British Journal of Nutrition (2012), **108**, 1246–1255
doi:10.1017/S0007114511006520

Soya protein hydrolysates modify the expression of various pro-inflammatory genes induced by fatty acids in ovine phagocytes

Ioannis Politis[1]*, Georgios Theodorou[1], Antonios D. Lampidonis[1], Roubini Chronopoulou[1] and Antonella Baldi[2]

[1]*Department of Animal Science and Aquaculture, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece*
[2]*Department of Veterinary Science and Technologies for Food Safety, Università degli Studi di Milano, Milan, Italy*

(Submitted 16 August 2011 – Final revision received 27 October 2011 – Accepted 31 October 2011 – First published online 15 December 2011)

Abstract

The objective of the present study was to test the hypothesis that fatty acids are the circulating mediators acting in a pro-inflammatory manner towards activated circulating ovine monocyte/macrophages and neutrophils. Furthermore, whether soya protein hydrolysates (SPH) inhibit the fatty acid-induced increase in the production of pro-inflammatory responses by ovine phagocytes was tested *in vitro*. All the fatty acids tested (myristic, palmitic, palmitoleic, stearic and oleic) increased ($P<0{\cdot}01$; $C_{18} > C_{16} > C_{14}$) membrane-bound urokinase plasminogen activator (u-PA) and u-PA free binding sites in cell membranes of activated ovine blood monocytes/macrophages, but only the C_{18} fatty acids (stearic, oleic) were effective towards blood neutrophils. The C_{18} fatty acids up-regulated ($P<0{\cdot}05$) the gene expression of u-PA, u-PA receptor, intercellular adhesion molecule 1 and inducible NO synthase (in monocytes) but not that of cyclo-oxygenase-2, integrin α X and plasminogen activator inhibitor types 1 and 2 by ovine phagocytes. SPH blocked completely or partially all C_{18} fatty acid-induced changes in the expression of various pro-inflammatory genes. In conclusion, fatty acids selectively 'activate' ovine phagocytes, suggesting that these cells 'sense' metabolic signals derived from adipocytes. Soya protein peptides inhibit all changes in gene expression induced by fatty acids in ovine phagocytes *in vitro*. This constitutes a novel mechanism of action.

Key words: Inflammation: Fatty acids: Phagocytes: Soya protein hydrolysates

The worldwide epidemic of obesity and its strong association with insulin resistance and type 2 diabetes have elicited considerable interest in the underlying mechanisms of these pathologies. The hallmark of obesity includes excess development of adipose tissue (mainly ectopic lipid deposition), reduced fatty acid oxidation and a low-grade inflammation orchestrated by metabolic signals produced in response to excess nutrients and energy[1].

A growing body of literature implicates the adipose tissue in playing a crucial role as the source and the main site of inflammation. Adipose tissue is now recognised as a key endocrine organ because it releases several bioactive substances, known as adipose-derived secreted factors or adipokines, that have mainly pro-inflammatory but also anti-inflammatory actions[2,3]. Elevated levels of adipokines such as leptin, TNFα, IL-1, IL-8, C-reactive protein, fibrinogen and plasminogen activator inhibitor (PAI) type 1 are generally increased during obesity. Increased production of PAI-1 may serve as an autocrine/paracrine feedback loop to limit adipose tissue expansion[4]. A general dysfunction of the adipose tissue occurs during obesity and it is related to impaired blood perfusion[5]. Furthermore, accumulation of immune cells, mainly monocytes, occurs within the adipose tissue in obese conditions[6]. Accumulated monocytes/macrophages within the adipose tissue overexpress urokinase plasminogen activator receptor (u-PAR)[7], a protein present in the cell membranes of ovine phagocytes and which plays a major role in dictating cell motility. Dysregulated function of adipose tissue affects the function of both adipocytes and monocytes/macrophages and essentially constitutes the cellular basis that promotes inflammation during obesity[5,8].

Increasing evidence supports the hypothesis that a local and systemic cross-talk between adipocytes and monocytes/macrophages is mediated by fatty acids. Most medium- to long-chain fatty acids promote inflammation in cell culture studies. NEFA dynamically regulate the expression of PAI-1 by monocytes/macrophages, suggesting that these cells 'sense' metabolic signals derived from fat cells[9,10]. Saturated

Abbreviations: BSA, bovine serum albumin; CD11c, integrin α X; COX-2, cyclo-oxygenase-2; HBSS, Hanks balanced salt solution; ICAM-1, inter-cellular adhesion molecule 1; iNOS, inducible NO synthase; LPS, lipopolysaccharide; PAI, plasminogen activator inhibitor; PMA, phorbol myristate acetate; SPH, soya protein hydrolysates; u-PA, urokinase plasminogen activator; u-PAR, urokinase plasminogen activator receptor.

* **Corresponding author:** Dr I. Politis, fax +30 210 529 4442, email i.politis@aua.gr

(palmitate) and unsaturated (oleate and linoleate) fatty acids up-regulated the expression of u-PAR in phorbol myristate acetate (PMA)-differentiated human monocyte/macrophage-like cells (U937)[11]. Palmitate, stearate, but not oleate increased the expression of Toll-like receptors 2 and 4, reactive oxygen species production, NFκB activity and IL-1β expression in a dose- and time-dependent manner in monocytes, thus, amplifying the onset of inflammation[12]. Palmitate, especially when combined with insulin, up-regulated the expression of IL-6 and TNFα in human monocytes[13]. Altogether, eleven fatty acids exerted differential effects on cytokine production by monocytes/macrophages, with the main finding being that eicosapentanoic acid acts in an anti-inflammatory manner[14]. PUFA (linoleic, α-linoleic and docosahexaenoic) elicited anti-inflammatory effects in THP-1 (human acute monocytic leukaemia cell line) cells[15]. The effects of fatty acids on the functional properties of neutrophils are less well investigated. Saturated and unsaturated fatty acids appear to activate neutrophils as shown by increased reactive oxygen species production[16] as well as increased cellular adhesion and degranulation[17]. To the best of our knowledge, there are no studies on the effect of fatty acids on the expression of pro-inflammatory genes by neutrophils.

Several studies suggest that soya proteins and their isoflavones may play a beneficial role in obesity by reducing body weight and fat mass. Whether soya proteins can down-regulate pro-inflammatory responses is not known. There is only one study suggesting that soya protein hydrolysates (SPH) reduce the production of NO and PGE_2 and the expression of inducible NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2) by lipopolysaccharide (LPS)-induced macrophages (RAW 264.7)[18]. This study implicates soya protein peptides generated by enzymatic proteolysis as acting in an anti-inflammatory manner. In a similar fashion, de Mejia & Dia[19] reported that lunasin (a peptide present in soyabeans) inhibits inflammation through the suppression of the NF-κB pathway in the macrophage. To the best of our knowledge, there is a total lack of studies investigating whether soya protein peptides can down-regulate pro-inflammatory responses elicited by fatty acids. If this hypothesis is proven to be correct, this will constitute a novel mechanism of action.

