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Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways

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ARTICLE INFO

Article history:
Received 4 May 2006
Accepted 2 July 2006

Keywords:
Citrus pectin
Inducible nitric oxide synthase (iNOS)
Cyclooxygenase-2 (COX-2)
Lipopolysaccharide (LPS)
Mitogen-activated protein kinases (MAPKs)
Nuclear factor-κB (NF-κB)

ABSTRACT

Pectin is composed of complex polysaccharides rich in galactoside residues, and it is most abundant in citrus fruits. Pectin and modified pectin have been found to exhibit anti-mutagenic activity and inhibit cancer metastasis and proliferation, with no evidence of toxicity or other serious side effects. In this study, we investigated the inhibitory effect of pectin at different degrees of esterification (DEs) on the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-activated macrophages. Western blot and RT-PCR analyses demonstrated that 30% esterified pectin (DE30), DE60 pectin, and DE90 pectin significantly inhibited the protein and mRNA expressions of iNOS and COX-2 in LPS-activated macrophages, and DE90 pectin was the most-potent inhibitor. To clarify the mechanisms involved, DE90 pectin was found to inhibit the phosphorylation of MAPKs and IKK kinase activity. In addition, DE90 pectin inhibited the activation of NF-κB and AP-1 by electrophoretic mobility shift assay and transient transfection experiments. Finally, we found that DE90 pectin could bind with LPS, and might result in decreased binding of LPS to its receptor. These results suggest that modulation of iNOS and COX-2 expressions by dietary pectin may be important in cancer chemoprevention and anti-inflammation.

1. Introduction

Overexpression of either inducible nitric oxide synthase (iNOS) or cyclooxygenase 2 (COX-2) has been implicated in the pathogenesis of many disease processes. For example, increases in prostaglandins and COX activities are associated with development of multiple epithelial cancers [1,2], and high levels of nitric oxide contribute to a variety of pathophysiological processes including various forms of circulatory shock [3], inflammation [4], and carcinogenesis [5]. Levels of COX-2 and iNOS are mainly regulated at the transcriptional level [6,7]. The murine iNOS promoter contains consensus sequences for

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doi:10.1016/j.bcp.2006.07.001

the binding of nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), which can be activated by LPS. NF-κB activity is induced by a cascade of events leading to the activation of inhibitor κB (IκB) kinase (IKK), which phosphorylates IκB, leading to its degradation, and free NF-κB is translocated to the nucleus and induces certain gene transcription [8]. AP-1 activity is stimulated by a complex network of signaling pathways that involves external signal (for example, growth factors) stimulation and the activation of mitogen-activated protein kinase (MAPK) members: extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) [9].

Epidemiological studies have shown that the consumption of vegetables and fruits is associated with a decreased risk of cancer. The ingestion of citrus fruit has been reported to be beneficial for the reduction of certain types of human cancer [10]. Pectin is a family of complex polysaccharides that function as hydrating agents and cementing materials for the cellulosic network [11]. Although pectin occurs in a majority of plant cell walls, it is most abundant in citrus (lime, lemon, grapefruit, and orange) fruits. The structure of pectin is primarily composed of repeating units of galacturonic acid joined by $\alpha\,1 \rightarrow 4$ glycosidic linkages, which create a linear polymer (Fig. 1). The regular linear structure is interrupted by the presence of neutral sugar side chains. Furthermore, the carboxyl groups of galacturonic acid may either remain as free acids, be esterified with methanol, or be neutralized with cations. The chemical structure of pectin varies by fruit species and also during the different developmental stages of the fruit [12].

Pectin has been found to exhibit antimutagenic activity against nitroaromatic compounds [13]. Rats fed a pectin-supplemented diet exhibited a higher apoptotic index in the colon [14]. A diet supplemented with 20% apple pectin significantly decreased the number and incidence of azoxymethane-induced colon tumors in rats, and reduced prostaglandin E_2 (PGE_2) levels in the distal colonic mucosa and blood of the portal vein. The ability of apple pectin to decrease PGE_2 is dose-dependent, and those results suggested an anti-inflammatory effect in the bowel. Apple pectin oligosaccharides showed a lower incidence of hepatic metastasis with antioxidant activity and portal scavenging functions [15]. The modification of citrus pectin to modified citrus pectin (MCP) by changing the pH has also been found to bind to the carcinogen, 1,2-dimethylhydrazine (DMH) [16]. In addition, MCP is believed to adhere to tumor cells through cell surface carbohydrate-binding proteins called lectins, preventing aggregation of tumor cells and adhesion to normal cells [17,18]. MCP prevents human umbilical vein endothelial cell migration and capillary tube formation in vitro, either by binding to the galectin-3 present in the matrix and/or on the endothelial cells or interfering with its binding to the receptor. Nude mice fed MCF showed a marked reduction in the density of tumor-associated blood vessels [19]. All of these results suggest that pectin exhibits many healthy effects, and it exists in large quantities in our plant-derived diet.

