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Soy protein inhibits inflammation-induced VCAM-1 and inflammatory cytokine induction by inhibiting the NF- κ B and AKT signaling pathway in apolipoprotein E-deficient mice

Ramona L. Burris,

Arkansas Children's Nutrition Center, Little Rock, AR, USA

Hang-Pong Ng, and

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA; Department of Pathology and Vascular Medicine Institute, University of Pittsburgh, BST E1256, 200 Lothrop Street, Pittsburgh, PA 15261, USA

Shanmugam Nagarajan

Arkansas Children's Nutrition Center, Little Rock, AR, USA; Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA; Department of Pathology and Vascular Medicine Institute, University of Pittsburgh, BST E1256, 200 Lothrop Street, Pittsburgh, PA 15261, USA

Shanmugam Nagarajan: nagarajans@upmc.edu

Abstract

Purpose—Inflammation is a hallmark of many diseases, such as atherosclerosis, autoimmune diseases, obesity, and cancer. Isoflavone-free soy protein diet (SPI[−]) has been shown to reduce atherosclerotic lesions in a hyperlipidemic mouse model compared to casein (CAS)-fed mice, despite unchanged serum lipid levels. However, possible mechanisms contributing to the athero-protective effect of soy protein remain unknown. Therefore, we investigated whether and how SPI[−] diet inhibits inflammatory responses associated with atherosclerosis.

Methods—Apolipoprotein E knockout (apoE^{−/−}) mice (5-week) were fed CAS or SPI[−] diet for 1 or 5 week to determine LPS- and hyperlipidemia-induced acute and chronic inflammatory responses, respectively. Expression of NF- κ B-dependent inflammation mediators such as VCAM-1, TNF- α , and MCP-1 were determined in aorta and liver. NF- κ B, MAP kinase, and AKT activation was determined to address mechanisms contributing to the anti-inflammatory properties of soy protein/peptides.

Results—Isoflavone-free soy protein diet significantly reduced LPS-induced VCAM-1 mRNA and protein expression in aorta compared to CAS-fed mice. Reduced VCAM-1 expression in SPI[−]-fed mice also paralleled attenuated monocyte adhesion to vascular endothelium, a critical and primary processes during inflammation. Notably, VCAM-1 mRNA and protein expression in lesion-prone aortic arch was significantly reduced in apoE^{−/−} mice fed SPI for 5 weeks compared with CAS-fed mice. Moreover, dietary SPI[−] potently inhibited LPS-induced NF- κ B activation and the subsequent upregulation of pro-inflammatory cytokines, including TNF- α , IL-6, IL-1 β , and MCP-1. Interestingly, SPI[−] inhibited NF- κ B-dependent inflammatory responses by targeting I- κ B phosphorylation and AKT activation with no effect on MAP kinase pathway. Of the five putative

soy peptides, four of the soy peptides inhibited LPS-induced VCAM-1, IL-6, IL-8, and MCP-1 protein expression in human vascular endothelial cells in vitro.

Conclusions—Collectively, our findings suggest that antiinflammatory properties of component(s) of soy protein/peptides may be a possible mechanism for the prevention of chronic inflammatory diseases such as atherosclerosis.

Keywords

Soy peptides; Inflammation; VCAM-1; Hyperlipidemia; Atherosclerosis

Introduction

Inflammation has been implicated in many human diseases including arthritis [32], obesity [22], cancer [15, 41], and atherosclerosis [21]. The essential cellular event for the initiation of inflammatory processes associated with atherosclerosis is monocyte migration to the inflammation site and its subsequent adhesion to endothelial cells [21]. The interaction of monocytes to endothelial cells is a multi-step process involving rolling, firm adhesion, and finally, transmigration of monocytes into arterial intima [12]. These processes are controlled by interaction between number of vascular cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1)¹ and leukocyte integrins CD49d, counter receptor for VCAM-1 [12, 27]. In vitro studies have established pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and IL-1 β , upregulates VCAM-1 expression in vascular endothelial cells [18, 23, 25, 45]. Moreover, deletion of VCAM-1 reduced atherosclerotic lesions in hyperlipidemic mouse models [11, 36]. These studies collectively indicate that VCAM-1 expression is essential for the initiation of the inflammatory process associated with atherosclerosis [12].

Epidemiological studies have shown lower incidence of cardiovascular disease in Asia than in Western countries [3]. These epidemiological reports have also been supported by animal studies using atherosclerosis-prone hyperlipidemic apolipoprotein E knockout (apoE^{-/-}) mice [1, 2, 28, 31]. Recently, we [28] and others [1, 2] showed isoflavone-free soy protein (SPI⁻)-fed apoE^{-/-} mice had reduced atherosclerotic lesions compared to casein (CAS)-fed mice. Moreover, attenuated lesion in SPI⁻-fed apoE^{-/-} mice was independent of serum cholesterol levels. As atherosclerosis has been recognized as a chronic inflammatory disease, these findings suggest that the protein component of soy diet may contribute to dampening the inflammatory processes associated with atherosclerosis. However, the mechanism(s) of soy proteins inhibiting inflammatory responses associated with atherosclerosis remain unknown. We hypothesized that anti-inflammatory function of SPI⁻ diet inhibits the inflammatory processes associated with atherosclerosis.

In this report, we showed that dietary SPI⁻ inhibits NF- κ B-dependent expression of inflammatory mediators such as VCAM-1, TNF- α , and MCP-1. We also showed reduced expression of VCAM-1 in aortic tissue during chronic inflammation in a hyperlipidemic apoE^{-/-} mouse model. The inhibitory effect of SPI⁻ on NF- κ B signaling is exerted via inhibition of I- κ B phosphorylation. Interestingly, the inhibitory effect of SPI⁻ on NF- κ B signaling is mediated via regulating AKT activation. These studies thus identify soy protein/peptides as a unique anti-inflammatory agent that may have a potential to prevent and/or treat chronic inflammatory diseases.