We want to examine further and in greater detail the hypothesis that fatty acids are the circulating metabolic factors that induce the expression of various pro-inflammatory responses in ovine monocytes/macrophages and neutrophils. We have selected a total of nine genes that play a crucial role in activation/deactivation of both cell types, urokinase plasminogen activator (u-PA), u-PAR, PAI-1, PAI-2, inter-cellular adhesion molecule 1 (ICAM-1), iNOS, integrin α X (CD11c), COX-2 and IL-10. Furthermore, we wish to test the hypothesis that soya protein peptides generated during enzymatic hydrolysis act in an anti-inflammatory manner by reducing the level of activation of monocytes/macrophages and neutrophils conferred by fatty acids. Therefore, the objectives of the present study were: (1) to study in detail the effect of myristic (C_{14}), palmitic, palmitoleic (both C_{16}), stearic and oleic (both C_{18}) fatty acids on the expression of *u-PA*, *u-PAR*, *PAI-1*, *PAI-2*, *ICAM-1*, *iNOS*, *CD11c*, *COX-2* and *IL-10* genes, along with membrane-bound u-PA activity and free u-PA binding sites in ovine monocytes/macrophages and neutrophils; and (2) to evaluate whether soya protein peptides produced by alcalase or by simulated gastrointestinal digestion can down-regulate the effects of fatty acid-induced effects on the expression of the aforementioned genes by ovine monocytes/macrophages and neutrophils.

Materials and methods

Animals

A total of ten ewes of the Chios breed and a synthetic breed (50% Boutsiko, 25% Arta and 25% Chios) were used to obtain blood samples for isolation of blood monocytes and neutrophils. Animals were housed within the premises of the experimental farm of the Agricultural University of Athens. A total of four blood samples were obtained from each animal at weekly intervals during the non-lactating period. This period was selected because it coincides with minimal fatty acid mobilisation in lactating animals. The guidelines of 'Council Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes' were followed throughout the experiment.

For this study, eight defatted soyabean flour samples were provided by Soya Hellas. All reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Preparation of soya protein hydrolysates

SPH were obtained using (1) treatment with alcalase and (2) simulated gastrointestinal digestion. The hydrolysates with alcalase treatment were prepared as described by Martinez-Villaluenga *et al.*[20]. The liquid hydrolysates were concentrated using stirred ultra-filtration cell 1 kDa membrane (Millipore). The protein content of the hydrolysate was determined using the Protein DC assay kit II (Bio-Rad). The hydrolysate using simulated gastrointestinal digestion was obtained following treatment with pepsin–pancreatin of defatted soya flours as described by others[21]. The dialysate containing the soluble compounds of low molecular weight from soya protein was collected and used as described next.

Cell isolation

Monocytes and neutrophils were isolated using methods previously described by Politis *et al.*[22,23]. The purity of monocytes was 92% (88–97%) and the purity of neutrophils was 95% (92–98%). Cell viability was assessed by the trypan blue dye exclusion methodology (typically >95% of total cells obtained were viable). All reagents and water used were endotoxin-free to avoid phagocyte activation.

Cell culture – treatment with NEFA

The first set of experiments examined the effect of NEFA on various enzymatic determinations of the u-PA system. Following isolation, cells (monocytes or neutrophils) were

cultured in cell culture medium (RPMI 1640 containing 2 mM-L-glutamine, 1·5 g/l HCO_3 and 10 mM-HEPES supplemented with 5·5 mM-glucose, 1 mM-sodium pyruvate, 50 mg/ml penicillin and streptomycin and 10 % fetal bovine serum) in the presence or absence of various concentrations (0–250 μM) of the following NEFA: myristic, palmitic, palmitoleic, stearic and oleic acids. Preliminary experiments indicated that higher concentrations of NEFA (375–500 μM) were no more effective than the 125 or 250 μM concentrations. NEFA were bound to bovine serum albumin (BSA) in a 10 mM stock solution following the procedure initially described by Brun *et al.*[24]. The molar ratio of NEFA to BSA in the 10 mM stock solution was 6:1. The NEFA stock solutions were diluted in cell culture medium before the experiments to obtain the final concentrations. The final concentrations of BSA in test media were 0·5 or 1 %. Control cultures contained the highest concentration of unbound BSA, even though addition of unbound BSA in preliminary experiments showed that it had no effect on any of the parameters measured. Cells were incubated with NEFA for 24 h. Preliminary experiments examined the optimal incubation between three time points (12, 24 and 48 h). NEFA were not effective at 12 h of incubation. The pattern of effect of NEFA was similar between 24 and 48 h. Thus, the incubation time of 24 h was selected for all subsequent experiments. At the end of incubation, cell culture medium was removed, cells were washed three times with Hanks balanced salt solution (HBSS) containing 20 μM-HEPES and PMA (81 μM) was added to activate the cells. After incubation for 30 min, cells were washed again three times with HBSS and then processed for various determinations of u-PA activity. Politis *et al.*[25] indicated that the concentration of 81 μM of PMA and the incubation time of 30 min resulted in optimal stimulation of phagocytes. Incubation of cells with PMA for periods longer than 60 min produced cytotoxic effects.

The second set of experiments examined the ability of SPH: (1) to block the induction of various genes or the induction of the u-PA system at the protein level in monocytes and neutrophils activated by PMA or LPS and (2) to block the induction of nitrate production by monocytes activated with LPS. Cells (monocytes or neutrophils) were cultured in culture medium in the presence of stearic or oleic acid (250 μM) and combinations of stearic or oleic acid (250 μM) with SPH (100 μM) for 24 h. At the end of incubation, cell culture medium was removed, cells were washed three times with HBSS containing 20 μM-HEPES and then PMA (81 μM) or LPS (100 ng/ml) was added to activate the cells. After incubation for 30 min, cells were washed again three times with HBSS and then processed for RNA isolation, for various determinations of u-PA activity or for determination of nitrate production (monocytes).