The role of dietary components in the etiology of various human cancers is of considerable interest, because their use as chemopreventive agents has important implications for cancer prevention. Although the anti-inflammatory and anticarcinogenic characteristics of pectin have been studied, the mechanisms of action are not fully understood. Identification and characterization of such pectins might provide us with additional new agents for cancer chemoprevention. In this study, we examine the effect of pectin with different degrees of esterification on the expressions of iNOS and COX-2 in LPS-activated macrophages and seek to elucidate possible reaction mechanisms.

2. Materials and methods

2.1. Materials

LPS (*Escherichia coli* 0127:B8) and citrus pectin (Fig. 1) with different degrees of esterification (~30%, ~60%, ~90% esterified pectin) were purchased from Sigma Chemical (St. Louis, MO). Purity of the pectin exceeded 97% of its dry weight.

2.2. Cell culture and treatment

The primary peritoneal macrophages were elicited by thioglycollate from specific pathogen-free male BALB/c mice at 5–8 weeks of age as described previously [20]. Both peritoneal macrophages and the mouse macrophage cell lines, RAW264.7 (BCRC 60001, Food Industry Research and Development Institute, Hsinchu, Taiwan), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. For all assays except the transient transfection experiments, cells were plated in 60-mm dishes at a density of 5×10^6 cells, allowed to grow for 18–24 h, and pretreated with citrus pectin for 30 min and then treated with LPS. Test compounds and LPS were prepared with sterile water.

2.3. Measurement of cell viability

RAW264.7 macrophages and peritoneal macrophages were treated with various concentrations of pectin for 24 h. Cell viability was evaluated by counting trypan blue dye-excluded cells in a hemacytometer.

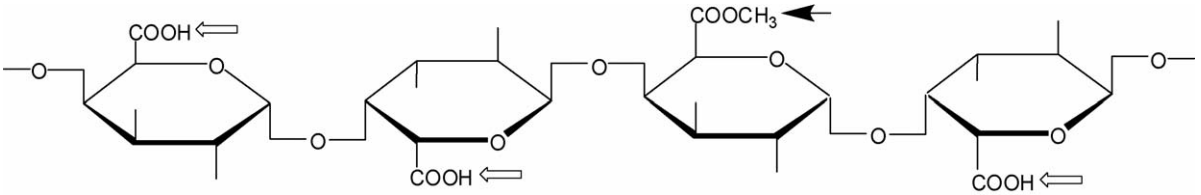

Fig. 1 – Chemical structure of pectin. The close arrow indicates the esterified carboxyl group, and the open arrow indicates possible esterification sites.

2.4. Western blot analysis

Equal amounts of total cellular protein (50 μg) were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described previously [21]. The membrane was then incubated with an anti-COX-2 antiserum, anti-iNOS antiserum, anti-IκB antiserum, anti-phosphorylated IκB antiserum, anti-phosphorylated ERK antiserum, anti-p65 antiserum, anti-ERK, anti-JNK, anti-p38 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated JNK antiserum, or anti-phosphorylated p38 antiserum (Cell Signaling Technology Inc., Danvers, MA). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using enhanced chemiluminescence kits (ECL; Amersham, England). The densities of the bands were quantitated with a computer densitometer (IS-1000 Digital Imaging System).

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR

Total RNA was isolated as described by Chomczynski and Sacchi [22]. The extracted RNA (2 μg) was reverse-transcribed at 37 °C for 1 h by adding 5 μM of random hexamer oligonucleotides (Gibco BRL), 200 U of reverse transcriptase, 2.5 mM deoxyribonucleotide triphosphates (dNTP), and 10 mM dithiothreitol. The semiquantitative PCR was carried out with 2 μl of cDNA and 23 μl of PCR mix buffer containing each primer (0.2 μM), dNTP (2.5 mM), and Taq DNA polymerase (1.25 U) (Takara). After the PCR, 10 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining.

