Parasitology Research (2019) 118:1239–1248
https://doi.org/10.1007/s00436-019-06230-z

IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Effect of norepinephrine treatment on *Haemonchus contortus* and its excretory products

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Received: 26 June 2018 / Accepted: 24 January 2019 / Published online: 20 February 2019

Abstract

Haemonchus contortus is a highly pathogenic gastrointestinal nematode of small ruminant animals. In modern intensive farming, livestock often suffer from different types of stress. However, whether host stress hormones influence *H. contortus* infection is largely unknown. Therefore, we treated *H. contortus* with norepinephrine (NE) and analyzed the changes in its excretory/secretory products (ESPs). Label-free quantitative proteomic analysis was used to identify differences in body proteins and ESPs between the control and NE-treated groups. We also investigated the changes in ESP action by analyzing cytokine secretion and goat peripheral blood mononuclear cell (PBMC) proliferation after incubation with ESPs secreted by NE-treated *H. contortus*. Thirty-two proteins in the body samples and 137 in the ESPs were differentially expressed between the groups. Gene ontology (GO) annotation showed that the functions of these different proteins might be involved in energy metabolism, protein metabolism, lipid metabolism, redox homeostasis, ion channel, and cell structure. NE treatment caused oxidative stress in *H. contortus* and changed the expression levels of some immunogenic proteins, such as the 15-kDa ESP. Meanwhile, the ESPs secreted by NE-treated *H. contortus* significantly decreased PBMC proliferation and the interleukin (IL)-2, IL-4, and interferon-gamma contents. Thus, NE treatment significantly affected the *H. contortus* body and ESP expression, and changes in the ESPs influenced PBMC function. The results reveal a relationship between host hormones and parasites and provide new clues to explain some of the variation in individual responses to infection.

Keywords *Haemonchus contortus* · Excretory/secretory products · Label-free · Comparative proteomics · PBMC

Handling Editor: Una Ryan

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-019-06230-z) contains supplementary material, which is available to authorized users.

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Introduction

Haemonchus contortus is a highly pathogenic gastrointestinal nematode (Peter and Chandrawathani 2005) that resides in the abomasum of small ruminants and feeds on host blood. This parasite causes anemia and infirmity, which can be lethal (Wang et al. 2016). *H. contortus* has a global distribution and produces enormous economic losses (Troell et al. 2006). Anthelmintic drugs are used to treat infection, but disease control and eradication are dependent on irradiation (Kaplan 2004; Kaplan and Vidyashankar 2012). Further elucidating the mechanisms of *H. contortus* infection is necessary to improve health of the host and the economic damage caused by the parasite.

Parasites dwell in the host, and the host's metabolic status and immune responses influence parasitic growth and the infection processes (Wang et al. 2017). In modern intensive culture, sheep typically suffer from stressors derived from the environment and transport (Nwunuji et al. 2014; Papanastasiou et al. 2016). In these conditions, the host

increases the amount of stress hormones, mainly norepinephrine (NE) and cortisol, in response to stress. A recent study reported that parasites might be able to directly respond to host stress (Gleichsner et al. 2016). Other studies reported that dexamethasone treatments applied to mimic host stress states increased host susceptibility to *H. contortus* infection by suppressing the immune response (Peña et al. 2004).

NE is a catecholamine that is selected rapidly after exposure to stress. It is secreted by the adrenal medulla as a hormone and by nerve cells as a neurotransmitter, and a high NE concentration can suppress the host immune system (Chen et al. 2016; Qiao et al. 2018; Tzelos et al. 2016). During *H. contortus* infection, the host NE concentration increases, which may stimulate the function of excretory cells (Davey et al. 1982; Fleming 1993). However, the mechanism by which NE affects *H. contortus* infection is unclear.

H. contortus that develops to the L4 or adult stage in a host releases multiple molecule, which are termed excretory/secretory products (ESPs). ESPs contain a variety of proteins (at least 200) (Yatsuda et al. 2003). These proteins are closely associated with the parasite life cycle and pathogenicity. Several crucial proteins include the 15 and 24 kDa proteins (Schallig et al. 1997), 55 kDa protein (Anbu and Joshi 2008), and 66 kDa protein (Rathore et al. 2006), which induce varying degrees of immune protection against parasitism. ESPs can suppress host immune responses by interfering with antigen processing, modulating antigen-presenting cells, interfering with cytokines, and mimicry (Diliani and Dondji 2017). Interaction of the recombinant *H. contortus* 24 kDa excretory/secretory product (rHcESP-24) modulates the host immune response, which significantly suppresses IFN-γ production and peripheral blood mononuclear cell (PBMC) proliferation and increases interleukin (IL)-4 and IL-17β expression (Gadahi et al. 2016a).

Therefore, in the present study, *H. contortus* was cultured in RPMI 1640 medium containing 10 μM NE for 24 h, and the proteins from the *H. contortus* body and ESPs were analyzed using comparative proteomics. Furthermore, cytokine secretion and goat PBMC proliferation after treatment with ESPs secreted by NE-treated *H. contortus* were investigated. The results clarify the response of *H. contortus* to host stress and provide information for future studies of parasite invasion and their mechanisms of immune escape.

Materials and methods

Parasite

A pure strain of *H. contortus* was conserved in the College of Veterinary Medicine, Nanjing Agricultural University, by regular passage through helminth-free goats (Zhao et al. 2012). The adult parasites were collected from the abomasa of infected goats (5 months of age) 4 weeks after inoculation with 5000 infective L3s.