Real-time quantitative PCR

Total RNA was extracted from 5×10^6 cells from both purified populations of blood cells (monocytes and neutrophils) using the RNaqueous kit (Ambion) and was quantified. The quality and quantity of the RNA extracted were confirmed by spectrophotometry as well as gel electrophoresis. Reverse transcription of equal amounts of total RNA was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions, using a mix of random hexamers and oligo-dT primers. Relative levels of mRNA were quantified with real-time PCR using TaqMan (Applied Biosystems) chemistry for the PA-related genes (*u-PA*, *u-PAR*, *PAI-1* and *PAI-2*), as previously described by our group[26]. For the relative quantification of gene expression of *ICAM-1*, *iNOS*, *CD11c*, *COX-2* and *IL-10*, EvaGreen chemistry was selected. A pair of primers for each of the target genes and the housekeeping gene (β-actin) were constructed using PerlPrimer software (http://perlprimer.sourceforge.net)[27]. All primers were designed based on *Ovis aries* sequences, while β-actin primers were designed in highly homologous regions, between different species. PCR products from all primer pairs used were verified by sequencing. All primer pairs are presented in Table 1. The amount of sample RNA was normalised by using *β-actin* as a housekeeping gene, which was stably expressed in all samples tested. The real-time PCR was performed using the SsoFast™ EvaGreen Supermix (Bio-Rad) according to the manufacturer's protocol. Each reaction for the quantification of the housekeeping gene and the target genes contained 50 ng RNA equivalents as well as 450 nM of forward and reverse primers. The reactions were incubated at 95°C for 30 s followed by forty cycles of 5 s at 95°C and 10 s at 60°C. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in

Table 1. Sequences and relative positions of primers for ovine cyclo-oxygenase-2 (*COX-2*), inter-cellular adhesion molecule 1 (*ICAM-1*) and inducible NO synthase (*iNOS*) genes used in real-time PCR

Gene	Primer	Sequence	Position (bp)
COX-2	Ov-COX2_for	AGGAGTACAACTATCAGCAG	1238–1257
	Ov-COX2_rev	GACTGGTATTTCATCTCTCTG	1404–1424
ICAM-1	Ov-ICAM1_for	CAACGGTGACTCTGTCTTGG	816–835
	Ov-ICAM1_rev	GGTCAGGGTGGGAAGTGG	946–963
iNOS	Ov-iNOS_for	CAAGCACCACATTGAGATCC	1538–1557
	Ov-iNOS_rev	GATTCTGCTGCGATTTGAGC	1682–1701
CD11c	Ov-CD11c_for	CAGACCCACTAAACCTCCTG	628–647
	Ov- CD11c _rev	ATGACCTCCTTGTAATCCACC	794–814
IL-10	Ov-IL-10_for	AGCTGTACCCACTTCCCA	141–158
	Ov- IL-10_rev	ACCCAGGTAACCCTTAAAGTC	273–293

CD11c, integrin α X.

Table 2. Effect of a 24 h incubation with various fatty acids on membrane-bound urokinase plasminogen activator (u-PA) activity and free u-PA binding sites on cell membranes of ovine monocytes and neutrophils activated with phorbol myristate acetate (81 μM)

(Mean values with their standard errors)

	Membrane-bound u-PA (ΔA/h per 10^6 cells)				Free u-PA binding sites (ΔA/h per 10^6 cells)			
	Monocytes		Neutrophils		Monocytes		Neutrophils	
Fatty acid (μM)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control (BSA)	0·26[a]	0·03	0·52[a]	0·07	0·62[a]	0·08	0·84[a]	0·10
Myristic (125)	0·28[a]	0·03	0·55[a]	0·07	0·60[a]	0·08	0·77[a]	0·10
Myristic (250)	0·34[b]	0·03	0·53[a]	0·07	0·75[b]	0·08	0·86[a]	0·10
Palmitic (125)	0·29[a]	0·03	0·58[a]	0·07	0·60[a]	0·08	0·75[a]	0·10
Palmitic (250)	0·42[c]	0·03	0·49[a]	0·07	0·88[c]	0·08	0·88[a]	0·10
Palmitoleic (125)	0·29[a]	0·03	0·61[a]	0·07	0·64[a]	0·08	0·90[a]	0·10
Palmitoleic (250)	0·42[c]	0·03	0·49[a]	0·07	0·87[c]	0·08	0·79[a]	0·10
Stearic (125)	0·40[c]	0·03	0·60[a]	0·07	0·85[c]	0·08	0·87[a]	0·10
Stearic (250)	0·60[d]	0·03	0·75[b]	0·07	1·05[d]	0·08	1·22[b]	0·10
Oleic (125)	0·42[c]	0·03	0·63[a]	0·07	0·87[c]	0·08	0·76[a]	0·10
Oleic (250)	0·61[d]	0·03	0·77[b]	0·07	1·05[d]	0·08	1·25[b]	0·10

BSA, bovine serum albumin.
[a,b,c,d] Mean values within the same column with unlike superscript letters are significantly different ($P<0·05$)

triplicates. The comparative C_t method[28] was used for relative quantification. The amount of target, normalised to β-actin and relative to a calibrator, is given by $2^{-\Delta\Delta C_t}$. All real-time PCR reactions were performed in the 7500 Real-Time PCR System (Applied Biosystems).

Determination of various forms of urokinase plasminogen activator activity and nitrate production

Total, membrane-bound u-PA activity and free u-PA binding sites were determined in monocytes and neutrophils following the methods developed by Politis *et al.*[25,29]. Nitrate production was measured using the method described by others[18,30].

Statistical analysis

Data represent the means with their standard errors of at least three independent experiments. Within each experiment, each treatment was performed in triplicate. The effect of the various treatments was assessed by ANOVA. Fisher's least significant difference test was used *post hoc*, with a 95 % CI. All analyses were performed using the PASW Statistics 18 release 18.01 (SPSS, Inc.) program.

Results

The effects of various saturated (myristic, palmitic, stearic) and unsaturated (palmitoleic, oleic) fatty acids on membrane-bound u-PA activity and free u-PA binding sites of activated ovine monocytes are presented in Table 2. Membrane-bound u-PA was always more than 95 % of total u-PA activity and for this reason data on total u-PA activity will not be presented in detail. Data show that all fatty acids increased membrane-bound u-PA activity and free u-PA binding sites in ovine monocytes. Stearic and oleic acids were effective in both concentrations (125 and 250 μM), while myristic, palmitic and palmitoleic acids were effective only at the higher concentration (250 μM). For the same concentration (250 μM), effectiveness increased with increasing carbon-chain length ($C_{18} > C_{16} > C_{14}$). For the same carbon-chain length, there were no differences between saturated and unsaturated fatty acids.

