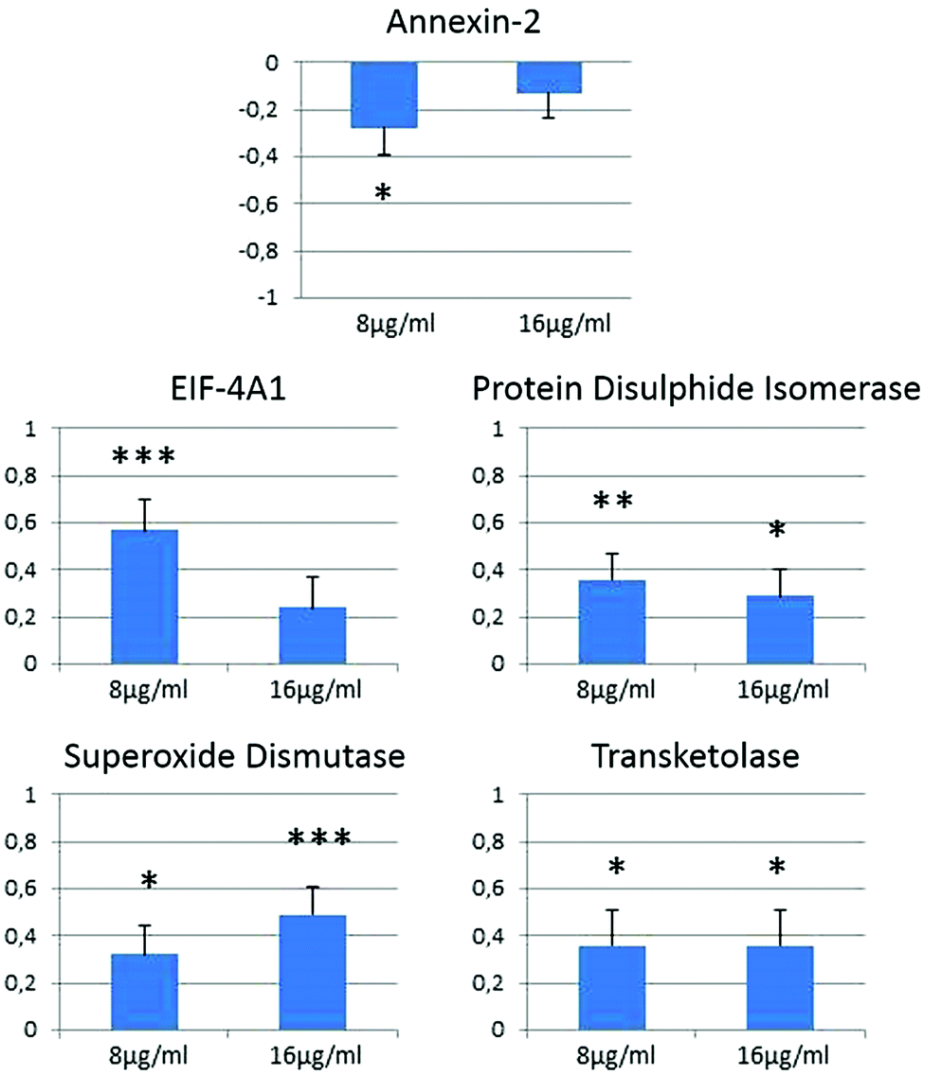

Fig. 4 Mean response for 5 differentially expressed proteins following 3 series of LPS challenge with 8 and 16 µg ml−1 LPS (each bar from 9 individual cows). The results are expressed as ratios when compared to controls and for each LPS dosage, significance of differences are tested against 0; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For all proteins, differences between 8 and 16 µg ml−1 LPS are non-significant.