Body protein and ES product preparation

Six infected goats were used in this experiment. All *H. contortus* parasites in each abomasum were collected into a buffer containing 5 mM $NaHCO_3$ (pH 6.4), 132 mM NaCl, and 5 mM KCl that was prewarmed to 38 °C (Kuang et al. 2009). Then, the pooled parasites were washed three times in the same buffer and once in sterile PBS containing 100 IU of penicillin and 0.1 mg/ml of streptomycin. Clean worms were incubated in RPMI 1640 medium (100 worms/ml) with final concentrations of 100 IU of penicillin, 0.1 mg/ml streptomycin, and 20% fetal bovine serum (Gibco, Life Technology) at 37 °C under 5% CO_2 (Gadahi et al. 2016b). All nematodes were allocated to one of two groups (control and 10 μM NE treatment) (Aladdin, Japan), each with three repeats (100 worms/repeat). The media were collected after 24 h and filtered through 0.2-μm filters into sterile tubes. A protease inhibitor cocktail (Complete EDTA-free, Roche, Penzberg, Germany) was added, and the samples were frozen at − 80 °C. Additionally, the nematode body was collected and frozen in liquid nitrogen. The frozen samples were ground and homogenized in 1 ml each of ice-cold homogenization buffer (RIPA Lysis Buffer containing the protease inhibitor cocktail). The protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA).

Label-free quantitative proteomics analysis

Whole worm protein and ESP samples ($n = 3$) that were isolated previously and contained the same quantity of protein (250 μg) were used for the proteomics analysis based on Filter-Aided Sample Preparation (FASP) of Proteins for Label-Free Mass Spectrometry (Mahadevan et al. 2016). The tryptic peptide fragments were analyzed by LC-MS/MS (Waters, Milford, MA, USA). The above work was carried out by the College of Life Science in Nanjing Agricultural University. Raw data were searched using Sequest HT and Proteome Discoverer with Percolator to filter at a 1% FDR peptide confidence with a precursor ion area detector for label-free quantification. The spectra were filtered at 350–5000 Da and a signal-to-noise threshold of 1.5 with a minimum peptide length of six amino acids. The strict parsimony principle was applied to the dataset, which required that protein groups have at least one unique peptide to be labelled as a distinct protein group. The peak area used to quantify the protein groups was calculated based on an average of up to three of the most abundant peptides for a given protein group. Peak area data were summed for each injection, and each sample was normalized to the run with the greatest total peak area. Spectral counts were normalized to the total spectral counts for each

sample and filtered prior to the analysis by removing any protein groups below an average of three normalized spectral counts (NSpC) from both the CON (control group, no treatment) and NE-treated samples. Finally, the MaxQuant software package was used to analyze the results, and LFQ signal intensity difference multiples above 1.5-fold (fold change > or ≤ 1.5 = 0.667) were considered the ultimate difference in protein expression. The proteome data analysis was conducted with the *H. contortus* UniProt database (http://www.uniprot.org) and BLAST by the NCBI database (http://www.ncbi.nlm.nih.gov).

Bioinformatics analysis

Gene ontology (GO) annotations for the identified proteins based on the BLAST results were analyzed using Blast2GO (www.blast2go.de). The identified protein sequences were searched against the UniProtKB databases to find homologous sequences for which the functional annotation could be transferred to the studied sequences. The enrichment *p* value was obtained from the Benjamin–Hochberg test to control the rate of false positives, and differences in GO terms and KEGG pathway annotations were screened using the *p* value ($p < 0.01$). The Blast2GO software was used to complement multiple identified proteins within the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway database (http://www.genome.ad.jp/kegg/ pathway.html).

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was extracted from the parasite samples (50 mg) with the Total RNA Isolation Reagent (3101-100, Shanghai Pufei Biotech). The RNA was quantified by the NanoDrop ND-1000 spectrophotometer (Thermo, USA). The absorption ratios (260/280 nm) of all samples were between 1.8 and 2.0. Aliquots of the RNA samples were subjected to electrophoresis using a 1.4% agarose–formaldehyde gel to verify the RNA integrity. Two micrograms of RNA was used to generate cDNA with the PrimeScript® First-Strand cDNA Synthesis Kit (D6110A, Takara). Two microliters of diluted cDNA (1:20, vol/vol) was used for real-time PCR in the Mx3000P (Stratagene, USA). All primers were designed with the "Primer Premier 6" software and then blasted against the NCBI database. All primers were synthesized by Tsingke (Nanjing, China) with the following sequences: Primer 1: 5′-CCCTTATCATCTCGTGGCA-3′ and 5′-AATATCGCTTCGCAGTCG-3′ for U6P3X5; Primer 2: 5′-TGCGGAAATCTGAACTCT-3′ and 5′-ACAGCACCCATCCAATCT-3′ for U6NVC5; Primer 3: 5′-CGAATACACCACCAACGA-3′ and 5′-TTCTTATCACCGGCAACC-3′ for U6P6E3; Primer 4: 5′-GGGCAGACCATCAGTTAC-3′ and Primer 5: 5′-AAATGTCGGAGAAGTCGT-3′ for U6P8H9; and Primer 6: 5′-AGGTGAAGGTATGGAAGAAG-3′ and 5′-TTGGAATAGGCGGAACTG-3′ for Hc-α-tubulin. The real-time PCR reaction system used 5 μl of the AceQ® qPCR SYBR® Green Master Mix, 0.2 μl of primers 1 and 2, 0.2 μl of ROX Reference Dye 1, 1 μl of cDNA, and 10 μl of ultrapure water. The $2^{-\Delta\Delta Ct}$ method was used to analyze the real-time PCR results, and all genes were normalized against the housekeeping gene Hc-α-tubulin and expressed as the fold change relative to the mean value of the control samples. All experiments were performed in triplicate.

Isolation of goat PBMCs

Three parasite-free goats were used in each biological replicate. Peripheral venous blood was collected in negative pressure vacuum anticoagulant tubes from dewormed healthy goats. PBMCs were separated with the standard Ficoll-Hypaque (GE Healthcare, USA) gradient centrifugation method within 2 h (Wang et al. 2014a). After washing twice with Ca^{2+}/Mg^{2+}-free PBS (pH 7.4), cell viability was assessed using the trypan blue exclusion test. The result was consistently > 95% in all tests. Resuspended cells were incubated in RPMI 1640 medium with final concentrations of 100 IU of penicillin, 0.1 mg/ml of streptomycin, and 10% fetal bovine serum (Gibco, Life Technology) at 37 °C under 5% CO_2. The experiment was performed in triplicate.

