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DIPARTIMENTO DI SCIENZE VETERINARIE PER LA SALUTE, LA PRODUZIONE ANIMALE E LA SICUREZZA ALIMENTARE

Association of dietary soy with expression of various pro-inflammatory genes in porcine phagocytes.

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ABSTRACT.

Soybean and whey are two common protein sources used in piglet feeding; however, their effects on pro-inflammatory responses remain unclear. The present study investigated the expression of various genes implicated in the activation/deactivation of porcine phagocytes post-weaning. Eighteen piglets were divided into two groups based on the main protein source of their diet; soybean (SB) or whey proteins (WP). Blood phagocytes were isolated at 72 days of age. Expression of urokinase plasminogen activator (u-PA), u-PA receptor (u-PAR), plasminogen activator inhibitors 1 and 2, intercellular adhesion molecule 1 (ICAM-1), inducible NO synthase (iNOS), cyclo-oxygenase-2 and interleukin-10 (IL-10) in activated monocytes and neutrophils (except IL-10) was determined by quantitative PCR. Expression of u-PAR, ICAM-1 and iNOS were lower in both cell types obtained from SB-fed piglets compared to WP-fed piglets. In conclusion, a SB-based diet, compared with a WP diet, is associated with reduced expression of crucial pro-inflammatory genes in porcine phagocytes.

KEYWORDS

Soy; phagocytes; inflammation.

PAGES

1 – 8

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1 Introduction

Conflicting data concerning the effects of soy foods on expression of cellular adhesion molecules and production of pro-inflammatory cytokines have been published. A review of 14 independent studies concluded that soy-based diets have mixed effects on expression of cellular adhesion molecules, and do not affect production of crucial pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) (Beavers *et al.* 2009). However, there is evidence suggesting that soy protein peptides (SPP) exert anti-inflammatory activity (using a dextran sodium sulfate-induced pig model of intestinal inflammation) by down-regulating pro-inflammatory responses in vivo (Young *et al.* 2012), and can reduce production of nitric oxide (NO) and prostaglandin E2 (PGE2) and the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) genes by macrophages activated by lipopolysaccharide in vitro (Martinez-Villaluenga *et al.* 2009). Lunasin (a SPP) inhibits inflammation through the suppression of the nuclear factor-kappaB (NF-kB) pathway in the macrophage in vitro (De Mejia and Dia, 2009). Our group has also shown that SPP down-regulated the expression of various pro-inflammatory genes in ovine phagocytes induced by fatty acids in vitro (Politis *et al.* 2012).

To the best of our knowledge, there is lack of studies investigating whether dietary soy proteins can modify the expression of various genes implicated in activation/deactivation of porcine phagocytes in vivo. Taking into account the wide use of soybean meal and whey concentrates as protein sources in pig feeding (Yang et al., 2007; Yun et al., 2005), the investigation of their effects on pro-inflammatory responses would be of great importance; particularly, in the critical post-weaning period. Therefore, the present study aimed to compare the expression patterns of urokinase-plasminogen activator (u-PA), u-PA receptor (u-PAR), plasminogen activator inhibitor type 1 and 2 (PAI-1, PAI-2), intercellular adhesion molecule 1 (ICAM-1), interleukin 10 (IL-10), iNOS and COX-2, in activated monocytes and neutrophils obtained from piglets fed diets with either soybean meal (SB-based diet) or whey proteins (WP-based diet) as the main crude protein source post-weaning.

2 Materials and Methods

2.1 Animals and diets

Eighteen castrated male Large White × Duroc × Landrace weaned piglets (29±2 days old) were used in compliance with the guidelines of the Faculty of Animal Science and Aquaculture of Agricultural University of Athens. Animals were divided into 2 groups (with average body weight of 8.4 ± 0.68 kg per group). They were fed ad libitum one of two isocaloric (15.5 MJ/Kg dry matter) and isonitrogenous (230 g/Kg dry matter) diets (NRC, 1998), which contained either soybean meal (SB) or a mixture of whey proteins [WP, 70% WheyPro65 (650 g CP/kg) + 30% WheyPro 80 (800 g CP/kg); Hellenic Proteins S.A., Veria, Greece] as the main crude protein (CP) source (table 1).

ISSN: 2283-3927

Table 1. Ingredients and chemical composition of the soybean meal-based (SB) and whey protein-based (WP) experimental diets.