Materials and methods

Acute inflammation model

Female apoE^{-/-} mice [47] in a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) were housed in micro-isolator cages and maintained on a 12-h light/dark cycle in a temperature-controlled room. Mice were fed semi-purified diets made according to the AIN-93G diet formula [28] (except that corn oil replaced soybean oil) and the protein source was either CAS (New Zealand Milk Products) or SPI⁻ (Solae Company). Mice consumed food and water ad libitum throughout the study period. As female apoE^{-/-} mice have been shown to develop more atherosclerotic lesions than age-matched male apoE^{-/-} mice [8, 33, 34], only female mice were used in this study. In experiment 1, mice (5-week female) were fed CAS diet for 1 week. Ultrapure *E. coli* 0111:B4 LPS (Invivogen, at indicated concentration/mouse, $n = 4/\text{concentration}$) was injected intraperitoneally. Animals were killed after 5 h; heart and aorta samples were collected. PBS-injected mice were used as controls. Based on LPS dose-response experiment, in subsequent experiments, LPS at 20 $\mu\text{g}/\text{mouse}$ was used. In experiments 2–4, apoE^{-/-} mice (5-week female) fed the CAS or SPI⁻ diets ($n = 4\text{--}5/\text{diet}$) for 1 week followed by LPS (20 $\mu\text{g}/\text{mouse}$) challenge for 5 h. Aorta samples from experiments 2 and 3 were used to determine VCAM-1 protein, mRNA expression. Aorta from experiments 1 and 4 were used to determine monocyte adhesion to mouse aorta. Livers from experiments 2 and 3 were used to determine inflammatory gene expression. Blood collected from experiments 2 and 3 were used to determine plasma TNF- α and serum amyloid antigen (SAA) levels. In experiment 5, apoE^{-/-} mice (5-week female) fed the CAS or SPI⁻ diets ($n = 4/\text{diet}$) for 1 week was challenged with LPS (20 $\mu\text{g}/\text{mouse}$) for 3 h. Aorta and liver from experiment 5 were used to determine NF- κB and MAP kinase activation. We have chosen 3 h as NF- κB , and MAP kinase transcriptional factor activation precedes inflammation-associated gene expression.

Hyperlipidemia-induced chronic inflammation

Twelve female mice (5 weeks) were randomly assigned to 2 groups ($n = 6$) and fed CAS or SPI⁻ diets for 5 weeks. Atherosclerotic lesion was not determined in this report because the objective of this report is to determine the effect of soy proteins on molecular events preceding to the fatty streak lesion formation. Moreover, we have previously reported atherosclerotic lesion analyses in SPI⁻-fed apoE^{-/-} mice [28]. Animals were housed for a 3-day period (at 7 weeks) under conditions of 12:12-h light-dark cycle in metabolic chambers using the complete Lab Animal Monitoring System to assess food intake (Columbus Instruments, Columbus, OH) as described [7]. Animals were killed at 10 week of age; aorta was collected and preserved in RNAlater (Invitrogen) for RNA isolation and quantitative RT-PCR analysis of VCAM-1 mRNA expression. Aortic sinus cryosections were used to determine VCAM-1 protein expression. These studies were conducted under the guidelines and protocols approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Immunohistochemical analysis

Serial aortic sinus cryosections (10 μm) were stained with goat anti-mouse VCAM-1 IgG (10 $\mu\text{g}/\text{ml}$, RND systems) followed by Vectastain ABC reagent (Vector Laboratories Inc.). The sections were developed with DAB (3,3'-diaminobenzidine) and counterstained with Mayer's hematoxylin. Images were captured using Olympus microscope. Sections stained with goat IgG were used as a non-specific IgG control. Percentage of VCAM-1+ staining area was determined by measuring the total aortic sinus area.

Quantitative RT-PCR analysis

The liver was perfused with nuclease-free PBS and total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, tissue samples (100 mg) were homogenized in 1 ml of TRIZOL reagent and the homogenized sample incubated for 5 min at room temperature. Chloroform (0.2 ml) was added and mixed for 15 s and incubated for 3 min. Samples were centrifuged at 12,000 ×g for 15 min at 4 °C and RNA was precipitated from the colorless upper aqueous phase by the addition of 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. RNA pellet was washed once with 75 % ethanol and then dissolved in RNase-DNase-free water. RNA quality was determined by BioRad Experion RNA analysis kits prior to gene expression analyses. Expression of inflammatory response genes (TNF- α , IL-6, IL-1 β , and MCP-1) was determined by quantitative real-time RT-PCR analysis using Toll-like receptor signaling pathway real-time RT-PCR array (SABiosciences, Frederick, MD), which is designed to have the genes involved in inflammatory responses. Furthermore, arrays include five housekeeping genes (β -actin, GAPDH, β -glucuronidase, hypoxanthine guanine phosphoribosyl transferase 1, heat shock protein 90- α class B1 member) to normalize the mRNA expression. The RT-PCR gene expression array also contains following controls: genomic DNA contamination, RT-PCR, and PCR controls, to validate the array. RNA ($n = 4$ /group, 0.25 μ g/sample) was pooled, and first strand cDNA was synthesized using cDNA synthesis kit (SA Biosciences). A two-step PCR with denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min for 40 cycles was conducted in an iCycler (BioRad) to determine the threshold cycle (Ct) value. Expression of inflammation-associated genes was calculated using $\Delta\Delta$ CT method using threshold cycles for five housekeeping genes (mentioned above) as normalization references. RT-PCR arrays were repeated four times with two independent RNA preparations from liver samples from two independent experiments (experiment 2 and 3). To determine aortic VCAM-1, TNF- α , and MCP-1 mRNA expression, the aorta was perfused with nuclease-free PBS, and total RNA was isolated using Trizol reagent (Invitrogen). VCAM-1 mRNA expression was determined by realtime RT-PCR using a total RNA (0.5 μ g) as described earlier [42]. Quantitative RT-PCR primer pairs for *tnf- α* (cat# PPM03113G), *il1beta* (cat# PPM03109F), *il6* (cat# PPM03015A), *mcp1* (cat# PPM03151G), and *vcam1* (cat# PPM03208C) were purchased from SA Biosciences. Expression of inflammatory response genes was calculated using $\Delta\Delta$ CT method [42] using threshold cycles for β -actin as normalization reference. All real-time PCR was carried out at least twice from independent cDNA preparations from individual aorta RNA samples. RNA without reverse transcriptase served as a negative control.