Detection of cytokine levels by ELISA

The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). PBMCs were separated from fresh peripheral venous blood, and then 1.0×10^6 cells were seeded into 24-well plates (1 ml/well). Each well was allocated to one of the four following experimental treatments: cells treated with 10 μM NE, 100 μg/ml of ESPs, and 100 μg/ml of NE treated ESPs and untreated (the control group). The PBMCs were cultured in complete RMPI 1640 medium (containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS) at 37 °C with 5% CO_2. After 48 h of culture, the supernatants were collected by centrifugation at 800×*g* for 10 min, and the protein concentrations were detected using the BAC kit. All samples were diluted to the same protein concentration. The IFN-γ and IL-4 levels in the supernatants were determined using commercially available goat ELISA kits (h005 and h025, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The experiment was performed in triplicate.

Cell proliferation assay

Cell proliferation assays were performed as described previously (Ehsan et al. 2017). Briefly, fresh cells were allocated to one of the four groups (CON, NE, CON-ESP, and NE-ESP). Prepared PBMCs were seeded into 96-well plates (1×10^4 cells/well) and incubated at 37 °C in 5% CO_2 for 48 h. A

total of 10 μl of the cell counting kit-8 (CCK-8) reagent (Beyotime Biotechnology, Haimen, Jiangsu, China) was added to each well. After 4 h, the harvesting absorbance was measured at 450 nm (OD450) using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cells with control buffer were set as the control, and the OD450 in the control group was set as 100%. The experiment was performed in triplicate.

Statistical analysis

All RT-PCR data are presented as the mean ± SEM. The data were tested for a normal distribution, and statistical significance was assessed by the independent samples *t* test using the Statistical Program for Social Sciences (SPSS) software, version 20.0, for Windows. Differences with p values < 0.05 were considered statistically significant.

Results

Identification of differentially expressed proteins in the *H. contortus* body and ESPs

Following high-dose NE treatment, 32 proteins were differentially expressed in the worm body, including 22 that were upregulated and 10 that were downregulated. In addition, the ESP composition, but not the amount of overall ESPs released, was changed. Of the 137 differentially expressed proteins in the ESPs, 48 were upregulated and 89 were downregulated (Supplemental file 1; Supplemental Fig. 2; Table 1). A heat map of the proteins is presented in Fig. 1.

RT-PCR verification

A RT-PCR was performed to verify the proteomics results. The results are presented in Table 2. Differentially expressed genes included those encoding a lipid transport protein (U6P6E3), halo acid dehalogenase hydrolase, and ATPase domain-containing protein (U6P8H9). The expression of the genes encoding the disulfide–isomerase (U6P3X5) and aminotransferase domain-containing (U6NVC5) proteins was consistent with the protein expression levels. U6P6E3, U6P8H9, and U6P3X5 were decreased by 0.75-fold, 0.80-fold, and 0.87-fold, respectively (Supplemental Fig. 1; Table 2).

GO annotation

The GO annotation results are presented in Fig. 2a, b. The 32 differentially expressed body proteins were annotated to 11 biological process, 14 molecular function, and eight cellular component categories. Identical annotation of 137 differentially expressed ESPs was evident for 10 biological process, 11 molecular function, and seven cellular component categories.

In the body proteins, the biological functions of the upregulated proteins predominantly involved the extracellular region and lipid binding. The downregulated proteins were enriched in transaminase activity, threonine catabolic processes, and biosynthetic processes (Table 3).

In the ESPs, the biological functions of the upregulated proteins predominantly involved the glycolytic process and cell redox homeostasis. The downregulated proteins were enriched in chitin metabolism and peptidase, aspartic-type endopeptidase, and threonine-type endopeptidase activities (Table 3). Detailed information is provided in Supplemental file 2. These proteins were classified into seven categories based on their functions (Fig. 3a, b).

KEGG annotation

In the KEGG database, 38 differentially expressed proteins in the parasite body were annotated to 233 signal/metabolic pathways. The upregulated genes were involved in taurine and hypo-taurine metabolism, arachidonic acid metabolism, cyan amino acid metabolism, and glutathione metabolism. The downregulated genes were involved in glycine, serine, and threonine metabolism. The 132 ESP species were annotated to 161 signal/metabolic pathways. Only genes involved with protein processing in the endoplasmic reticulum (ER) were upregulated (Supplemental file 3).

Effect of ESP and NE-ESP on the secretion of individual cytokines by PBMCs

The effects of the ESPs and NE-treated ESPs on the secretion of individual cytokines were investigated by ELISA. IL-2, IL-4, IL-10, and IFN-γ were significantly reduced to 79.51%,

Table 1 Common differentially expressed proteins in the nematode body and ESPs

Protein ID	Protein name	Protein	ESP
U6NR36	Nematode fatty acid retinoid binding domain-containing protein	↑	↑
U6P3X5	Protein disulfide–isomerase	↑	↑
U6P6E3	Lipid transport protein and vitellogenin and von Willebrand factor domain-containing protein	↓	↓