The effects of various saturated (myristic, palmitic, stearic) and unsaturated (palmitoleic, oleic) fatty acids on membrane-bound u-PA activity and free u-PA binding sites of activated ovine neutrophils are presented in Table 2. Only the C_{18} fatty acids (stearic and oleic) were effective at the high concentration (250 μM). In contrast, myristic, palmitic and palmitoleic acids caused no effect on membrane-bound u-PA activity and free u-PA binding sites of neutrophils.

SPH at both concentrations (100 and 200 μM) inhibited the stearic and oleic acid-induced increase in membrane-bound u-PA activity and free u-PA binding sites of both monocytes and neutrophils (Table 3). SPH produced with both methodologies (simulated gastrointestinal digestion and treatment with alcalase), were equally effective and therefore, data with only the simulated gastrointestinal digestion will be presented throughout.

The effect of SPH on the relative expression of u-PA, u-PAR, PAI-1 and PAI-2 in activated ovine monocytes and neutrophils cultured in the presence of stearic or oleic acids is presented in Fig. 1. Data indicate that both stearic and oleic acid increased 4-fold ($P<0·001$) the expression of u-PA and u-PAR in ovine monocytes (Fig. 1(A)) and by 2-fold ($P<0·05$) in ovine neutrophils (Fig. 1(B)). SPH, when added together with the respective fatty acid in ovine monocytes and neutrophils, blocked partially (Fig. 1(A)) and totally (Fig. 1(B)) the up-regulation of u-PA and u-PAR expression observed when fatty acids alone were added. In contrast, none of the fatty acids alone or when combined with SPH affected the expression of PAI-1 and PAI-2 by ovine monocytes (Fig. 1(A)) and neutrophils (Fig. 1(B)).

Table 3. Effect of soya protein hydrolysate (SPH) on membrane-bound urokinase plasminogen activator (u-PA) activity and free u-PA binding sites on cell membranes of ovine monocytes and neutrophils activated with phorbol myristate acetate (81 μM) in the presence or absence of various fatty acids

(Mean values with their standard errors)

	Membrane-bound u-PA (ΔA/h per 10^6 cells)				Free u-PA binding sites (ΔA/h per 10^6 cells)			
	Monocytes		Neutrophils		Monocytes		Neutrophils	
Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control (BSA)	0·24[a]	0·04	0·50[a]	0·08	0·65[a]	0·10	0·85[a]	0·12
Stearic (250 μM)	0·49[b]	0·04	0·81[b]	0·08	0·98[b]	0·10	1·26[b]	0·12
Stearic (250 μM) + SPH (100 μM)	0·32[c]	0·04	0·62[c]	0·08	0·77[c]	0·10	0·88[a]	0·12
Stearic (250 μM) + SPH (200 μM)	0·34[c]	0·04	0·65[c]	0·08	0·73[c]	0·10	0·85[a]	0·12
Oleic (250 μM)	0·50[b]	0·04	0·80[b]	0·08	1·02[b]	0·10	1·30[b]	0·12
Oleic (250 μM) + SPH (100 μM)	0·34[c]	0·04	0·63[c]	0·08	0·79[c]	0·10	0·93[a]	0·12
Oleic (250 μM) + SPH (200 μM)	0·31[c]	0·04	0·60[c]	0·08	0·74[c]	0·10	0·90[a]	0·12

BSA, bovine serum albumin.
[a,b,c] Mean values within the same column with unlike superscript letters are significantly different ($P<0·05$).

The effect of SPH on the relative expression of ICAM-1, iNOS, CD11c and COX-2 in activated ovine monocytes and neutrophils cultured in the presence of stearic or oleic acids is presented in Fig. 2. Data indicate that both fatty acids caused a 2-fold increase in the expression of ICAM-1 by ovine monocytes (Fig. 2(A)) and neutrophils (Fig. 2(B)) and an increase by 50 % in the expression of iNOS by ovine monocytes (Fig. 2(A)). The increase in expression caused by fatty acids in both cell types was totally negated with respect to ICAM-1 when SPH was added to the culture medium (Fig. 2). With respect to the iNOS, there was a trend for inhibition of the up-regulation caused by fatty acids in monocytes (Fig. 2(A)) in the presence of SPH, but this effect was not statistically significant ($P>0·05$). None of the treatments tested had any effect on the expression of COX-2 and CD11c by ovine monocytes (Fig. 2(A)) and neutrophils (Fig. 2(B)).

To further understand the role of SPH, we examined whether SPH could modify the effect of stearic and oleic acid on nitrate production by LPS-activated monocytes. Only the high concentration of SPH (200 μM) inhibited the stearic and oleic acid-induced increase in nitrate production (Table 4).

The effect of SPH on the relative expression of IL-10 in activated ovine monocytes cultured in the presence of stearic or oleic acids was also examined. However, none of the treatments tested had any effect on the expression of IL-10 by ovine monocytes (data not shown).

Discussion

The first finding emerging from the present study is that all the fatty acids used (C_{14}–C_{18}) increased total and membrane-bound u-PA activity and u-PA free binding sites in ovine monocytes. The up-regulation of membrane-bound u-PA contributes to the overall migratory potential of monocytes, because membrane-bound u-PA is catalytically active and thus, it can convert the inactive zymogen plasminogen to active plasmin. Plasmin, in turn, is capable of degrading certain matrix components, in addition to activating other matrix-degrading enzymes such as metalloproteinases[31]. Carbon-chain length is the pivotal factor in the interaction of fatty acids and membrane-bound u-PA as well as the free u-PA binding sites in ovine monocytes. More specifically, with respect to the action of myristic (C_{14}), palmitic or palmitoleic (C_{16}) acid, only the high concentration (250 μM) was effective. In contrast, the C_{18} fatty acids (stearic and oleic) were effective in both concentrations tested and they acted in a dose-dependent manner (see Table 2). Thus, the effectiveness of the fatty acids follows the order $C_{18} > C_{16} > C_{14}$. It is important to note that saturated and unsaturated fatty acids of the same carbon-chain length were equally effective. Thus, the presence and the position of double bonds within the fatty acid molecule play no further role in determining its effectiveness.