Western blot analysis

Aorta was lysed in RIPA buffer with protease and phosphatase inhibitors and lysates were centrifuged at 15,000 rpm at 4 °C to remove undigested tissue debris. VCAM-1 expression in the descending aorta lysate (20 μ g protein) was assessed by Western blot analysis using goat anti-mouse VCAM-1 IgG (RND Systems) followed by HRP-conjugated rabbit anti-goat IgG (RND Systems). Blots were probed for β -actin and GAPDH with HRP-conjugated anti- β -actin antibody (Sigma, experiment 1) or HRP-conjugated anti-GAPDH IgG (in all other experiments) to normalize the protein expression and used as loading control. The blots were developed using ECL-plus substrate (GE Healthcare), and band intensity was quantified by QuantityOne software (BioRad).

TNF- α and SAA ELISA

Plasma collected from acute inflammation model (experiment 2 and 3) was used to determine TNF- α and SAA by sandwich ELISA using kits specific for mouse TNF- α (BD-Biosciences) and SAA (Invitrogen), respectively.

Ex vivo monocyte adhesion

ApoE^{-/-} mice (5-week female, $n = 5/\text{diet}$) were fed with CAS or SPI⁻ for 1 week followed by LPS challenge (experiment 4). A human monocytic cell line (HL-60) was labeled with PKH-26, a lipophilic fluorescent dye [29] and adhesion to mouse aorta was carried out as described previously [6]. Human monocytic cell line was used as human monocytic cell line binds to mouse VCAM-1 [5, 26, 40], and earlier studies have shown that interaction between VCAM-1 and CD49d, its counter receptor, is conserved between species [5, 26, 40]. Photographs were taken using Olympus fluorescent microscope and number of monocytic cells adhered to mouse aorta was counted by three individuals in a blinded manner.

Endothelial cell activation

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in EGM-2 media supplemented with 2 % FBS and Penn/Strep. Cells between passage 3 and 5 were used for the experiments. Soy peptides (21st Century Biochemicals, Marlboro, MA) were >98 purity and peptide amino acid sequence was confirmed by MS-TOF (21st Century Biochemicals, Marlboro, MA). To determine soy peptide effect on LPS-induced endothelial cell inflammatory responses, HUVEC (1×10^5 cells/well) were plated in a 96-well plate and cultured overnight. HUVEC were treated with soy peptides (Table 1) at indicated concentration for 1 h, followed by the addition of LPS (20 ng/ml). Cells were incubated at 37 °C CO₂ incubator for 6 h for cytokine analyses and 18 h for VCAM-1 protein expression. For cytokine analyses, supernatant was collected 6 h after LPS treatment and levels of pro-inflammatory cytokine and chemokines (human IL-6, IL-8 and MCP-1) were determined by cytokine bead array from BD-Biosciences using BD-LSR Fortessa flow cytometer followed by analysis using FCAParray v3 software (BD-Biosciences).

LPS-induced VCAM-1 expression was determined by cell ELISA. Briefly, after LPS activation, cells were washed in cold PBS and fixed in PBS/2 % paraformaldehyde for 30 min. Cells were then washed and incubated with PBS/1 % BSA overnight. Cells were incubated with mouse anti-human VCAM-1 IgG (STA, eBioscience, 3 $\mu\text{g}/\text{ml}$) for 1 h at 22 °C. Cells were washed in PBS/1 % BSA and further incubated with streptavidin-HRP (1:10,000 diluted, Jackson ImmunoResearch) and incubated at 45 min at 22 °C. Cells were then washed twice with PBS/1 % BSA buffer, 100 μL HRP substrate TMB-1 component (KPL Inc., Gaithersburg, MD) was added to each well, and color developed was stopped by the addition of equal volume of 2 N sulfuric acid. Absorbance was measured using BioTek microplate reader at 450 nm. Cells treated without LPS was used as a baseline control. Cells treated with soy peptides alone without LPS was also included to rule out direct effect of soy peptides on endothelial cells.

NF- κ B activation

Nuclear and cytosolic fractions were prepared from aorta and liver lysates (Experiment 5). Total- and phospho-NF- κ B activation was determined using NF- κ B activation Pathscan assay kit (Cell Signaling) according to manufacturer's instruction. To confirm NF- κ B activation, I κ B degradation was determined in the cytosolic fractions by Western blot analysis using anti-I- κ B antibody (Cell Signaling Technology) according to the manufacturer's instruction. Blots were probed with HRP-conjugated mouse anti-GAPDH IgG (Sigma) at 1:10,000 dilutions to normalize the protein expression.