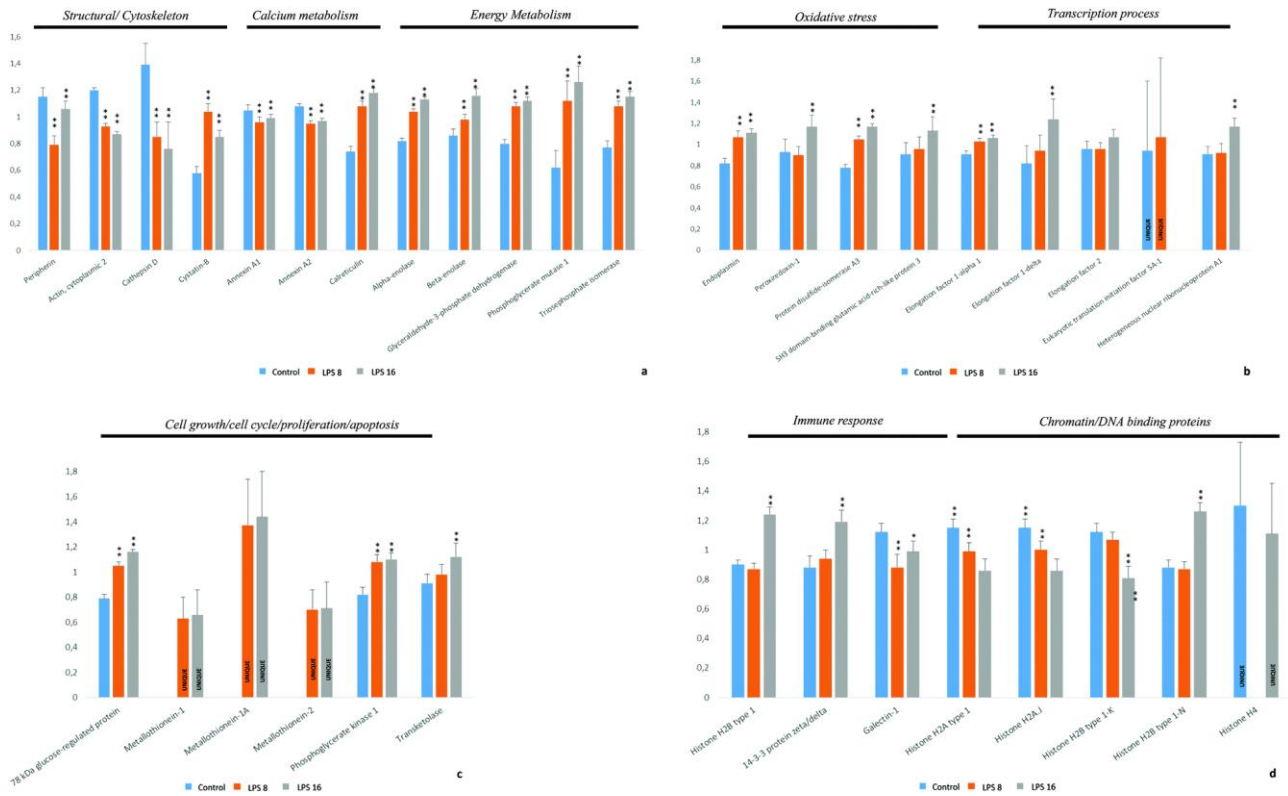

Fig. 5 Results from shotgun analysis, proteins significantly over-expressed and under-expressed following challenge of endometrial epithelial cells with 8 (orange bars) or 16 µg ml−1 LPS (grey bars) when compared to controls (blue bars), * $p < 0.05$, ** $p < 0.01$.