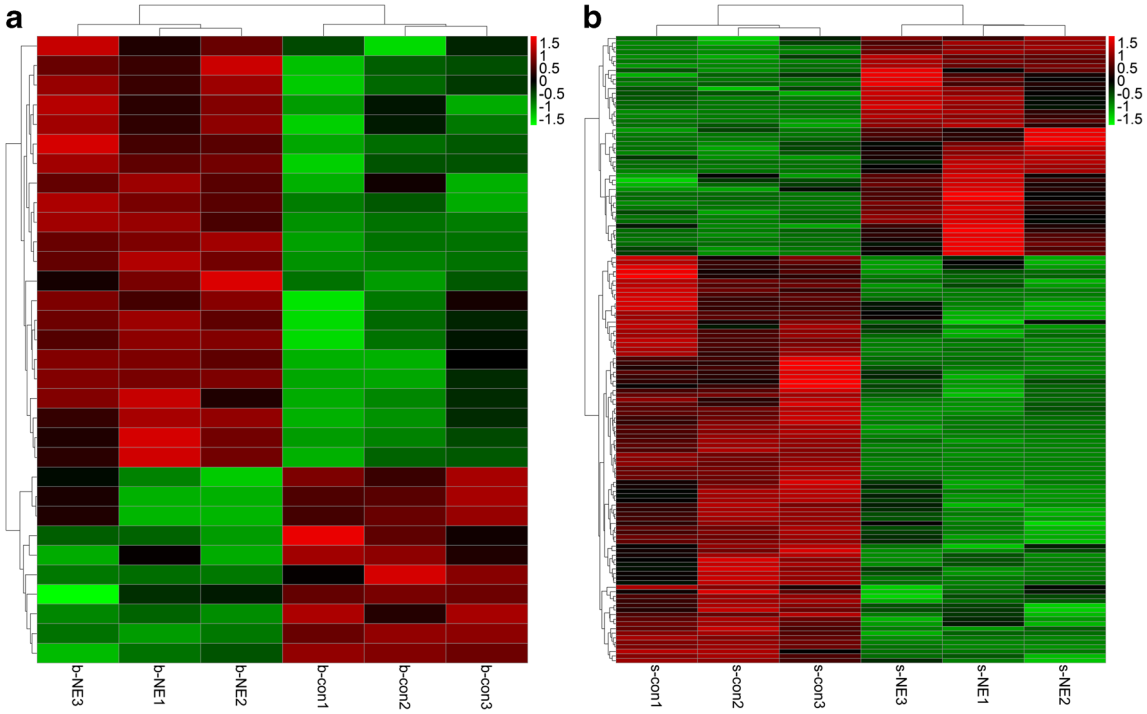

Fig. 1 Heat map of differentially expressed proteins from the label-free proteomics analysis. **a** Heat map of differentially expressed body proteins. **b** Heat map of differentially expressed ESP proteins. The line at the top is a cluster of samples, and the line on the left is a cluster of genes. By comparing differences between samples, we can see changes in gene expression in the different samples. A deeper color indicates higher gene expression

84.26%, 84.24%, and 80.42% by ESP treatment (100 μg/ml), respectively. Unsurprisingly, the reduction trend of the NE-ESP group was more significant, and IL-2, IL-4, IL-10, and IFN-γ productions were reduced to 67.56%, 66.38%, 72.62%, and 65.75%, respectively. No significant difference was found between the CON-ESP and NE groups. However, IFN-γ expression was decreased by NE-ESP treatment compared to that of the NE-CON group (Fig. 4).

ESP and NE-ESP treatment decreases PBMC proliferation

CCK8 analysis was used to evaluate the effect of ESPs and NE-ESPs on PBMC proliferation. Cell proliferation was significantly suppressed by 100 μg/ml of ESP treatment compared with that of the control group, with the effect of the NE-ESPs being especially pronounced (Fig. 5).

Discussion

Our study shows that NE may influence the body proteins and ESPs excreted by the parasite. Since NE is an important hormone that is secreted abundantly after stress, our results indicate that the host stress condition may impact the parasite state and thereby possibly influence parasite evasion. Most previous studies on the relationship between the host and parasite have focused predominantly on the ability of the host immune system to recognize and destroy the parasite (Gadahi et al.

Table 2 Regulation of differential protein expression and gene transcription during NE treatment

Protein ID	Protein name	Protein expression	Protein *p* value	mRNA expression	mRNA *p* value
U6P3X5	Protein disulfide–isomerase	↑	0.02^{*}	↓	0.04^{*}
U6NVC5	Aminotransferase domain-containing protein	↓	$<0.01^{**}$	↑	0.05^{*}
U6P6E3	Lipid transport protein	↓	0.03^{*}	↓	$0.08^{\#}$
U6P8H9	Haloacid dehalogenase hydrolase and ATPase domain-containing protein	↓	0.04^{*}	↓	0.05^{*}

Data represent the mean ± SEM. Data are considered statistically significant when $^{*}p < 0.05$, $n = 4$

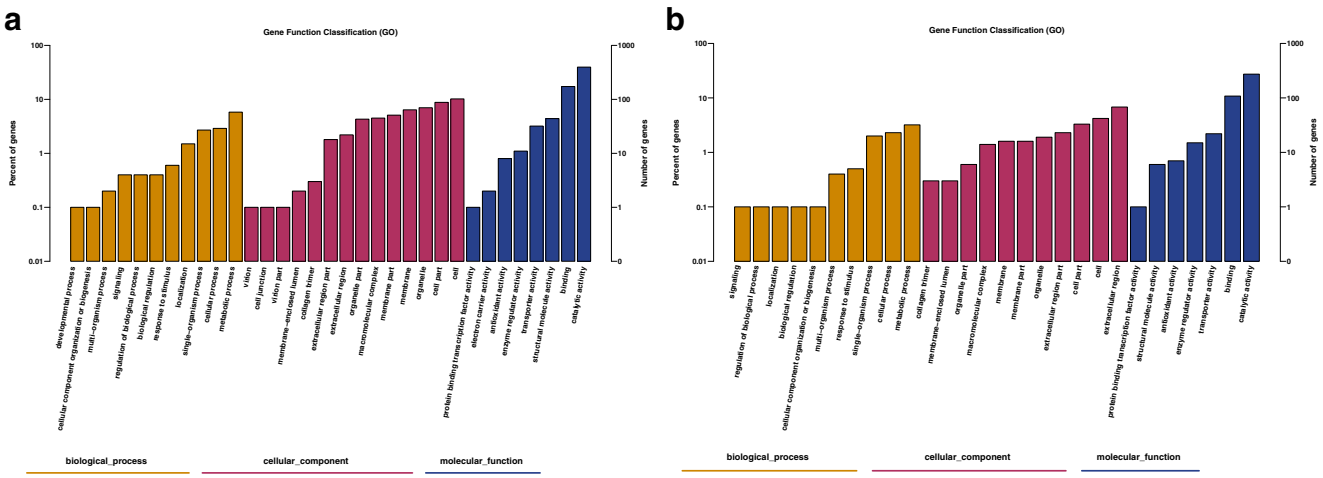

Fig. 2 Total protein GO analysis and function annotation. **a** Nematode body proteins were annotated to 11 biological process, 14 molecular function, and eight cellular component categories. **b** ESPs were annotated to 10 biological process, 11 molecular function, and seven cellular component categories

2016c). This present study presumed that the host's physiological state might also influence parasite invasion.