NF- κ B immunofluorescence

Aortic sinus and liver sections (10 μ m) were fixed in PBS/10 % formalin and incubated overnight at 4 °C with rabbit anti-NF- κ B p65 IgG (Cell Signaling, 1:50 dilution in antibody dilution in antibody dilution buffer). Sections were washed and further incubated for 1 h at room temperature with biotinylated anti-rabbit IgG (Sigma, 1:800 dilution). After washing, the sections were incubated for 30 min with fluorescein-avidin DCS (Vector Laboratories) at 20 μ g/ml. After washing the sections in PBS, the slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Photomicrographs were taken in Olympus fluorescent microscope and number of nucleated (DAPI+) and NF- κ B-positive cells were counted in 3 separate sections/mouse.

MAP kinase and AKT activation

Total lysates were prepared from aorta and liver ($n = 4$ /group, experiment 5). Activation of MAP kinase was determined using PathScan multi-target sandwich ELISA kit (Cell signaling) according to the manufacturer's instructions. Protein concentration was determined by Biorad dye binding method, and lysate (20 μ g protein) was used to determine phospho-p44/42 (Erk1/2), phospho-p38, phospho-MEK1, total-JNK, phospho-JNK, and total-AKT and phosphorylated-AKT protein levels.

Data analysis

Results are expressed as mean $\pm$ SD. Data were analyzed by two-tailed Student's t test. Differences were considered significant at $P < 0.05$. All analyses were carried out with inStat statistical program (Graphpad Inc.).

Results

Isoflavone-free soy protein diet inhibits hyperlipidemia-induced VCAM-1 expression

First, we determined whether SPI⁻ diet inhibits VCAM-1 expression during a chronic inflammatory condition using hyperlipidemic apoE^{-/-} mice. At 10 week, body weight of CAS (20.4 $\pm$ 0.32)- and SPI⁻-fed (20.1 $\pm$ 0.22) mice was not different. Moreover, food intake of CAS- and SPI⁻-fed mice was 4 g/day and 3.9 g/day, respectively. Similarly, plasma cholesterol levels were not different between CAS- and SPI⁻-fed apoE^{-/-} mice (data not shown). Real-time quantitative RT-PCR analyses showed 50 % reduced mRNA expression of VCAM-1 in aortic samples from apoE^{-/-} mice fed SPI⁻ in comparison with CAS-fed mice (Fig. 1a). Immunohistochemical analyses showed reduction in immunopositive staining area for VCAM-1 expression in aortic sections of SPI⁻ compared to CAS-fed apoE^{-/-} mice (Fig. 1b and c). These findings suggest that the athero-protective effect of SPI⁻ diet in part may be mediated via regulating endothelial cell activation associated with hyperlipidemia-induced chronic inflammatory conditions.

Isoflavone-free SPI⁻ diet inhibits aortic tissue inflammatory response in vivo

We next evaluated the effects of SPI⁻ diet on acute inflammation-induced aortic VCAM-1 expression. VCAM-1 protein expression was very low in PBS-injected mice, while LPS at 20 μ g upregulated VCAM-1 protein expression in aorta (Fig. 2a). Initial experiments showed a dose-dependent increase in VCAM-1 protein expression in mouse aorta in LPS-injected mice (Fig. 2b). Based on the dose response, LPS at 20 μ g/mouse was used in subsequent experiments. LPS-induced VCAM-1 protein expression was high in CAS-fed mice (Fig. 2c, d). Interestingly, LPS-induced VCAM-1 protein expression was significantly reduced ($P < 0.01$) in mice fed SPI⁻ diet compared with CAS-fed group (Fig. 2c, d).

SPI⁻ diet inhibits monocyte adhesion to mouse aorta

We then evaluated whether LPS-induced VCAM-1 upregulation results in monocyte adhesion and dietary SPI⁻ inhibits this adhesion. Fluorescent-labeled monocytic cells adhered to mouse aorta from apoE^{-/-} mice challenged with LPS (Fig. 3a). HL-60 cells express CD11a and CD49d, counter receptor for ICAM-1 and VCAM-1, respectively (data not shown). Monocytes adhesion to vascular endothelium is mediated through the interaction between endothelia ICAM-1 and VCAM-1 and CD11a and CD49d expressed on monocytes, respectively. Hence to determine the relative contribution of VCAM-1 in mediating monocytic cell adhesion to mouse aorta, monocytic cells were pre-treated with anti-CD49d mAb. Pre-treatment of monocytic cells with anti-CD49d mAb blocked >70 % of their adhesion to mouse aorta of LPS-injected mice (Fig. 3a, b), suggesting interactions between VCAM-1 and CD49d integrins expressed on monocytes principally contribute to the monocyte adhesion to mouse aorta. We then determined whether SPI⁻-mediated inhibition of LPS-induced VCAM-1 expression results in reduced monocyte adhesion to mouse aorta. Ex vivo monocyte adhesion assay showed that number of monocytic cells adhered to aorta from CAS-fed mice was high (Fig. 3c, d). However, in SPI⁻-fed mice, number of monocytic cells adhered to aorta were 50 % reduced (Fig. 3c, d).

Reduced plasma inflammatory markers in SPI⁻-fed mice

Then, we determined whether dietary SPI⁻ affect overall plasma inflammatory markers such as TNF- α and SAA. Notably, the plasma TNF- α (Fig. 4a) and SAA (Fig. 4b) levels were 60 % lower in SPI⁻-fed compared with CAS-fed mice. As SAA is synthesized primarily in liver [19, 37] and expression is upregulated by the pro-inflammatory cytokines IL-1 β and TNF- α [13], these findings indicated that dietary SPI⁻ inhibits inflammatory responses not only in vascular tissue but also in other tissues such as liver.