The quantitative real-time PCR was carried out with 2 μl of cDNA and 500 μM of primer in a FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Taipei, Taiwan) using a Light Cycler^{TM} (Roche Diagnostics). After initial heat denaturation at 95 °C for 5 min, PCR cycles were repeated 40 times under the following conditions: denaturation at 95 °C for 5 s, annealing at 62 °C for 5 s, and extension at 72 °C for 10 s. The quantification of the cDNA copy number was performed using control samples according to the manufacture's protocol. iNOS and COX-2 mRNA expressions were quantified in relation to the expression of β-glucuronidase.

Semiquantitative PCR primers for the mouse iNOS and COX-2 cDNA probes were synthesized according to the following oligonucleotide sequences: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′; and COX-2, forward primer 5′-TGATGACTGCCCAACTCCCATG-3′, reverse primer 5′-AATGTTGAAGGTGTCCGGCAGC-3′. Quantitative real-time PCR primers for the mouse iNOS and COX-2 cDNA probes were synthesized according to the following oligonucleotide sequences: iNOS, forward primer 5′-CCTCCACCCTACCAAGT-3′, reverse primer 5′-CAGCTCCAAGGAAGAGTGA-3′; and COX-2, forward primer 5′-CGTGGTCACTTTACTACGAG-3′, reverse primer 5′-AGGTACATAGTAGTCCTGAGC-3′.

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins and a ^{32}P-labeled double-stranded oligonucleotide probe were prepared as described previously [20]. For the EMSA, 2 μg of each nuclear extract was mixed with the labeled double-stranded NF-κB and AP-1 consensus oligonucleotide (Santa Cruz Biotechnology) and incubated at room temperature for 20 min. The DNA–protein complex was separated on 5% non-denaturing acrylamide gels before vacuum drying and autoradiography.

To determine the specificity of NF-κB or AP-1 binding to its consensus motif, supershift experiments were conducted using specific antibodies (anti-p65 NF-κB or anti-c-Jun and anti-c-Fos, Santa Cruz Biotechnology). Briefly, the probe was incubated with 5 μg nuclear extracts in a 15-μl volume of binding reaction for 20 min on ice, at which time 2 μg specific antibodies were added, followed by incubation on ice for an additional 30 min. Reaction mixtures were resolved on 5% non-denaturing acrylamide gels before vacuum drying and autoradiography.

2.7. Plasmids, transient transfection, and luciferase assay

The mouse iNOS promoter plasmid (pGL2-iNOS), generously provided by Dr. Charles J. Lowenstein (Johns Hopkins University), and mouse COX-2 promoter plasmid (pGL2-COX2) were prepared as described previously [23]. Cells (5×10^4) were seeded in 12-well plates. After 24 h of incubation, the medium was replaced with the Opi-MEM medium (Invitrogen, Carlsbad, CA) and co-transfected with the pGL2-iNOS, pGL2-COX2, pAP-1-Luc (Stratagene, La Jolla, CA), or pNFκB-Luc (Stratagene) plasmid plus the phRL-TK (Promega, Madison, WI) plasmid as an internal control using Lipofectamine2000^{TM} (Invitrogen). After 48 h of incubation, cells were treated with pectin and LPS (100 ng/ml) for 4 or 8 h. Each well was washed with cold-PBS and harvested in 200 μl of passive lysis buffer (PLB). Aliquots of 100 μl of cell lysate were used to assay luciferase activity with the Dual-Luciferase Reporter Assay kit (Promega).

2.8. IκB kinase (IKK) assay

An IKK kinase activity assay was performed as described in our previous report [24]. Briefly, equal amounts of total cellular protein (200 μg) were immunoprecipitated with an IKK-α-specific antibody (Santa Cruz Biotechnology) and protein A/G-PLUS agarose for 12 h at 4 °C. The kinase assay was carried out in 45 μl of kinase buffer containing 5 μM cold ATP, 10 μCi [γ-^{32}P] ATP (5000 Ci/mmol, Amersham), and 1 μg GST-IκBα fusion protein (Santa Cruz Biotechnology) as the substrate, and incubated for 20 min at 25 °C. Each sample was mixed with 8 μl of 5× Laemmli's loading buffer to stop the reaction, heated for 10 min at 100 °C, and subjected to 8% SDS-PAGE. Gels were dried and visualized by autoradiography.