Initially, NE treatment changes a series of the parasite's body proteins related to metabolism. These changes were mainly concentrated in protein metabolism (five proteins changed), fat metabolism (three proteins changed), and energy metabolism (two proteins changed). For example, among lipid metabolism, the nematode fatty acid retinoid-binding protein (U6NR36, FAR) was upregulated, whereas the lipid transport protein (U6P6E3) was downregulated. FAR is a small lipid-binding protein that is secreted by nematodes and is required for their metabolic and developmental demands (Kuang et al. 2009; Garofalo et al. 2003). In addition, the proteins related to protein metabolism included the peptidase A1 domain-containing protein, energy metabolism-related protein, and ATPase domain-containing protein (Supplemental file 1). These changes may have an effect on the parasite's metabolism.

At the same time, differential protein expression was also detected in the ESPs. A number of changes were observed in the ESPs of *H. contortus* following NE treatment, and some of these proteins could have an effect on immune evasion. In the present study, 137 differentially expressed proteins were identified in the ESPs, of which 48 were upregulated and 89 were downregulated. Notably, the expression of a 15 kDa excretory/secretory product (15 kDa E/S, O18518), which is an effective antigenic substance in the ESPs, was decreased during NE treatment. A prior study reported that hyperimmune sera from infected sheep differentially and strongly reacted with the 15 kDa E/S product, suggesting a possible role in the infectious process (Schallig et al. 1994). Furthermore, the galectin (Hco-gal-1, O7664) level was also

Table 3 GO significant enrichment analysis

Term	*p* value	numDEInCat	numInCat	Regulation
Body				
Threonine catabolic process	0.01*	1	2	↓
Biosynthetic process	0.03*	1	7	↓
Extracellular region	0.04*	1	5	↑
Lipid binding	0.02*	2	17	↑
Transaminase activity	0.03*	1	6	↓
ESPs				
Glycolytic process	0.03*	2	10	↑
Cell redox homeostasis	0.04*	2	12	↑
Chitin metabolic process	0.02*	2	2	↓
Peptidase activity	< 0.01**	4	5	↓
Aspartic-type endopeptidase activity	0.01*	5	22	↓
Chitin binding	0.02*	2	2	↓
Threonine-type endopeptidase activity	0.04*	2	6	↓

Term is the GO annotation information. NumInCat is the total number of proteins with the GO annotation. NumDEInCat is the number of differentially expressed proteins with the GO annotation. Data are considered statistically significant when $*p < 0.05$, $n = 3$

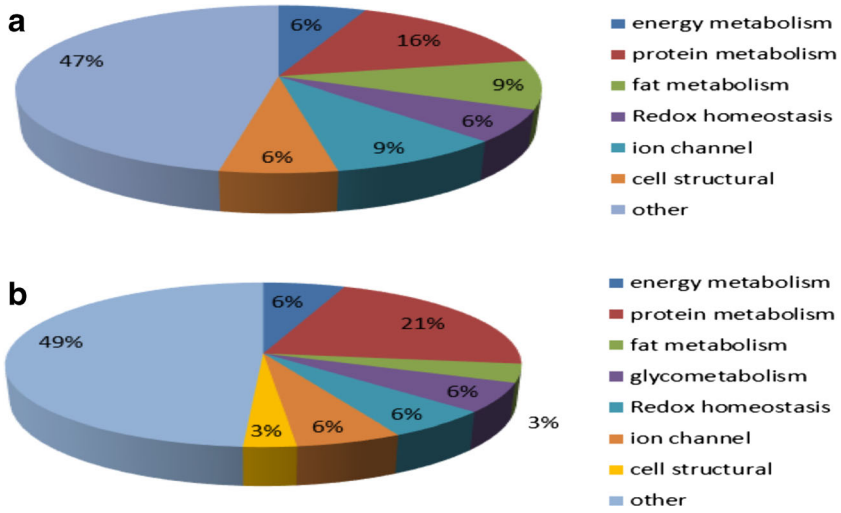

Fig. 3 Classification of differentially expressed proteins in the label-free proteomics analysis. **a** The differentially expressed body proteins were classified to six functions, including energy metabolism (two proteins), protein metabolism (five proteins), fat metabolism (three proteins), redox homeostasis (two proteins), ion channel (three proteins), cell structure (two proteins), and unknown (one protein). **b** The differentially expressed ESP proteins were classified to seven functions, including energy metabolism (eight proteins), protein metabolism (29 proteins), fat metabolism (five proteins), glycometabolism (eight proteins), redox homeostasis (eight proteins), ion channel (eight proteins), cell structure (four proteins), and unknown (one proteins)

increased in the ESPs (Supplemental file 1). As another effective antigenic substance in ESPs, galectin can suppress the host immune response by combining with goat PBMCs to suppress transcription of IL-1β, IL-4, IFN-γ, and TNFα (Wang et al. 2014b, c). Thus, galectin in the parasite can mimic galectin in the host to participate in host immune regulation and achieve the goal of immune escape (van Die and Cummings 2010). This hypothesis suggests that NE treatment may influence parasite immune evasion.