Dietary SPI⁻ inhibits LPS-induced pro-inflammatory cytokines and VCAM-1 mRNA expression

Inflammatory stimuli such as LPS or TNF- α increase the VCAM-1 protein expression by transcriptional regulation by NF- κ B activation [10, 14, 20]. Hence, the effect of SPI⁻ diet on LPS-induced upregulation of NF- κ B-dependent pro-inflammatory cytokines, chemokine, and VCAM-1 mRNA expression was investigated. Quantitative RT-PCR analyses showed LPS-induced VCAM-1 mRNA expression in aorta was decreased 50 % in SPI⁻-fed mice compared with CAS-fed mice (Fig. 5a). SPI⁻ also inhibited LPS-induced TNF- α and MCP-1 mRNA expression in the aorta (Fig. 5b, c). Then, we determined whether dietary SPI⁻ also induced anti-inflammatory responses in liver. Dietary SPI⁻ inhibited LPS-induced upregulation of TNF- α (Fig. 5d), MCP-1 (Fig. 5e), IL-6 (Fig. 5f), and IL-1 β (Fig. 5g) mRNA expression in liver, suggesting isoflavone-free soy protein potently inhibited inflammatory response in vivo.

SPI⁻ inhibits NF- κ B activation

Then, we investigated whether SPI⁻ diet induces an anti-inflammatory response by inhibiting NF- κ B activation. Total NF- κ B levels in the aortic lysate were not different in CAS- or SPI⁻-fed mice (Fig. 6a). However, LPS-induced phosphorylation of NF- κ B p65 subunit in the nuclear lysates of aorta was 50 % reduced ($P < 0.01$) in SPI⁻-fed mice compared with CAS-fed mice (Fig. 6a). LPS-induced phosphorylation of NF- κ B p65 subunit in the nuclear lysates of liver was also 50 % reduced ($P < 0.01$) in mice fed SPI⁻ versus CAS diet (Fig. 6b). As IKK-dependent phosphorylation and degradation of I- κ B α precedes NF- κ B phosphorylation and translocation, we evaluated the effects of dietary SPI⁻ on LPS-induced I- κ B degradation. LPS induced I- κ B degradation as measured by Western blot analysis using an antiphospho-Ser32 I- κ B α antibody. I- κ B protein expression was low in

cytosolic fraction of aortic lysates of CAS-fed mice (Fig. 6c, d). However, cytosolic I- κ B levels were about twofold high in aortic lysates of SPI⁻-fed mice (Fig. 6c, d). Then, we determined whether decreased NF- κ B phosphorylation results in impaired nuclear translocation. SPI⁻ diet inhibited nuclear translocation of p50/p65 heterodimeric NF- κ B in liver from mice fed SPI⁻ compared with mice fed CAS diet (Fig. 6e, f). Similar findings were observed in aortic sinus of SPI⁻-fed mice (data not shown). These findings suggest that SPI⁻ diet blocks LPS-induced acute inflammatory responses by inhibiting NF- κ B activation.

Soy protein diet does not inhibit LPS-induced MAP kinase activation

Since activation of MAP kinases ERK1/2, JNK, and p38 has been shown to contribute to LPS-induced pro-inflammatory response [17], we next determined whether SPI⁻ inhibits MAP kinase activation in aorta and liver. LPS induced activation of MAP kinases; p38 and JNK in liver (Fig. 7a) and aorta (Fig. 7b) were not different between CAS- or SPI⁻-fed mice. Similarly, there was no significant inhibitory effect of SPI⁻ on LPS-induced activation of MEK1 in liver and aorta (Fig. 7a, b), an upstream kinase regulating MAP Kinase activation. These findings demonstrate that inhibition of LPS-induced inflammatory mediators by isoflavone-free SPI⁻ diet is independent of MAP kinase activation.

Soy protein diet inhibits LPS-induced AKT phosphorylation

AKT phosphorylation has been implicated as one of the downstream signaling components of LPS/TLR4 interaction [4, 35]. We then determined whether SPI⁻ diet regulates LPS-induced NF- κ B activation via AKT phosphorylation. Total-AKT and the phosphorylated-AKT protein at Ser473 was significantly decreased in liver (Fig. 7a) aorta (Fig. 7b) of SPI⁻-fed compared with CAS-fed mice. These findings suggest that SPI⁻ diet blocks LPS-induced acute inflammatory responses by inhibiting NF- κ B activation in part via AKT phosphorylation.

Putative soy peptides with anti-inflammatory function

Dietary β -conglycinin, one of the major proteins present in soybeans, have shown to have a pronounced inhibitory effect on the development of atherosclerosis in the LDL receptor knockout mouse model [2]. Interestingly, our findings showing isoflavone-free SPI⁻ diet inhibits LPS-induced inflammatory mediators' expression suggesting that either some endogenous factor produced by SPI⁻ diet or peptide or bioactive small peptide fractions produced by the digestion of soy protein may have favorable anti-inflammatory effects of SPI⁻ diet. Hence, we determined effect of soy peptides (Table I) on LPS-induced endothelial cell activation resulting in VCAM-1, and pro-inflammatory cytokine/chemokine expression was determined. Soy peptides alone without the addition of LPS did not show any detectable levels of cytokine secretion or VCAM-1 expression (data not shown). Of the five soy peptides, four of them inhibited LPS-induced IL-6 (Fig. 8a), IL-8 (Fig. 8b), and MCP-1 (Fig. 8c) secretion by vascular endothelial cells. Moreover, LPS-induced VCAM-1 expression (Fig. 8d) was also inhibited by these four soy peptides. These findings suggest that some of the putative soy peptides that may be generated in vivo may inhibit inflammation-induced vascular endothelial cell activation.

Discussion

Recent studies have implicated that chronic inflammation is an important contributing factor for number of human diseases including arthritis, obesity, cancer, and atherosclerosis [15, 21, 22, 32, 41]. In the current study, we tested the hypothesis that anti-inflammatory functions of soy proteins/peptides contributes to the athero-protective effect of isoflavone-free SPI⁻ diet. We showed dietary SPI⁻ significantly inhibited NF- κ B-dependent expression of inflammatory cytokines and VCAM-1 in vivo in acute (LPS-induced) and chronic

(hyperlipidemia-induced) inflammation models. Thus, our findings identify soy protein diet as an attractive anti-inflammatory candidate for the development of nutritional strategies for the treatment and/or prevention of various chronic inflammatory diseases including atherosclerosis.