	Diets	
	SB	**WP**
Ingredients (g/kg)		
Maize	621.0	754.0
Soybean meal (440 g CP/kg)	342.0	-
Whey proteinsa (660 g CP/kg)	-	210.0
L-Lysine 80%	2.0	2.0
DL-Methionine 99%	1.0	1.0
Sodium chloride	5.0	4.0
Calcium carbonate	13.0	14.0
Monocalcium phosphate	13.0	12.0
Mineral-vitamin premixb	3.0	3.0
Analyzed chemical composition (g/kg DM)		
Dry matter (g/kg)	888.0	903.0
Crude protein	232.0	228.0
Ether extract	33.1	40.4
Digestible energyc (MJ/kg)	15.5	15.4

a. Mixture of whey proteins (Hellenic Proteins S.A., Veria, Greece); 70% WheyPro 65 (650 g CP/kg) + 30% WheyPro 80 (800 g CP/kg) designed to have a content of 660 g of crude protein/kg.

b. Mineral-vitamin premix (Nuevo S.A., N. Artaki, Greece) provided per kg of diet: 15000 IU vitamin A (retinyl acetate), 2000 IU vitamin D3 (cholecalciferol), 100 mg vitamin E (DL-α-tocopheryl acetate), 3.5 mg menadione (vitamin K3), 2.5 mg vitamin B1, 6 mg vitamin B2, 3 mg vitamin B6, 25 μg cyanocobalamin, 25 mg nicotinic acid, 20 mg pantothenic acid, 2 mg folic acid, 250 μg biotin, 2 mg Co, 4 mg I, 600 μg Se, 300 mg Fe, 100 mg Mn, 100 mg Mg, 320 mg Cu and 240 mg Zn.

c. Digestible energy values were calculated according to tabulated data (NRC, 1998).

2.2 Chemical analyses

Feed samples were milled through 1-mm screen prior to analyses. Feed DM was assessed in 5 g samples by oven drying at 105 °C overnight. Routine procedures of AOAC (1984) were used

ISSN: 2283-3927

for ether extract (EE; 7.063). Crude protein (CP) was determined as 6.25×Kjeldahl nitrogen, using a Kjeltec autoanalyzer unit (Foss, Sweden). All analyses were performed in duplicate.

2.3 Blood sampling and cell isolation, activation

Blood samples were collected from all animals at the last day of the experiment (72 days of age). Monocytes and neutrophils were isolated (Politis et al., 2012), washed (×3) with Hank's balanced salt solution, and then activated by adding phorbol myristate acetate (PMA; 81 μM). After 30 min, cells were washed (×3) with Hank's balanced salt solution (HBSS) and kept at -80 °C pending RNA extraction and RT-PCR.

2.4 RNA extraction and RT-PCR analysis

Total RNA was extracted from 5 x 10^6 cells from both purified populations of blood cells. Total RNA extraction and reverse transcription were performed as described previously (Politis et al., 2012). Relative levels of mRNA were quantified with real-time, quantitative RT-PCR using SYBR Green chemistry. A pair of primers for each of the genes used in this study was constructed using PERLprimer software (Marshall, 2004). All primer pairs are presented in table 2. The amount of sample RNA was normalised by using b-actin as a housekeeping gene. Real time PCR was performed in the MyiQ2 cycler (BioRad) using the KAPA SYBR® FAST qPCR Kit (Kapa biosystems) according to the manufacturer's protocol. Each reaction contained 12.5 ng RNA equivalents as well as 150-300 nM of forward and reverse primers for each gene. The reactions were incubated at 95 °C for 30 s followed by 40 cycles of 5 s at 95 °C and 15 s at 60 – 64 °C. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. The comparative Ct method (Livak & Schmittgen, 2001) was used for relative quantification.

2.5 Statistical analysis

Data were analyzed using the SPSS statistical package (version 17.0). Comparisons between diets were conducted using a two-tail unpaired t-test and values are presented as means ± SEM.

3 Results

The expression of u-PAR was lower (P<0.001) in both monocytes and neutrophils obtained from the SB fed piglets when compared to those obtained from the WP fed piglets, whereas the expression of u-PA, PAI-1 and PAI-2 did not differ between the two experimental groups (figure 1). The expression of ICAM-1 and iNOS was also lower (P < 0.05) in both monocytes and neutrophils obtained from SB fed piglets when compared with the corresponding values in phagocytes obtained from piglets fed the WP-based diet (figure 2). There were no differences

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in the expression of the COX-2 and IL-10 between the two experimental groups (data not shown).