During NE treatment, we observed that the expression of stress-related proteins in both the parasite body and ESPs was increased as expected. The KEGG enrichment analysis revealed upregulated protein processing in the endoplasmic reticulum. One of the related proteins (protein disulfide–isomerase (U6P3X5, PDI)) was increased in both the parasite body and ESPs, although contrasting changes were evident at the transcriptional level. With strong evolutionary conservation, PDI is required in vertebrates and invertebrates because

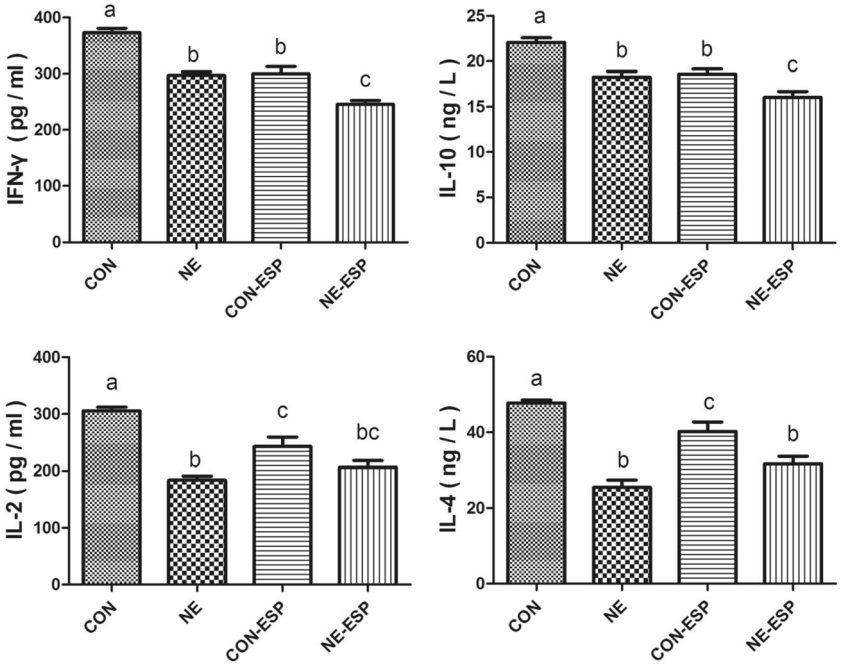

Fig. 4 Analysis of the cytokine levels produced by the PBMCs in vitro. The PBMCs were separated into four groups and treated with NE, CON-ESP, or NE-ESP for 24 h and control group. Cytokine secretion in the cell culture supernatant was detected by ELISA. **a** Cytokine IFN-γ level. **b** Cytokine IL-10 level. **c** Cytokine IL-2 level. **d** Cytokine IL-4 level.All data are presented as the mean ± SEM ($n = 4$), and values with different letters are significantly different ($p < 0.05$)

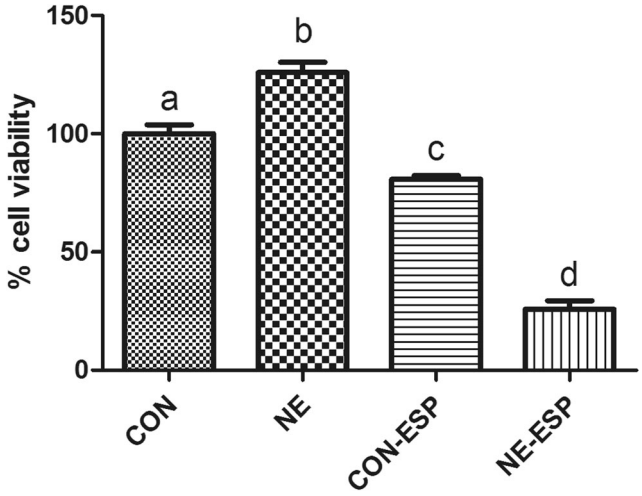

Fig. 5 Effect of ESP and NE-ESP treatments on PBMC proliferation. Cells were treated with NE, CON-ESP, and NE-ESP in a 96-well plate. All data are presented as the mean ± SEM ($n = 4$), and values with different letters are significantly different ($p < 0.05$)

they all contain disulfide linkages (Winter et al. 2007). PDI is an oxidoreductase in the ER that plays an important role in the unfolded protein response (UPR) by protecting cells against oxidative damage and maintaining ER-related redox homeostasis (Galligan and Petersen 2012; Gruber et al. 2006; Wilkinson and Gilbert 2004). The increased PDI expression under ER stress conditions indicates that NE treatment can induce nematode stress. Furthermore, several stress-related proteins, such as the heat shock proteins hsp70 and hsp90 (Supplemental file 1), are significantly increased during stress; these proteins are immunodominant antigens during infections (Gillan et al. 2009; Zhang et al. 2013). The results indicate that host stress hormones may influence parasite invasion by changing stress-related proteins.

Finally, we observed that the ESPs obtained in the NE treatment group influenced the function of host immune cells. We detected the levels of specific types of cytokine and found lower IL-2, IL-4, and IFN-γ expression in the NE-treated group than in the control group. Unsurprisingly, the NE-ESP-treated group showed a stronger inhibitory effect than the other groups (Fig. 4). IL-2 and IFN-γ are produced by TH1 cells and mediate an inflammatory immune response. IL-4 is produced by TH2 cells; it participates in the humoral immune response and plays a critical role in the antiparasitic infection response induced by activated mast cells, which release histamine, causing diarrhea and intestinal peristalsis and thereby eliminating the parasites. Previous reports have demonstrated increased IL-2, IL-4, and IFN-γ expression in lambs experimentally infected with *H. contortus* (Buendia-Jimenez et al. 2015). Meanwhile, PBMC proliferation was suppressed by the ESP and NE-ESP treatments, especially in the NE-ESP group (Fig. 5). Cell proliferation is an important indicator of the immune response. Proliferation is controlled by complex regulatory activities, such as cytokine secretion. The immune response can stimulate cell proliferation, which in turn affects the numbers of immunoeffector cells (Turner et al. 2008). These results may suggest that the ESPs secreted by *H. contortus* during stress conditions that are mimicked by the high-dose NE treatment can suppress host immune responses. This phenomenon could potentially provide a mechanism of immunosuppression that contributes to immune evasion by *H. contortus*.