Regarding the ability of fatty acids to modulate the expression of various pro-inflammatory and one anti-inflammatory gene (*IL-10*), the experiments were done using the two most effective fatty acids, i.e. stearic (saturated) and oleic (unsaturated), based on the results presented in Table 2. Our results clearly indicate that both fatty acids selectively up-regulated the expression of crucial pro-inflammatory genes in ovine monocytes, thus, providing support for the notion that fatty acids constitute the metabolic mediators in a cross-talk between adipocytes and immunocompetent cells (monocytes), ending in a 'low-grade inflammation'. Both fatty acids used (stearic and oleic) up-regulated the expression of u-PA and u-PAR, two genes involved in cell migration. The work of others suggests that the u-PAR molecule plays a central role in the plasminogen-activating cascade. Typically, u-PAR clusters are observed at the leading edges of migrating cells[32,33]. The u-PA produced by phagocytic cells can bind to this receptor and, thus, can convert the abundant proteolytically inactive proenzyme plasminogen to active plasmin[31]. However, u-PAR can regulate cell migration in the absence of functional u-PA, suggesting that u-PAR has a protease-independent function[34]. Our data regarding the ability of the C18 fatty acids to up-regulate the u-PAR system have certain similarities with those of Assmann *et al.*[11] who demonstrated that palmitate, oleate and linoleate increased

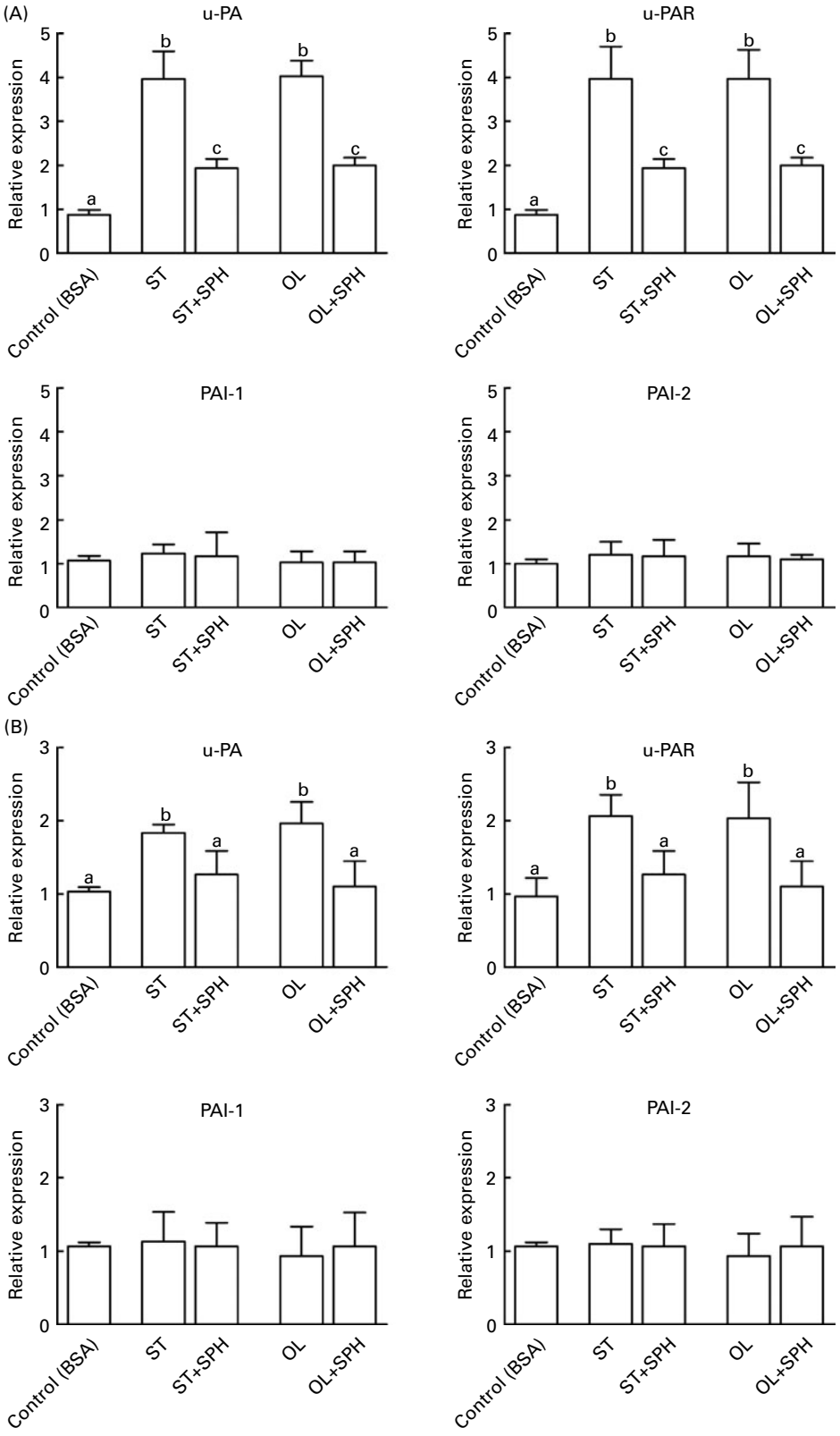

Fig. 1. Effect of soya protein hydrolysate (SPH) on the relative expression of urokinase plasminogen activator (u-PA), u-PA receptor (u-PAR) and plasminogen activator inhibitor types 1 and 2 (PAI-1 and PAI-2) of ovine (A) monocytes and (B) neutrophils activated with phorbol myristate acetate (81 μM) or lipopolysaccharide (100 ng/ml) cultured in the presence of stearic (ST) or oleic (OL) acid. Values are means, with their standard errors represented by vertical bars of at least three independent experiments. Within each experiment there were three replicates for each treatment. Differences between treatments were assessed using Fisher's least significant difference test following an ANOVA analysis. [a,b,c] Mean values with unlike letters were significantly different ($P<0{\cdot}05$). BSA, bovine serum albumin.