Using a hyperlipidemic mouse model, others [1, 2] and we [28] have shown that attenuated lesions in SPI⁻-fed mice were independent of plasma lipid levels, suggesting alternative mechanisms may be contributing to the athero-protective effect of soy protein. In this report, we showed SPI⁻ diet inhibited chronic (hypercholesterolemia) and acute (LPS-induced) inflammation-induced VCAM-1 expression in mouse aorta. Moreover, attenuated LPS-induced VCAM-1 expression by SPI⁻ diet also paralleled reduced monocyte adhesion. We have chosen 10 weeks of age to determine VCAM-1 expression, as it has been reported that aortic VCAM-1 expression is upregulated in apoE^{-/-} mice at 10 weeks of age [30], and VCAM-1 expression precedes fatty streak lesion formation in apoE^{-/-} mice. Our findings suggest an inverse relationship between isoflavone-free dietary SPI⁻ and VCAM-1 expression and subsequent monocyte adhesion to vascular endothelium in acute and chronic inflammatory conditions. Subcutaneous administration of genistein (0.3 mg/kg/daily) has been shown to inhibit atherosclerosis and decreased VCAM-1 expression in LDL receptor knockout mice (LDLR^{-/-}) [44], suggesting soy isoflavone may contribute to the athero-protective effect of soy-based diets. However, in this report, circulating levels of genistein or genistein metabolites were not determined. It is estimated that the circulating levels of soy isoflavones (genistein, daidzein, and equol) is about 1 μ M after feeding isoflavone-containing soy protein isolate diet in rats [16] with a major proportion of that amount as genistein [38]. Notably, at this circulating level of soy isoflavones (1 μ M), including genistein did not inhibit LPS- or TNF- α -induced VCAM-1 expression in human endothelial cells in vitro (unpublished observation). However, genistein, one of the principal soy isoflavones, at a high concentration (100 μ M) significantly inhibited LPS-induced VCAM-1 expression in human endothelial cells in vitro (unpublished observation). These findings are in accordance with earlier studies showing genistein at circulating levels (1 μ M) did not inhibit TNF- α -induced vascular cell adhesion molecule expression [9]. Interestingly, recent studies by Ronis et al. [38] have demonstrated that expression of PPAR- α target genes (hepatic acyl Co-A oxidase and carnitine palmitoyl transferase) and PPAR- γ target genes (glucokinase, and CD36) were upregulated in isoflavone-containing SPI⁺-fed rats, while the same genes are downregulated in rats fed casein diet supplemented with genistein [38]. These findings suggest that downstream cellular and molecular events to the dietary administration of genistein or soy protein isolate containing genistein are not the same.

NF- κ B is a key transcriptional factor that plays a critical role in mediating inflammatory responses by regulating expression of VCAM-1 [20] and pro-inflammatory cytokines and chemokines such as TNF- α and MCP-1 [10, 14]. Hence, it is possible that SPI⁻ diet-mediated downregulation of VCAM-1, MCP-1, and TNF- α expression could be mediated by blocking NF- κ B activation. We showed that SPI⁻ inhibits NF- κ B-dependent transcriptional activity as evidenced by reduced phosphorylation of NF- κ B p65 subunit and subsequent nuclear translocation. These results suggest that isoflavone-free soy protein inhibits LPS-induced NF- κ B activation by preventing I- κ B α phosphorylation and degradation, thus acting at or upstream of I- κ B α . Earlier studies have shown that activation of MAP kinases ERK1/2, JNK, and p38 contribute to LPS-induced pro-inflammatory response [17]. Notably, our data also showed that SPI⁻ does not alter the phosphorylation status of p38 and JNK, ERK1/2 and MKK an upstream kinase regulating MAP kinases. Together, these data suggest that the inhibitory effect of soy protein/peptides on inflammatory response is NF- κ B dependent and is likely to be independent of MAP kinase activation.

Stimulation of human endothelial cells with LPS, a TLR4 ligand, has been shown to induce phosphorylation of AKT [4, 35]. Moreover, inhibition of TNF- α -induced VCAM-1 expression and subsequent monocyte adhesion to endothelial cells have been shown to be dependent on inhibition of AKT and NF- κ B activation [35]. Notably, a dominant-negative mutant of AKT inhibited LPS or IL-1 β induced activation of NF- κ B in human endothelial cells [20]. These findings suggest that AKT positively regulates NF- κ B activation upstream to IKK. We showed that isoflavone-free SPI⁻ reduced LPS-induced activation of AKT in vivo. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a natural negative regulator of PI3 K/Akt signaling [43]. Earlier studies have shown that rats fed SPI diet showed increased expression of PTEN in mammary glands [39]. Hence, it is possible that decreased AKT phosphorylation could be due to increased expression of PTEN in SPI⁻ fed mice. Further studies are necessary to address such a possibility. These findings collectively suggest that LPS-induced NF- κ B activation is in part mediated by negatively regulating AKT signaling upstream of NF- κ B activation.