2.9. LPS binding assay

For cell binding, cells were pretreated with various concentrations of DE90 and then with 100 ng/ml Alexa Fluor-LPS (Molecular Probes Inc., Eugene, OR) for 30 min at 37 °C. The

cells were washed thrice with PBS, and unbound Alexa Fluor-LPS was then removed before they were collected for determination of fluorescence intensity. The fluorescence intensity of Alexa Fluor-LPS that remained associated with the cells was measured by a F-4500 fluorescence spectrophotometer (Hitachi) at the emission of 519 nm and excitation of 488 nm. For DE90 binding, various concentrations of DE90 were placed in 2 ml tubes and added with 200 ng/ml Alexa Fluor-LPS for 30 min at 37 °C. The incubations were added with equal volume of 100% ethanol to precipitate DE90. Unbound Alexa Fluor-LPS was removed by centrifugation, and the pellet was washed thrice with 75% ethanol and re-dissolved in distilled water for determination of fluorescence intensity.

2.10. *Statistical analysis*

Data are presented as the mean of S.E. for the indicated number of independently performed experiments. Statistical analysis was done using one-way Student's *t*-test.

3. Results

3.1. *Pectin inhibited iNOS and COX-2 expression in LPS-activated macrophages*

DE30, DE60, and DE90 are pectins consisting of about 30%, 60%, and 90% methoxylated polygalacturonic acid, respectively, and these were used in this study. Macrophage is a suitable model to evaluate the anti-inflammatory effect of various natural products or other drugs. To examine whether these pectins could inhibit inflammation, mouse macrophage cell line RAW264.7 was chosen to evaluate the effects. According to previous reports [18,19], the pectins were used at the concentration range from 0.1 to 1 mg/ml to examine the anti-inflammatory effect. First, RAW264.7 cells were treated with the three types of pectin (0.5 mg/ml) and LPS (100 ng/ml) for 6 or 24 h. Western blot and RT-PCR analyses indicated that both iNOS and COX-2 proteins as well as mRNA expression were significantly inhibited by the three types of pectin at 0.5 mg/ml (Fig. 2). Among the pectins tested, DE90 strongly inhibited iNOS and COX-2 expression and was the most potent inhibitor. The next experiment was to determine the effective concentration of DE90 pectin on LPS-activated macrophages. As shown in Fig. 3, DE90 pectin inhibited iNOS and COX-2 protein and mRNA expressions in a concentration-dependant manner, and the significantly effective concentration was between 0.25 and 0.5 mg/ml. Peritoneal macrophages were also used to test the inhibitory effects of DE90 pectin on the expression of iNOS and COX-2 induced by LPS. The results showed that DE90 pectin was able to inhibit the activation of peritoneal macrophages induced by LPS, indicating by downregulation of iNOS and COX-2 protein expression (Fig. 3D). To exclude the inhibitory effect resulting from the cytotoxic effect of pectin on RAW264.7 macrophages and peritoneal macrophages, cells were treated with various concentrations (0.25–1 mg/ml) of pectin for 24 h, and the cell viability was determined using trypan blue dye in a hemacytometer. The results revealed that pectin had no significant cytotoxicity to RAW264.7 macrophages or peritoneal macrophages, even at a concentration of 1 mg/ml (data not shown).