British Journal of Nutrition

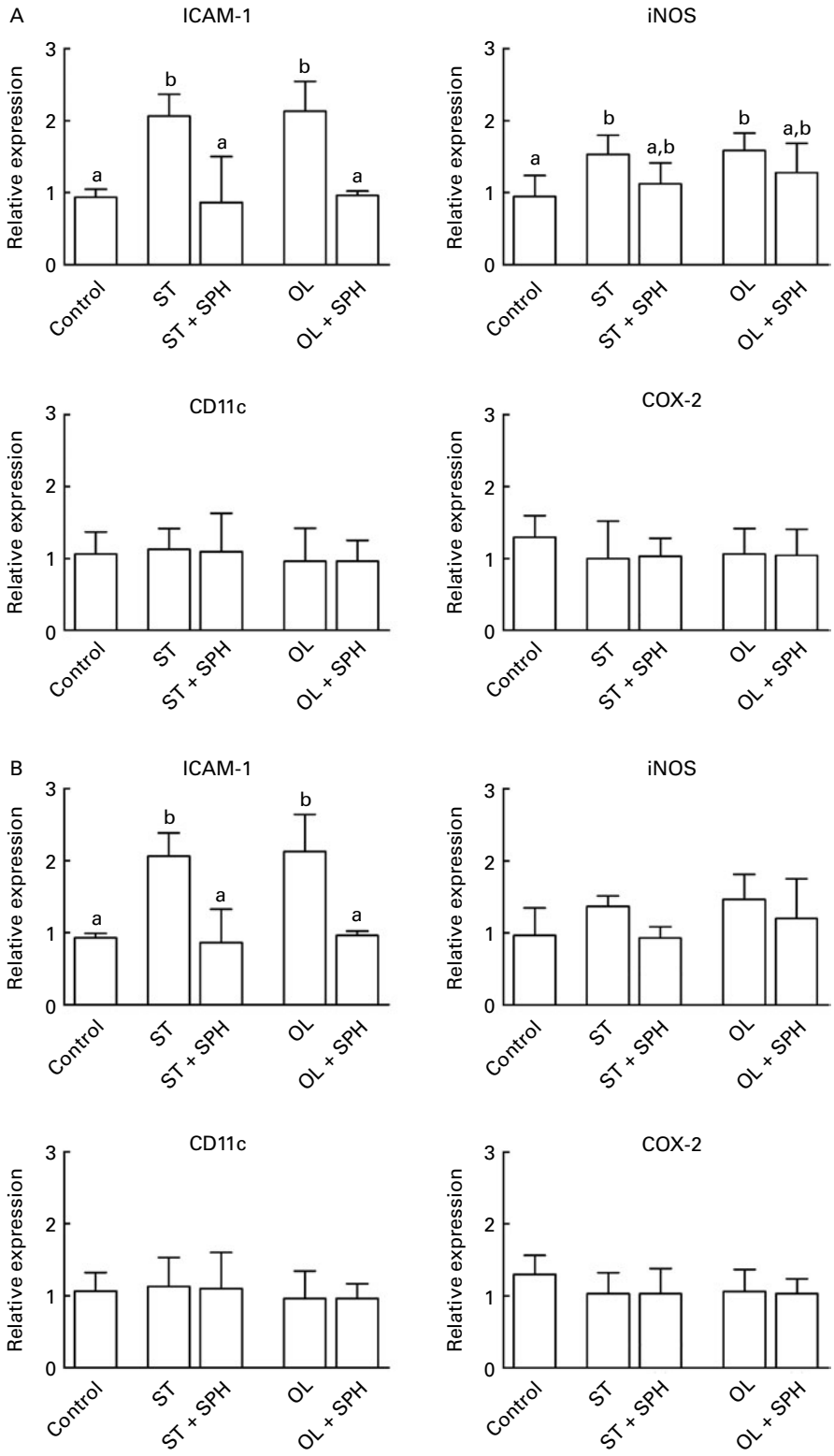

Fig. 2. Effect of soya protein hydrolysate (SPH) on the relative expression of inter-cellular adhesion molecule 1 (ICAM-1), inducible NO synthase (iNOS), integrin, α X (CD11c) and cyclo-oxygenase-2 (COX-2) of ovine (A) monocytes and (B) neutrophils activated with phorbol myristate acetate (81 μM) or lipopolysaccharide (100 ng/ml) cultured in the presence of stearic (ST) or oleic (OL) acid. Values are means, with their standard errors represented by vertical bars of at least three independent experiments. Within each experiment there were three replicates for each treatment. Differences between treatments were assessed using Fisher's least significant difference test following an ANOVA analysis. [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}05$). BSA, bovine serum albumin.

Table 4. Effect of soya protein hydrolysate (SPH) on nitrate production by ovine monocytes activated by lipopolysaccharide (100 ng/ml) in the presence or absence of various fatty acids

(Mean values with their standard errors)

Treatment	Nitrate (μM)	
	Mean	SEM
Control (BSA)	26·1[a]	4·1
Stearic (250 μM)	43·7[b]	4·1
Stearic (250 μM) + SPH (100 μM)	37·9[b]	4·1
Stearic (250 μM) + SPH (200 μM)	24·9[a]	4·1
Oleic (250 μM)	44·7[b]	4·1
Oleic (250 μM) + SPH (100 μM)	40·4[b]	4·1
Oleic (250 μM) + SPH (200 μM)	26·6[a]	4·1

BSA, bovine serum albumin.
[a,b] Mean values within the column with unlike superscript letters are significantly different ($P<0·05$).

the expression of u-PAR. However, palmitate was more potent than the other two fatty acids, suggesting that carbon-chain length is not the pivotal factor in monocyte/macrophage-like cells (U937). It should be noted that we have demonstrated that the C18 fatty acids up-regulate both u-PA and u-PAR, but not PAI types 1 and 2, providing a more holistic picture, while in the previous study[11], only u-PAR changes were investigated. Consistent with the notion that fatty acids act in a pro-inflammatory manner is the lack of effect of fatty acids on the expression of PAI-1 and PAI-2. These genes appear to play a crucial role in limiting the plasminogen-activating cascade, thus, contributing to the successful localisation of an infection as proposed by us[26].

The C_{18} fatty acids selectively up-regulated the expression of ICAM-1 and iNOS, two genes acting in a pro-inflammatory manner. ICAM-1 encodes a cell surface adhesion glycoprotein, which is typically expressed by endothelial cells, as also by monocytic cells[35]. Expression of the *iNOS* gene results in NO production by activated monocytes. NO is one of the major pro-inflammatory compounds secreted by activated monocytes[36]. Consistent with this notion, NEFA elevated nitrate production by activated monocytes (see Table 4). There were, however, two genes the modulation of which was not consistent with the notion that fatty acids act in a pro-inflammatory manner. These two genes were *COX-2* and *CD11c*. COX-2 encodes an enzyme that catalyses the production of PG during the inflammatory process[37] and CD11c encodes for an adhesion molecule of crucial importance dictating cell migration[38]. Thus, fatty acids selectively up-regulated the expression of pro-inflammatory genes *in vitro* by monocytes. The reason behind the fact that the C_{18} fatty acids do not regulate all pro-inflammatory genes in the same manner is not known and it will be the topic of future studies.