β -Conglycinin (or 7S globulins) and glycinin (or 11S globulins) are the major proteins present in soybeans. β -Conglycinin is a trimeric protein with α , α' , and β sub-units. Studies by Adams et al. [2] have demonstrated that β -conglycinin had a pronounced inhibitory effect on the development of atherosclerosis in the LDL receptor knockout mouse model. Interestingly, our findings show that isoflavone-free SPI⁻ diet inhibits LPS-induced inflammatory mediators' expression in an in vivo acute inflammation model. These findings suggest that either endogenous factor(s) produced by SPI⁻ diet or peptide or bioactive small peptide fractions produced by the digestion of soy protein may have favorable anti-inflammatory effects of SPI⁻ diet. This inhibition could be mediated by soy peptides generated in vivo by protease/peptidases in the gut. We tested this possibility using purified synthetic soy peptides in LPS-induced endothelial cell activation. Notably, some of the putative soy peptides from β -conglycinin inhibited LPS-induced vascular cell activation resulting in blunted VCAM-1 and pro-inflammatory cytokine/chemokine expression. These peptides were selected since recent studies have suggested that these peptides have been shown to inhibit fatty acid synthase expressed in adipose tissue [24]. Moreover, we have selected peptides that are present in α , α' , and β subunits of β -conglycinin protein. Recent studies have implicated administration of soymophin-5, a pentapeptide derived from β -conglycinin improves lipid metabolism in vivo in a diabetic mouse model [46]. It will be interesting to determine whether these putative soy peptides are generated in vivo after consumption of soy protein. However, the molecular signature of peptides or protein components in the SPI⁻ diet generated in vivo that may have contributed to the inhibition of LPS-induced inflammatory mediators' expression is still unknown. We realize that this study did not address the components such as peptides or other minor components of SPI⁻ contributing to the anti-inflammatory effect of SPI⁻ diet in vivo. One approach is to generate enzymatically (proteases)-derived peptides of β -conglycinin or glycinin and test the anti-inflammatory functions of these peptides using in vitro cell culture model. A caveat in this approach is that even if inflammatory response is inhibited by SPI⁻-derived peptides, we may not know whether similar peptides are produced in vivo. Nevertheless, it is important to identify bioactive peptides of SPI⁻ contributing to the in vivo athero-protective and anti-inflammatory effect of SPI⁻ diet. Identification of the soy peptides contributing to the anti-inflammatory functions is the focus of current investigations in our laboratory.

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Abbreviations

ApoE	Apolipoprotein E
CAS	Casein
ICAM-1	Intercellular adhesion molecule-1
MCP-1	Monocyte chemoattractant protein
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
SPI⁻	Isoflavone-free soy protein isolate
TNF-α	Tumor necrosis factor- α
VCAM-1	Vascular cell adhesion molecule-1

Fig. 1.

Reduced VCAM-1 expression in hyperlipidemic apoE^{-/-} mice fed SPI⁻ diet. Relative VCAM-1 mRNA expression levels in aortic samples from apoE^{-/-} mice fed SPI⁻ or CAS diets for 5 weeks (**a**). Values are mean $\pm$ SD, $n = 6$. Data represent percent reduction in expression by taking expression in CAS-fed groups as 100 %. * $P < 0.05$ compared to CAS-fed mice. **b** Immunohistochemical analysis of aortic VCAM-1 expression in CAS- or SPI⁻-fed apoE^{-/-} mice ($\times 200$ magnification). VCAM-1 protein expression in aortic sinus was determined by immunohistochemical analysis using goat anti-mouse VCAM-1 antibody followed by Vectastain ABC kit. Under similar conditions, aortic sections stained with goat IgG (NS IgG) as a negative control was minimal. Arrows indicate regions of VCAM-1-positive staining. **c** Quantification of VCAM-1-positive areas was carried out in a blinded fashion as described. Mean-positive VCAM-1+ areas from three slides/mouse and $n = 5$ /diet group is represented. * $P < 0.05$ compared to CAS-fed mice

Fig. 2.

SPI⁻ diet inhibits LPS-induced VCAM-1 expression in mouse aorta. ApoE^{-/-} mice were injected with indicated concentration of LPS ($n = 4$ /concentration). After 5 h, descending aorta was excised (experiment 1) and lysates were used to determine VCAM-1 protein expression by Western blot. Mice ($n = 4$) injected with PBS were used as controls. VCAM-1 protein expression was determined by Western blot analysis and pixel intensity was normalized to β -actin levels (**a**). Representative blots from PBS ($n = 4$, numbered 1–4) LPS (20 μ g/mouse)-injected mice ($n = 4$, numbered 5–8) was presented (**b**). *** $P < 0.001$ compared to PBS-injected group. Effect of dietary SPI⁻ on LPS-induced aortic VCAM-1 protein (**c**, **d**) expression was determined by Western blot analysis (experiment 2). ApoE^{-/-} mice were fed CAS ($n = 4$, numbered 1–4) or SPI⁻ ($n = 4$, numbered 5–8) diet for 1 week followed by LPS (20 μ g/mouse) administration. Aorta from PBS-injected CAS- or SPI⁻-fed mice did not show detectable VCAM-1 protein expression (data not shown). Values are mean $\pm$ SD, $n = 4$. ** $P < 0.01$ compared to CAS-fed group

Fig. 3.

Reduced VCAM-1-dependent monocyte adhesion to aorta of SPI^{-/-} fed mice.

Photomicrograph showing monocyctic cell adhesion to mouse aorta isolated from LPS (20 μ g)-injected apoE^{-/-} mice (experiment 1) (**a**). PKH-labeled monocyctic cells were treated

with anti-CD49d antibody to determine VCAM-1- and CD49d-dependent monocyte adhesion. Monocyctic cells treated with mIgG1 isotype control were used as a negative control (NS). **b** Quantitative analysis of monocyctic cell line adhesion to mouse aorta.

Monocyctic cell adhesion to three individual areas of mouse aorta was counted. Values are mean $\pm$ SD, $n = 4$ mice/group. *** $P < 0.001$ compared to NS IgG-treated cells. ApoE^{-/-} mice were fed CAS or SPI^{-/-} diet for 1 week followed by LPS (20 μ g) challenge (**c**).