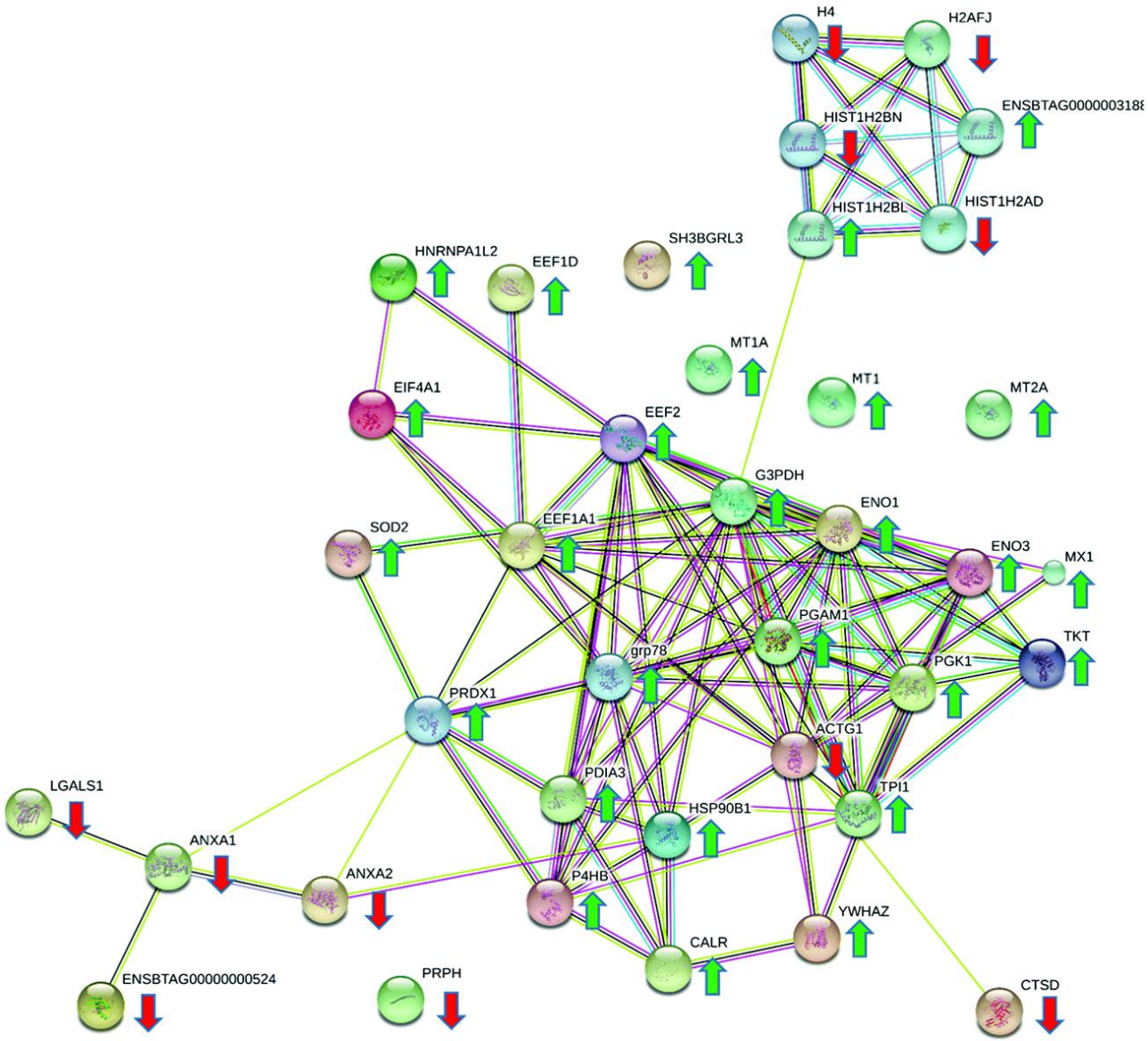

Fig. 6 Protein legend STRING protein–protein interaction analysis. The string name of each protein represented in the figure is indicated in the 4th column of Table 1.

Table 1 Table representing the whole dataset of the differentially expressed proteins identified through shotgun MS analysis and 2D electrophoresis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

					Ratio (shotgun MS)		Ratio (2DE)	
Metabolism	GO terms	Accession	String name	Description	LPS8/CTRL	LPS16/CTRL	LPS8 *vs.* CTRL	LPS16 *vs.* CTRL
Structural/ cytoskeleton	Structural constituent of cytoskeleton	P63258	ACTG1	Actin, cytoplasmic 2	↓0.771051593*	↓0.718923724*		
	Intermediate filament cytoskeleton organization, extracellular exosome	A6QQJ3	PRPH	Peripherin	↓0.683861412*	0.923116348		
	Proteolysis, hydrolase activity, extracellular region	P80209	CTSD	Cathepsin	↓0.612626388*			
	Endopeptidase inhibitor activity	P25417	ENSBTAG00000000524	Cystatin-B	↑2.339646908*			
Calcium metabolism	Calcium ion binding	P04272	ANXA2	Annexin A2	↓0.886920439*	↓0.895834136*	↓0.71564409*	
	Calcium ion transmembrane transport	P46193	ANXA1	Annexin A1	↓0.913931182*	↓0.93239382*		
	Calcium ion binding, protein folding	P52193	CALR	Calreticulin OS	↑1.462284582*	↑1.599994191*		
Energy metabolism	Gluconeogenesis, glycolytic process	Q3SZ62	PGAM1	Phosphoglycerate mutase	↑1.803988368*	↑2.033991215*		
	Glycolytic process	P10096	G3PDH	Glyceraldehyde-3-phosphate dehydrogenase	↑1.336427477*	↑1.390968147*		
	Glycolytic process	Q5E956	TPI1	Triosephosphate isomerase	↑1.40494759558807*	↑1.50681777972405*		
	Glycolytic process	Q3ZC09	ENO3	Beta-enolase OS	↑1.138828378*	↑1.349858824*		
	Glycolytic process	Q9XSJ4	ENO1	Alpha-enolase	↑1.271249144*	↑1.377127754*		
Oxidative stress response	Response to reactive oxygen species	Q5E947	PRDX1	Peroxiredoxin-1	0.970445534	↑1.258600015*		
	Cell redox homeostasis	P38657	PDIA3	Protein disulfide-isomerase A3	↑1.349858824*	↑1.50681778*	↑1.34654270*	
	Response to hypoxia, protein folding, response to stress	Q95M18	HSP90B1	Endoplasmin	↑1.296930074*	↑1.363425117*		
	Cell redox homeostasis	Q3ZCL8	SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3	↔	↑1.246076729*		
	Superoxide dismutase activity, oxidative stress response	P41976	SOD2	Superoxide dismutase			↑1.32768258*	↑1.491990**
	Protein folding	P05307	P4HB	Protein disulfide-isomerase	↑1.3771277544*	↑1.462284582*		
Translation process/protein synthesis	Protein biosynthesis	P68103	EEF1A1	Elongation factor 1-alpha	↑1.138828378*	↑1.173510867*		
	Translation, translational elongation, signal transduction	A5D989	EEF1D	Elongation factor 1-delta	↑1.15027379954284*	↑1.50681778*		
	Positive regulation of translation, translational elongation	Q3SYU2	EEF2	Elongation factor 2	↔	↑1.1162780*		
	Translational initiation, regulation of gene expression,	Q3SZ54	EIF4A1	Eukaryotic initiation factor 4A-I	↑*	↔	↑1.57531***	
	mRNA processing	P09867	HNRNPA1L2	Heterogeneous nuclear ribonucleoprotein A1	↔	↑1.271249144*		