Table 2. Sequences, corresponding accession numbers and relative positions of primers for urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2), intercellular adhesion molecule 1 (ICAM-1), cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin 10 (IL-10) and beta actin (ACTb) used in Real Time PCR.

	Primer	Sequence 5' to 3'	Accession number	Position
uPA	Sus_UPA_F	GTGGCTGTCTGAATGGAGG	NM_213945.1	202 - 318
	Sus_UPA_R	AGGTTTGCGATGTGTCTATCTC		
uPAR	Sus_UPAR_F	ATGGGAAGGAGGTGAGGA	XM_003127198.2	188 - 384
	Sus_UPAR_R	AAGCACATTCAAGGTAACGAC		
PAI-1	Sus_PAI1_F	TTCTGCCCAAGTTCTCCC	NM_213910.1	1027 - 1194
	Sus_PAI1_R	CATTCACCTCGATCTTCACCT		
PAI-2	Sus_PAI2_F	AACATCGGATACTTAGCAGACC	XM_003121697.1	739 - 939
	Sus_PAI2_R	ATACACCTCCACATCATCTTCAG		
ICAM-1	Sus_ICAM1_F	AACTTATGTCCTGCCAGCC	NM_213816.1	671 - 841
	Sus_ICAM1_R	CCATTATGCGTGATTGTTAGTGG		
COX-2	Sus_COX2_F	CTGTACTACACCTGAATTTCTGAC	NM_214321.1	279 - 385
	Sus_COX2_R	TGACAATGTTCCAGACTCCC		
iNOS	Sus_INOS_F	AAGTTTGACCATAGGACCCAG	NM_001143690.1	344 - 488
	Sus_INOS_R	CTTTGTTACCGCTTCCACC		
IL-10	Sus_IL10_F	CTGTCATCAATTTCTGCCCTG	HQ236499.1	363 - 526
	Sus_IL10_R	AGTTCTTCCTCATCTTCATCGT		
ACTb	Sus_ACTbF	CTACCAGTTCGCCATGGA	XM_003124280.2	78 - 252
	Sus_ACTbR	CACGTAGGAGTCCTTCTGG		

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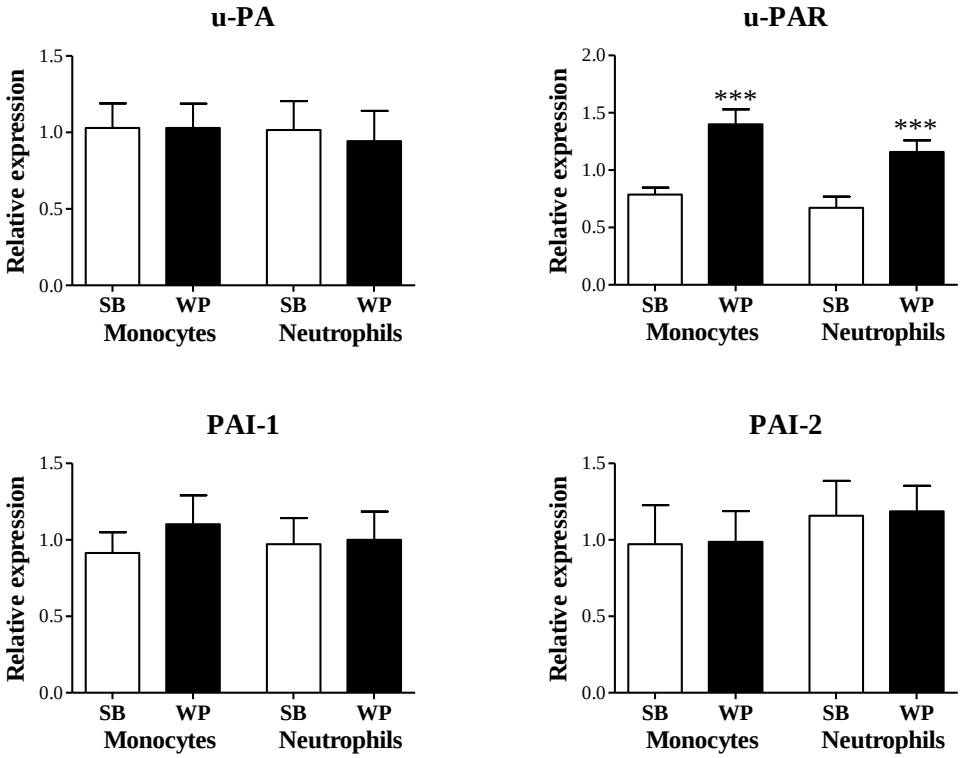
Figure 1. Relative mRNA levels (means±SEM) of urokinase plasminogen activator (u-PA), its receptor (u-PAR) and plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2) in blood monocytes and neutrophils from 72 day old pigs fed diets with soybean meal (SB) or whey proteins (WP). Asterisks indicate significant differences between SB and WP (***: P < 0.001).