Mirroring the Th1/Th2 concept of T-cell activation, a mirror image M1/M2 concept has been developed for macrophages[39]. M1 macrophages are the classical macrophages characterised by elevated expression of various cytokines, adhesion molecules and they display strong anti-bacterial killing ability manifested by increased NO production induced by iNOS. On the other hand, M2 macrophages are characterised by increased expression of the *IL-10* gene, they inhibit T-cell proliferation and they display poor anti-bacterial killing ability. The great majority of the gene effects caused by fatty acids in ovine monocytes in the present study are consistent with the notion that fatty acids promote the M1 phenotype. To document this further, we investigated the effect of fatty acids on *IL-10* gene expression, the key gene related to the M2 phenotype. Our results indicate that the levels of expression of IL-10 were low and were not up-regulated by fatty acids. Thus, the C_{18} fatty acid effects on the gene expression of ovine monocytes are consistent with promoting the M1 rather than the M2 phenotype. This observation is interesting. Recent data suggest that monocytes in the adipose tissue resemble those of the M2 phenotype, but high-fat diets appear to shift the expression of these monocytes from the M2 to the M1 phenotype[39]. It is important to emphasise that direct comparison of our data concerning blood macrophages with those related to the functional properties of fatty acids and monocytes in the adipose tissue cannot be made. However, there is an interesting analogy between our data supporting the notion that fatty acids mediate M1-like gene effects and those of others[39,40]- stating that high-fat diets increase the relative proportion of M1 monocytes in adipose tissue.

The second finding emerging from the present study is that only the C18 fatty acids (stearic and oleic) were effective towards ovine neutrophils. Therefore, carbon-chain length is the factor of paramount importance dictating the effectiveness of fatty acids towards neutrophils. With respect to mode of action, the C18 fatty acids acted towards ovine neutrophils in a pro-inflammatory manner and the general pattern of effects was very similar to that towards ovine monocytes. More specifically, both C18 fatty acids up-regulated the expression of u-PA, u-PAR and ICAM-1 but not that of iNOS, even though there was a trend for increased expression of the latter gene, but this trend did not reach the designated level of significance ($P>0·05$). To the best of our knowledge, our study is the first that documents that C_{18} fatty acids are neutrophil activators at the gene expression level. Our findings are along the same lines with those of others[16,17,41] who demonstrated that medium- and long-chain fatty acids increase cellular adhesion and degranulation, production of oxygen radicals, but at the same time decreased cell motility and impaired killing ability of neutrophils. The latter three studies also suggested that carbon-chain length was the pivotal factor in determining the effectiveness of medium- and long-chain fatty acids.

The third finding emerging from the present study is that peptides from soya protein produced by simulated gastrointestinal digestion or treatment with alcalase blocked completely or partially all effects on gene expression caused by the C_{18} fatty acids towards ovine monocytes and neutrophils. More specifically, soya protein peptides down-regulated the increase in the expression of u-PA and u-PAR caused by the C_{18} fatty acids. Increased expression of u-PA and u-PAR is thought to favour adhesion of both cell types to the endothelium and infiltration into the vessel wall. Furthermore, soya protein peptides down-regulated the increase in ICAM-1 expression caused by C_{18} fatty acids, thus, limiting another inflammatory pathway related to cell adhesion. Our data

have some similarities with those of others[18] who suggested that soya protein peptides inhibited LPS-induced increases in *iNOS* and *COX-2* gene expression. The unique element in the present study is that we have demonstrated that soya protein peptides block totally or partially the effects caused by fatty acids in ovine phagocytes. Anti-inflammatory effects of soyabean have been demonstrated by others, but were related to the presence of other non-protein compounds, such as isoflavones[42–44] and saponins[45]. Lunasin is a novel 43aa peptide derived from soyabean and other plant sources and it is reported to possess mainly anti-cancer, as also anti-inflammatory properties[19,46].

In conclusion, the present study documents that fatty acids up-regulated the expression of various genes implicated in the plasminogen-activating cascade (u-PA, u-PAR) along with that of ICAM-1 and iNOS, two genes that play a crucial role in cell adhesion and NO production, respectively. SPH differentially modify the expression of various pro-inflammatory genes in ovine phagocytes *in vitro*. These data, taken together, support the notion that soya should be classified in the category of 'healthy' foods. Future studies will focus on identifying the specific peptides mediating the observed effects and the potential mechanism of action of the respective peptides.

Acknowledgements

The authors' responsibilities were as follows: I. P. and A. B. designed the study; G. T., A. D. L. and R. C. conducted the study; G. T., A. D. L. and R. C. analysed the data; I. P., G. T. and A. B. wrote the manuscript; I. P. had the primary responsibility for the final content. All authors read and approved the final manuscript. The authors have no conflicts of interest. The present study was partially supported by the JSK, a company based in Greece, and specialised in communication strategy.