Descending aorta (experiment 4) was opened up longitudinally and monocyctic cell adhesion was determined as described in "Materials and methods." Number of monocyctic cells adhered to mouse aorta (**d**) was counted in three separate fields by individuals in a blinded manner. Values are mean $\pm$ SD, $n = 5$. ** $P < 0.01$ compared to CAS-fed group

Fig. 4. LPS-induced plasma inflammatory markers are reduced in SPI^{-/-}-fed mice. Plasma TNF-α (a) and SAA (b) levels in apoE^{-/-}-mice ($n = 5/\text{diet}$) fed CAS or SPI^{-/-} diet for 1 week followed by LPS (20 μg) challenge for 5 h. Plasma samples from experiment 2 and 3 were used to determine TNF-α and SAA levels by ELISA kits from RND systems and Invitrogen, respectively. Values are expressed as mean $\pm$ SD, $n = 5$. ** $P < 0.01$ compared to CAS-fed group

Fig. 5.

SPI⁻ feeding inhibits LPS-induced VCAM-1 and pro-inflammatory cytokines expression. ApoE^{-/-} mice ($n = 4/\text{diet}$) were fed CAS or SPI⁻ diet for 1 week followed by LPS (20 $\mu\text{g}/\text{mouse}$) administration. VCAM-1 (a) and TNF- α (b) and MCP-1 (c) mRNA expression in the descending aorta (experiment 3) was determined by quantitative RT-PCR analyses. Dietary SPI⁻ inhibits LPS-induced inflammatory cytokines and chemokines in liver. RNA was isolated from individual liver samples ($n = 4/\text{group}$) from mice fed CAS or SPI⁻ diets (experiments 2 and 3). RNA was pooled ($n = 4$) for each diet group, and quantitative RT-PCR array analyses of TNF- α (d), MCP-1 (e), IL-6 (f), and IL-1 β (g) mRNA expression in liver was determined as described in section “Materials and methods.” Quantitative RT-PCR was repeated four times with two independent RNA preparations from liver samples from two independent experiments. Inflammatory cytokine and chemokine mRNA expression was normalized to five housekeeping genes for each quantitative RT-PCR array. Values are expressed as mean $\pm$ SD from three different quantitative RT-PCR arrays. * $P < 0.05$, ** < 0.01 and *** < 0.001 compared to CAS-fed group

Fig. 6.

SPI⁻ inhibits LPS-induced NF-κB activation. ApoE^{-/-} mice ($n = 4/\text{diet}$) were fed CAS- or SPI⁻-diet for 1 week followed by LPS challenge for 3 h. Nuclear and cytosolic fractions were prepared from aorta and liver lysates (experiment 5). Total- and phospho-NF-κB in nuclear fractions of aorta (**a**) and liver (**b**) were determined by NF-κB Pathscan ELISA kit. Cytosolic I-κB protein levels (**c**, **d**) in aortic lysates from CAS- or SPI⁻-fed apoE^{-/-} mice were determined by Western blot. Blots probed with GAPDH were used to normalize I-κB protein levels. Values are mean $\pm$ SD, $n = 3$. Immunofluorescence analysis of NF-κB p65 nuclear translocation in liver CAS- or SPI⁻-fed mice ($n = 4$). Representative pictures from 2 mice per diet group are represented. Liver sections (10 μm) were fixed in PBS/10 % formalin and stained with rabbit anti-NF-κB p65 mAb as described under section "Materials and methods." Sections were mounted with DAPI to localize nucleus. Number of nucleated (DAPI+) and NF-κB-positive cells were counted in 3 separate sections/mouse. ** $P < 0.01$ compared to CAS-fed group

Fig. 7. SPI⁻ inhibits LPS-induced AKT activation. ApoE^{-/-} mice ($n = 4/\text{diet}$) were fed CAS- or SPI⁻-diet for 1 week followed by LPS (20 $\mu\text{g}/\text{mouse}$) challenge for 5 h (experiment 5). Total lysates were prepared from liver (**a**) and aorta (**b**), and phosphorylation status of MAP kinase, and AKT were determined using PathScan multi-target sandwich ELISA kit (cell signaling) as described under section “Materials and methods.” Values are mean $\pm$ SD, $n = 4$. * $P < 0.05$ and ** $P < 0.01$ compared to CAS-fed group

Fig. 8.

Soy peptides inhibit LPS-induced VCAM-1 and proinflammatory cytokine expression by endothelial cells in vitro. HUVEC were pre-incubated with soy peptides or vehicle (0.1 % DMSO) for 1 h at 37 °C and then treated with LPS (20 ng/ml) for indicated time. Supernatant collected after 6 h was used to determine IL-6 (a), IL-8 (b), and MCP-1 (c) secretion by Flowcytomix beads followed by analysis using Flow cytomic Pro 3.0 software (eBioscience, San Diego, CA, USA). Basal secretion of cytokines (cells treated without peptides or LPS) did not show any detectable levels of cytokines (data not shown). Cells after 24-h treatment were used to determine VCAM-1 expression (d) by cell ELISA. Values are mean $\pm$ SD of triplicate wells. Representation of two independent experiments are presented, * $P < 0.05$ and ** $P < 0.001$ compared to LPS-treated cells without soy peptides

Table 1
Soy peptides and sequences

Soy peptides	Sequence	Purity (%)	Soy subunit
S1	KNPQLR	91	Both α - and β -subunit of β -conglycinin
S2	EITPEKNPQLR	91	Both α - and β -subunits of β -conglycinin
S3	RKQEEDEDEEQRE	95	α subunit of β -conglycinin
S4	MITLAIPVNK PGRF	97	α' subunit of β -conglycinin
S5	QKEEKJEWQ	95	α' subunit of β -conglycinin