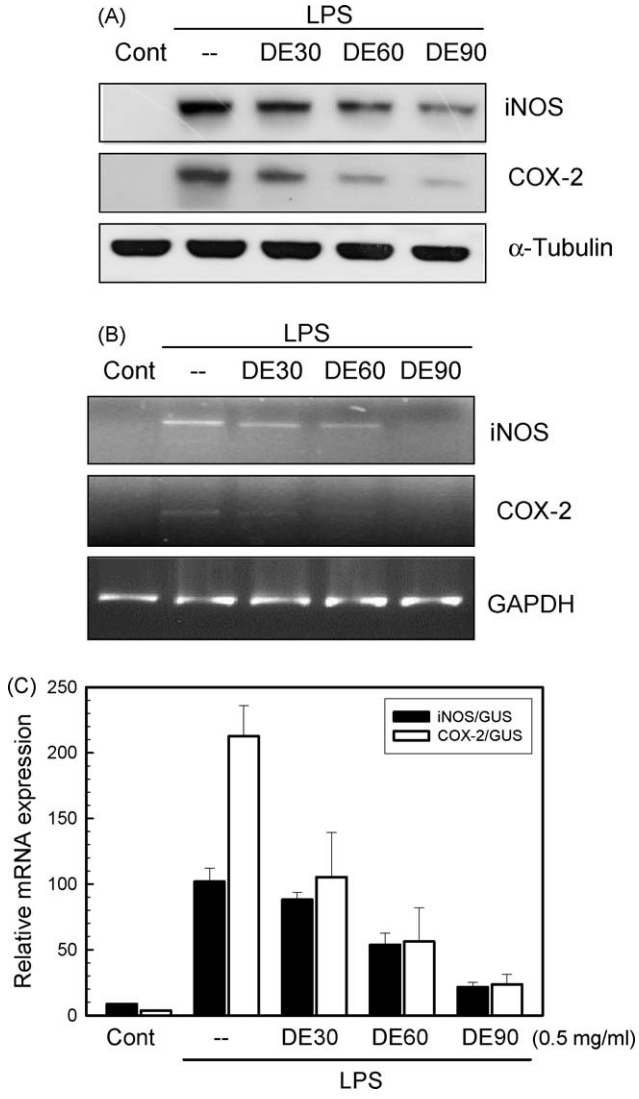

Fig. 2 – Effects of DE30, DE60, and DE90 pectins on the expressions of iNOS and COX-2 in LPS-activated macrophages. RAW264.7 cells were treated with three types of pectin (0.5 mg/ml) and LPS (100 ng/ml) for (A) 24 h or (B and C) 6 h. (A) Whole-cell lysates were prepared, and immunoblotted with specific antibodies for iNOS, COX-2, and α-tubulin. (B and C) Total mRNA was prepared, and the levels of iNOS, COX-2, and GAPDH were detected by RT-PCR (B) or quantified by real-time RT-PCR (C). Values are expressed as the mean ± S.E. of triplicate tests.

To further investigate the importance of DE90 pectin in modulating the expressions of iNOS and COX-2, transient transfection was performed using mouse COX-2 and iNOS luciferase promoter constructs. Transfected cells were treated with LPS, which led to approximately 4.2- and 3.4-fold increases in iNOS and COX-2 promoter activities, respectively. These were inhibited by DE90 pectin in a dose-dependent manner (Fig. 4). These results could potentially be explained by DE90 pectin's blocking of LPS-mediated signal transduction.