References

1. Gregor MF & Hotamisligil GS (2011) Inflammatory mechanisms in obesity. *Annu Rev Immunol* **29**, 415–445.
2. Ouchi N, Parker JL, Lugus JJ, *et al.* (2011) Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* **11**, 85–97.
3. Wellen KE & Hotamisligil GS (2003) Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* **112**, 1785–1788.
4. Alessi MC & Juhan-Vague I (2006) PAI-1 and the metabolic syndrome: links, causes, and consequences. *Arterioscler Thromb Vasc Biol* **26**, 2200–2207.
5. Ye J (2011) Adipose tissue vascularization: its role in chronic inflammation. *Curr Diab Rep* **11**, 203–210.
6. Lolmede K, Duffaut C, Zakaroff-Girard A, *et al.* (2011) Immune cells in adipose tissue: key players in metabolic disorders. *Diabetes Metab* **37**, 283–290.
7. Cancello R, Rouault C, Guilhem G, *et al.* (2011) Urokinase plasminogen activator receptor in adipose tissue macrophages of morbidly obese subjects. *Obes Facts* **4**, 17–25.
8. Mathis D & Shoelson SE (2011) Immunometabolism: an emerging frontier. *Nat Rev Immunol* **11**, 81.
9. Lumeng CN (2010) Adipose tissue macrophages: a piece of the PAI of metabolic syndrome. *Sci Transl Med* **2**, 20ps7.
10. Kishore P, Li W, Tonelli J, *et al.* (2010) Adipocyte-derived factors potentiate nutrient-induced production of plasminogen activator inhibitor-1 by macrophages. *Sci Transl Med* **2**, 20ra15.
11. Assmann A, Mohlig M, Osterhoff M, *et al.* (2008) Fatty acids differentially modify the expression of urokinase type plasminogen activator receptor in monocytes. *Biochem Biophys Res Commun* **376**, 196–199.
12. Dasu MR & Jialal I (2011) Free fatty acids in the presence of high glucose amplify monocyte inflammation via Toll-like receptors. *Am J Physiol Endocrinol Metab* **300**, E145–E154.
13. Bunn RC, Cockrell GE, Ou Y, *et al.* (2010) Palmitate and insulin synergistically induce IL-6 expression in human monocytes. *Cardiovasc Diabetol* **9**, 73.
14. Kopp A, Gross P, Falk W, *et al.* (2009) Fatty acids as metabolic mediators in innate immunity. *Eur J Clin Invest* **39**, 924–933.
15. Zhao G, Etherton TD, Martin KR, *et al.* (2005) Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells. *Biochem Biophys Res Commun* **336**, 909–917.
16. Wanten GJ, Janssen FP & Naber AH (2002) Saturated triglycerides and fatty acids activate neutrophils depending on carbon chain-length. *Eur J Clin Invest* **32**, 285–289.
17. Wanten GJ, Geijtenbeek TB, Raymakers RA, *et al.* (2000) Medium-chain, triglyceride-containing lipid emulsions increase human neutrophil beta2 integrin expression, adhesion, and degranulation. *JPEN J Parenter Enteral Nutr* **24**, 228–233.
18. Martinez-Villaluenga C, Dia VP, Berhow M, *et al.* (2009) Protein hydrolysates from beta-conglycinin enriched soybean genotypes inhibit lipid accumulation and inflammation *in vitro*. *Mol Nutr Food Res* **53**, 1007–1018.
19. de Mejia EG & Dia VP (2009) Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-kappaB pathway in the macrophage. *Peptides* **30**, 2388–2398.
20. Martinez-Villaluenga C, Bringe NA, Berhow MA, *et al.* (2008) Beta-conglycinin embeds active peptides that inhibit lipid accumulation in 3T3-L1 adipocytes *in vitro*. *J Agric Food Chem* **56**, 10533–10543.
21. Kapsokefalou M & Miller DD (1991) Effects of meat and selected food components on the valence of nonheme iron during *in vitro* digestion. *J Food Sci* **56**, 352–355.
22. Politis I, Zhao X, McBride BW, *et al.* (1991) Plasminogen activator production by bovine milk macrophages and blood monocytes. *Am J Vet Res* **52**, 1208–1213.
23. Politis I, Hidiroglou N, White JH, *et al.* (1996) Effects of vitamin E on mammary and blood leukocyte function, with emphasis on chemotaxis, in periparturient dairy cows. *Am J Vet Res* **57**, 468–471.
24. Brun T, Assimacopoulos-Jeannet F, Corkey BE, *et al.* (1997) Long-chain fatty acids inhibit acetyl-CoA carboxylase gene expression in the pancreatic beta-cell line INS-1. *Diabetes* **46**, 393–400.
25. Politis I, Zavizjon B, Cheli F, *et al.* (2002) Expression of urokinase plasminogen activator receptor in resting and activated bovine neutrophils. *J Dairy Res* **69**, 195–204.
26. Theodorou G, Daskalopoulou M, Chronopoulou R, *et al.* (2010) Acute mastitis induces upregulation of expression of plasminogen activator-related genes by blood monocytes and neutrophils in dairy ewes. *Vet Immunol Immunopathol* **138**, 124–128.
27. Marshall OJ (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20**, 2471–2472.

28. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta-delta C(T)) Method. *Methods* **25**, 402–408.
29. Politis I, Voudouri A, Bizelis I, *et al.* (2003) The effect of various vitamin E derivatives on the urokinase-plasminogen activator system of ovine macrophages and neutrophils. *Br J Nutr* **89**, 259–265.
30. Green LC, Wagner DA, Glogowski J, *et al.* (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* **126**, 131–138.
31. Politis I (1996) Plasminogen activator system: implications for mammary cell growth and involution. *J Dairy Sci* **79**, 1097–1107.
32. Blasi F (1993) Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. *Bioessays* **15**, 105–111.
33. Sitrin RG, Pan PM, Harper HA, *et al.* (2000) Clustering of urokinase receptors (uPAR; CD87) induces proinflammatory signaling in human polymorphonuclear neutrophils. *J Immunol* **165**, 3341–3349.
34. Waltz DA, Fujita RM, Yang X, *et al.* (2000) Nonproteolytic role for the urokinase receptor in cellular migration *in vivo*. *Am J Respir Cell Mol Biol* **22**, 316–322.
35. Martin J, Collot-Teixeira S, McGregor L, *et al.* (2007) The dialogue between endothelial cells and monocytes/macrophages in vascular syndromes. *Curr Pharm Des* **13**, 1751–1759.
36. Sharma JN, Al-Omran A & Parvathy SS (2007) Role of nitric oxide in inflammatory diseases. *Inflammopharmacology* **15**, 252–259.
37. Ghoshal S, Trivedi DB, Graf GA, *et al.* (2011) Cyclooxygenase-2 deficiency attenuates adipose tissue differentiation and inflammation in mice. *J Biol Chem* **286**, 889–898.
38. Arndt S, Melle C, Mondal K, *et al.* (2007) Interactions of TANGO and leukocyte integrin CD11c/CD18 regulate the migration of human monocytes. *J Leukoc Biol* **82**, 1466–1472.
39. Zeyda M & Stulnig TM (2007) Adipose tissue macrophages. *Immunol Lett* **112**, 61–67.
40. Lumeng CN, Bodzin JL & Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175–184.
41. Kruimel JW, Naber AH, Curfs JH, *et al.* (2000) With medium-chain triglycerides, higher and faster oxygen radical production by stimulated polymorphonuclear leukocytes occurs. *JPEN J Parenter Enteral Nutr* **24**, 107–112.
42. Kalhan R, Smith LJ, Nlend MC, *et al.* (2008) A mechanism of benefit of soy genistein in asthma: inhibition of eosinophil p38-dependent leukotriene synthesis. *Clin Exp Allergy* **38**, 103–112.
43. Kao TH, Wu WM, Hung CF, *et al.* (2007) Anti-inflammatory effects of isoflavone powder produced from soybean cake. *J Agric Food Chem* **55**, 11068–11079.
44. Hooshmand S, Soung dY, Lucas EA, *et al.* (2007) Genistein reduces the production of proinflammatory molecules in human chondrocytes. *J Nutr Biochem* **18**, 609–614.
45. Kang JH, Sung MK, Kawada T, *et al.* (2005) Soybean saponins suppress the release of proinflammatory mediators by LPS-stimulated peritoneal macrophages. *Cancer Lett* **230**, 219–227.
46. Dia VP, Wang W, Oh VL, *et al.* (2009) Isolation, purification and characterisation of lunasin from defatted soybean flour and *in vitro* evaluation of its anti-inflammatory activity. *Food Chem* **114**, 108–115.

British Journal of Nutrition