One caveat of our study design is that the NE concentration used was much higher than the physiologically relevant concentration in goats. Therefore, the present results suggest that the hormones secreted during host stress may influence ESP production by parasites. With this limitation in mind, currently, we are collecting *H. contortus* from goats in a long-term chronic stress state and assessing their metabolic condition and the ESP components. Here, we present initial evidence that high-dose NE can influence the ESP composition and may contribute to immune avoidance.

Conclusion

We identified many differentially expressed proteins in the nematode body and ESPs after NE treatment. Some of these proteins may be involved in *H. contortus* development and infection. The changed ESPs influence PBMC functions, as shown by the decreases in IL-2, IL-4, and IFN-γ expressions and PBMC proliferation. The results reveal a relationship between host hormones and parasite metabolism and provide new clues to explain differences between individual hosts during parasite invasion. However, the specific mechanism by which the whole worm proteins and ESPs are changed after NE treatment requires further studies.

Acknowledgements We especially thank Mingmin Lu and Yongqian Bu, Laboratory of Veterinary Parasitology, Nanjing Agricultural University, who provided valuable suggestions.

Availability of data and material The datasets supporting the conclusions of this article are included within the article.

Authors' contributions XiaoJing Yang guided the entire project and took part in the experimental design. Jinglong Chen performed the experiment, analyzed the data, and wrote the manuscript. Fuli Ma assisted with obtaining parasite and blood samples. Xiangrui Li and Ruqian Zhao provided inputs into the experimental design. All authors read and approved the final version of the manuscript.

Funding This study was supported by the National Basic Science Research Program (973 program) of China (2015CB150300) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Compliance with ethical standards This experiment was approved by the Animals Ethics Committee, Nanjing Agricultural

University, China, and abided by the guidelines of the Animal Welfare Council of China.

Conflict of interest The authors declare that they have no competing interests.

Consent for publication Not applicable.

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References

Anbu KA, Joshi P (2008) Identification of a 55 kDa Haemonchus contortus excretory/secretory glycoprotein as a neutrophil inhibitory factor. Parasite Immunol 30(1):23–30

Buendia-Jimenez JA, Munoz-Guzman MA, Vega-Lopez MA, Cuenca-Verde C, Martinez-Labat JP, Cuellar-Ordaz JA et al (2015) Partial protection and abomasal cytokine expression in sheep experimentally infected with Haemonchus contortus and pre-treated with Taenia hydatigena vesicular concentrate. Vet Parasitol 211(1–2): 60–66

Chen X, Kelly AC, Yates DT, Macko AR, Lynch RM, Limesand SW (2016) Islet adaptations in fetal sheep persist following chronic exposure to high norepinephrine. J Endocrinol 232(2):285–295

Davey KG, Sommerville RI, Rogers WP (1982) The effect of ethoxyzolamide, an analogue of insect juvenile hormone, noradrenaline and iodine on changes in the optical path difference in the excretory cells and oesophagus during exsheathment in Haemonchus contortus. Int J Parasitol 12(6):509–513

Diliani N, Dondji B (2017) Hookworm excretory/secretory products modulate immune responses to heterologous and species-specific antigens. Parasite Immunol 39(10)

Ehsan M, Gao W, Gadahi JA, Lu M, Liu X, Wang Y, Yan RF, Xu LX, Song XK, Li XR (2017) Arginine kinase from Haemonchus contortus decreased the proliferation and increased the apoptosis of goat PBMCs in vitro. Parasit Vectors 10(1):311

Fleming MW (1993) Catecholamines during development of the parasitic nematode, Haemonchus contortus. Comp Biochem Physiol C 104(2):333–334

Gadahi JA, Li B, Ehsan M, Wang S, Zhang Z, Wang Y, Hasan MW, Yan R, Song X, Xu L, Li X (2016a) Recombinant Haemonchus contortus 24 kDa excretory/secretory protein (rHcES-24) modulate the immune functions of goat PBMCs in vitro. Oncotarget 7(51): 83926–83937

Gadahi JA, Yongqian B, Ehsan M, Zhang ZC, Wang S, Yan RF, Song XK, Xu LX, Li XR (2016b) Haemonchus contortus excretory and secretory proteins (HcESPs) suppress functions of goat PBMCs in vitro. Oncotarget 7(24):35670–35679

Gadahi, JA, Yongqian, B, Ehsan, M, Zhang, ZC, WANG, S, Yan, RF et al (2016c) Haemonchus contortus excretory and secretory proteins (HcESPs) suppress functions of goat PBMCs in vitro. Oncotarget

Galligan JJ, Petersen DR (2012) The human protein disulfide isomerase gene family. Hum Genomics 6:6

Garofalo A, Kennedy MW, Bradley JE (2003) The FAR proteins of parasitic nematodes: their possible involvement in the pathogenesis of infection and the use of Caenorhabditis elegans as a model system to evaluate their function. Med Microbiol Immunol 192(1):47–52

Gillan V, Maitland K, McCormack G, Nik Him NAII, Devaney E (2009) Functional genomics of hsp-90 in parasitic and free-living nematodes. Int J Parasitol 39(10):1071–1081

Gleichsner AM, Cleveland JA, Minchella DJ (2016) One stimulus-two responses: host and parasite life-history variation in response to environmental stress. Evolution 70(11):2640–2646

Gruber CW, Cemazar M, Heras B, Martin JL, Craik DJ (2006) Protein disulfide isomerase: the structure of oxidative folding. Trends Biochem Sci 31(8):455–464

Kaplan RM (2004) Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol 20(10):477–481