Table 1 (*continued*)

Metabolism	GO terms	Accession	String name	Description	Ratio (shotgun MS) LPS8/CTRL	Ratio (shotgun MS) LPS16/CTRL	Ratio (2DE) LPS8 *vs.* CTRL	Ratio (2DE) LPS16 *vs.* CTRL
Regulation of cell cycle and proliferation	Regulation of growth	Q6B855	TKT	Transketolase	↑1.072508182	1.233678052↑	↑1.36185114*	↑1.36639*
	Epithelial cell differentiation	Q3T0P6	PGK1	Phosphoglycerate kinase 1	↑1.309964465*	↑1.349858824*	↑2.2031346*	
	Positive regulation of cell migration	Q0VCX2	grp78	78 kDa glucose-regulated protein	↑1.336427477*	↑1.476980773*		
	Negative regulation of growth, cellular response to zinc ion	P58280	MT1	Metallothionein-1	↑Unique LPS8*			
	Negative regulation of growth, cellular response to zinc ion	P67983	MT1A	Metallothionein-1A	↑Unique LPS8*			
	Negative regulation of growth, cellular response to zinc ion	P68301	MT2A	Metallothionein-2	↑Unique LPS8*			
Immune response	Innate immune response in mucosa, antibacterial humoral response, defense response to Gram-positive bacterium	P62808	ENSBTAG00000031889	Histone H2B type 1	0.970445534	↑1.377127754*		
	Protein domain specific binding	P63103	YWHAZ	14-3-3 protein zeta/delta	↔	↑1.349858824*		
	Myoblast differentiation, plasma cell differentiation, positive regulation of I-kappaB kinase/NF-kappaB signaling, T cell costimulation	P11116	LGALS1	Galectin-1	↓0.786627865286354*	↓0.886920439*		
	Innate immune response	P79135	MX1_BOVIN	Interferon-induced GTP-binding protein Mx1			↑2.519244**	↑2.843155**
Chromatin and DNA binding	DNA binding, protein heterodimerization activity	Q2M2T1	HIST1H2BN	Histone H2B type 1-K	0.96078944	↓0.726149042*		
	Chromatin organization, chromatin silencing	P0C0S9	ENSBTAG00000039492	Histone H2A type 1	↓ 0.852143792013715*	↓0.740818212*		
	Chromatin organization, chromatin silencing	Q3ZBX9	H2AFJ	Histone H2A.J	↓ 0.869358235*	↓0.740818212*		
	DNA binding, protein heterodimerization activity	Q32L48	HIST1H2BL	Histone H2B type 1-N	0.990049834	↑ 1.433329435*		
	Histone binding, extracellular exosome, DNA replication-dependent nucleosome assembly	P62803	ENSBTAG00000040277	Histone H4	↓Unique CTRL*			