4 Discussion

The main finding emerging from the present study is that SB-based diets, when compared to WP-based diets, are associated with lower levels of expression of three crucial pro-inflammatory genes (u-PAR, ICAM-1 and iNOS) in phagocytes obtained from piglets. The u-PAR molecule plays a central role in the plasminogen activating cascade and a crucial role in cell migration. Typically, u-PAR clusters are observed at the leading edges of migrating phagocytes. In an autocrine manner, the u-PA molecule produced by phagocytic cells themselves binds to its receptor (uPAR) and remains catalytically active; thus, it converts the inactive proenzyme plasminogen to active plasmin, which allows the migrating phagocytes to cross the endothelial barrier and reach the point of inflammation (De Mejia & Dia, 2009). The ICAM-1 encodes a cell surface adhesion glycoprotein, which is typically expressed by endothelial and phagocytic cells and it plays a crucial role in the process of diapedesis. On the other hand, expression of the iNOS gene results in the production of NO (free radical), which is one of the main pro-inflammatory compounds secreted by activated phagocytes (De Mejia &

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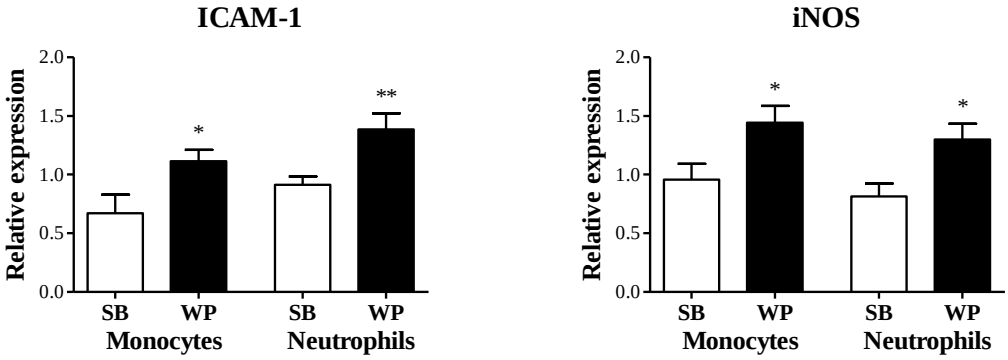
Dia, 2009). Interestingly, the results of the present in vivo study have certain similarities with those of a previous in vitro study, which showed that soy protein hydrolysates (peptides) down-regulated almost the same set of genes (u-PAR, ICAM-1 and iNOS), but not that of COX-2 and IL-10 in ovine phagocytes (Politis et al., 2012). The reduced expression of the 3 key pro-inflammatory genes in the SB fed piglets can be reasonably attributed to the soybean meal proteins, without excluding a possible contribution of other non- protein compounds, such as isoflavones (Chacko et al., 2005). Noteworthy, there were no differences between groups in the expression of PAI-1, PAI-2 and COX-2 in phagocytes, and IL-10 in monocytes, which are genes with well-established roles in the inflammatory process (De Mejia & Dia, 2009; Theodorou et al., 2010). The reasons behind the fact that certain pro-inflammatory genes were expressed in lower levels, whilst others were not, in phagocytes from piglets fed the SB-based diet are not known and require future research.

Figure 2. Relative mRNA levels (means±SEM) of inter-cellular adhesion molecule 1 (ICAM-1) and inducible nitric oxide synthase (iNOS) in blood monocytes and neutrophils from 72 day old pigs fed diets with soybean meal (SB) or whey proteins (WP). Asterisks indicate significant differences between SB and WP (*: P < 0.05, **: P < 0.01).



5 Conclusions

In conclusion, a SB-based diet, when compared with a WP diet, is associated with lower levels of three key pro-inflammatory genes (u-PAR, ICAM-1 and iNOS) in phagocytes obtained from piglets. Future studies will focus on the potential mechanism of action through which soy may exercise its effects on immunocompetent cells.

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ISSN: 2283-3927

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