Kaplan RM, Vidyashankar AN (2012) An inconvenient truth: global warming and anthelmintic resistance. Vet Parasitol 186(1–2):70–78

Kuang L, Colgrave ML, Bagnall NH, Knox MR, Qian M, Wijffels G (2009) The complexity of the secreted NPA and FAR lipid-binding protein families of Haemonchus contortus revealed by an iterative proteomics-bioinformatics approach. Mol Biochem Parasitol 168(1):84–94

Mahadevan C, Krishnan A, Saraswathy GG, Surendran A, Jaleel A, Sakuntala M (2016) Transcriptome-assisted label-free quantitative proteomics analysis reveals novel insights into Piper nigrum—Phytophthora capsici Phytopathosystem. Front Plant Sci 7

Nwunuji TP, Mayowa OO, Yusoff SM, Bejo S, Salisi S, Mohd EAW (2014) The ameliorative effect of ascorbic acid on the oxidative status, live weight and recovery rate in road transport stressed goats in a hot humid tropical environment. Anim Sci J 85(5):611–616

Papanastasiou DK, Bartzanas T, Panagakis P, Zhang G, Kittas C (2016) Study of heat-stress levels in naturally ventilated sheep barns during heat waves: development and assessment of regression models. Int J Biometeorol 60(11):1637–1644

Peña MT, Miller JE, Horohov DW (2004) Effect of dexamethasone treatment on the immune response of Gulf Coast Native lambs to Haemonchus contortus infection. Vet Parasitol 119(2–3):223–235

Peter JW, Chandrawathani P (2005) Haemonchus contortus: parasite problem no. 1 from tropics—polar circle. Problems and prospects for control based on epidemiology. Trop Biomed 22(2):131–137

Qiao G, Chen M, Bucsek MJ, Repasky EA, Hylander BL (2018) Adrenergic signaling: a targetable checkpoint limiting development of the antitumor immune response. Front Immunol 9:164

Rathore DK, Suchitra S, Saini M, Singh BP, Joshi P (2006) Identification of a 66 kDa Haemonchus contortus excretory/secretory antigen that inhibits host monocytes. Vet Parasitol 138(3–4):291–300

Schallig HD, van Leeuwen MA, Hendrikx WM (1994) Immune responses of Texel sheep to excretory/secretory products of adult Haemonchus contortus. Parasitology 108(Pt 3):351–357

Schallig HD, van Leeuwen MA, Cornelissen AW (1997) Protective immunity induced by vaccination with two Haemonchus contortus excretory secretory proteins in sheep. Parasite Immunol 19(10): 447–453

Troell K, Engstrom A, Morrison DA, Mattsson JG, Hoglund J (2006) Global patterns reveal strong population structure in Haemonchus contortus, a nematode parasite of domesticated ruminants. Int J Parasitol 36(12):1305–1316

Turner DG, Wildblood LA, Inglis NF, Jones DG (2008) Characterization of a galectin-like activity from the parasitic nematode, Haemonchus contortus, which modulates ovine eosinophil migration in vitro. Vet Immunol Immunopathol 122(1–2):138–145

Tzelos T, Matthews JB, Buck AH, Simbari F, Frew D, Inglis NF et al (2016) A preliminary proteomic characterisation of extracellular vesicles released by the ovine parasitic nematode, Teladorsagia circumcincta. Vet Parasitol 221:84–92

van Die I, Cummings RD (2010) Glycan gimmickry by parasitic helminths: a strategy for modulating the host immune response? Glycobiology 20(1):2–12

Wang W, Wang S, Zhang H, Yuan C, Yan R, Song X, Xu LX, Li XR (2014a) Galectin Hco-gal-m from Haemonchus contortus modulates goat monocytes and T cell function in different patterns. Parasit Vectors 7:342

Wang Y, Yang W, Cama V, Wang L, Cabrera L, Ortega Y, Bern C, Feng Y, Gilman R, Xiao L (2014b) Population genetics of Cryptosporidium meleagridis in humans and birds: evidence for cross-species transmission. Int J Parasitol 44(8):515–521

Wang W, Yuan C, Wang S, Song X, Xu L, Yan R et al (2014c) Transcriptional and proteomic analysis reveal recombinant galectins of Haemonchus contortus down-regulated functions of goat PBMC and modulation of several signaling cascades in vitro. J Proteomics 98:123–137

Wang F, Xu L, Song X, Li X, Yan R (2016) Identification of differentially expressed proteins between free-living and activated third-stage larvae of Haemonchus contortus. Vet Parasitol 215:72–77

Wang Y, Lu M, Wang S, Ehsan M, Yan R, Song X et al (2017) Characterization of a secreted macrophage migration inhibitory factor homologue of the parasitic nematode Haemonchus Contortus acting at the parasite-host cell interface. Oncotarget 8(25):40052–40064

Wilkinson B, Gilbert HF (2004) Protein disulfide isomerase. Biochim Biophys Acta 1699(1–2):35–44

Winter AD, McCormack G, Page AP (2007) Protein disulfide isomerase activity is essential for viability and extracellular matrix formation in the nematode Caenorhabditis elegans. Dev Biol 308(2):449–461

Yatsuda AP, Krijgsveld J, Cornelissen AW, Heck AJ, de Vries E (2003) Comprehensive analysis of the secreted proteins of the parasite Haemonchus contortus reveals extensive sequence variation and differential immune recognition. J Biol Chem 278(19):16941–16951

Zhang H, Zhou Q, Yang Y, Chen X, Yan B, Du A (2013) Characterization of heat shock protein 70 gene from Haemonchus contortus and its expression and promoter analysis in Caenorhabditis elegans. Parasitology 140(06):683–694

Zhao G, Yan R, Muleke CI, Sun Y, Xu L, Li X (2012) Vaccination of goats with DNA vaccines encoding H11 and IL-2 induces partial protection against Haemonchus contortus infection. Vet J 191(1):94–100