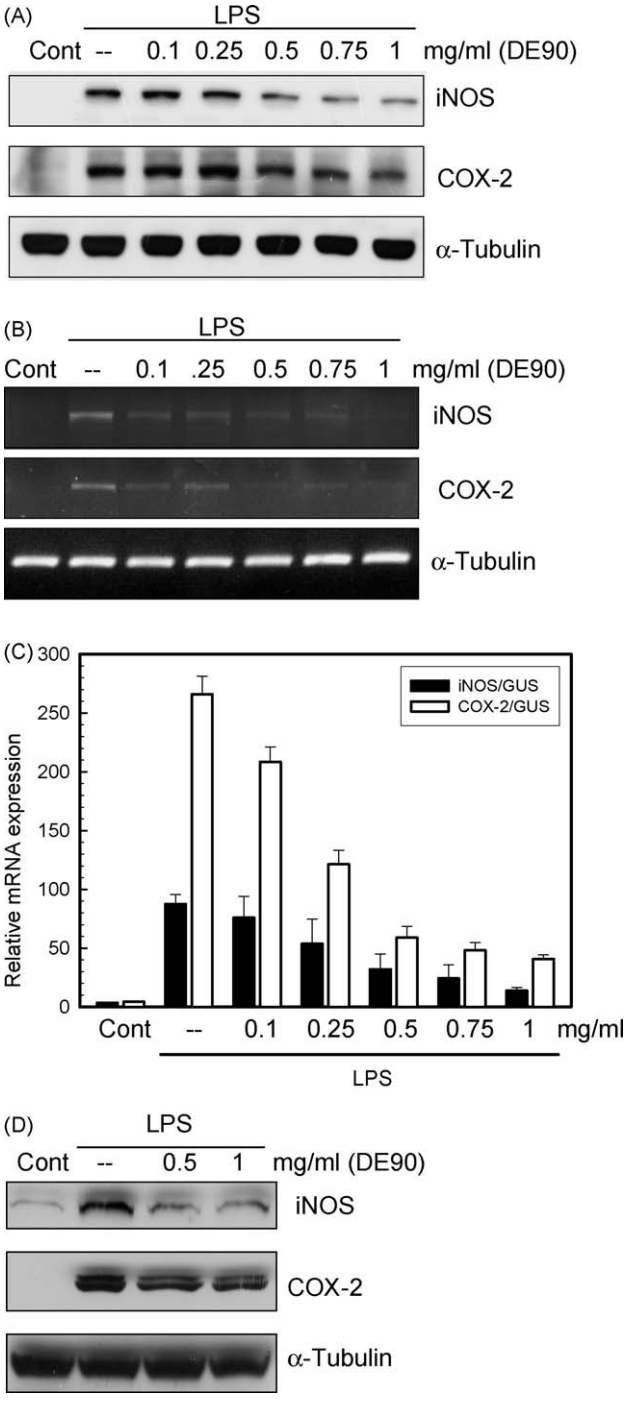

Fig. 3 – Effects of various concentrations of DE90 pectin on the expressions of iNOS and COX-2 in LPS-activated macrophages. RAW264.7 cells were treated with various concentrations of DE90 pectin (0.1–1 mg/ml) and LPS (100 ng/ml) for (A) 24 h or (B and C) 6 h. (A) Whole-cell lysates were prepared and immunoblotted with specific antibodies for iNOS, COX-2, and α-tubulin. (B and C) Total mRNA was prepared, and the levels of iNOS, COX-2, and GAPDH were detected by RT-PCR (B) or quantified by real-time RT-PCR (C). (D) Thioglycollate-elicited peritoneal macrophages were incubated with various concentrations of DE90 pectin (0.5 and 1 mg/ml) and LPS (100 ng/ml) for 24 h. Whole-cell lysates were prepared, and immunoblotted with specific antibodies for iNOS, COX-2, and α-tubulin.

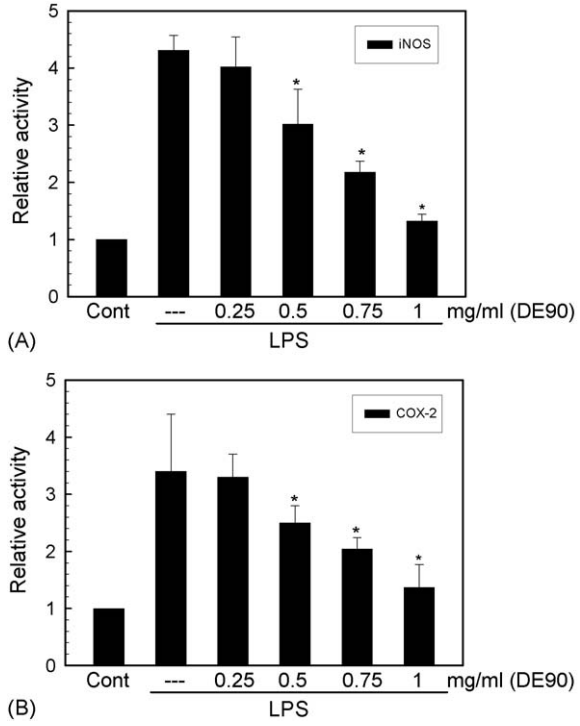

Fig. 4 – Effects of DE90 pectin on iNOS and COX-2 promoter activities in LPS-activated macrophages. RAW264.7 cells were transfected with a mouse COX-2 promoter construct (−996/+70) or mouse iNOS promoter (−1592/+160) with the phRL-TK plasmid as an internal control. After transfection, cells were treated with various concentrations of DE90 pectin (0.25–1 mg/ml) and LPS (100 ng/ml) for 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Section 2. Values are expressed as the mean ± S.E. of triplicate tests. $^{*}p < 0.01$ vs. LPS treatment.

3.2. *DE90 pectin inhibited MAPKs and NF-κB signals in LPS-activated macrophages*

To clarify the inhibitory mechanism involved, the most-potent inhibitor, DE90, was chosen to examine the effect of pectin on the activation of MAPKs (ERK, JNK, and p38) and NF-κB in LPS-activated macrophages. Cells were treated with DE90 pectin (1 mg/ml) and LPS for 15, 30, 60, and 120 min. The time-course experiment showed that phosphorylated levels of ERK, JNK, and p38 reached the maximum after 30 min of LPS treatment (Fig. 5), and DE90 significantly inhibited the phosphorylation within 30 min. However, DE90 slightly inhibited the phosphorylation of ERK at 1 and 2 h after LPS treatment.

It is known that NF-κB activation is mediated by the phosphorylation and degradation of IκB, and then translocation of NF-κB to the nucleus. To examine whether DE90 pectin can suppress NF-κB activity, cells lysates were collected for determination of the phosphorylation and degradation of IκB. After 30 min of LPS treatment, IκB degradation was found, but DE90 pectin significantly prevented that degradation (Fig. 6A, upper panel). Moreover, DE90 pectin also inhibited the phosphorylation of IκB after 30 min of LPS treatment (Fig. 6A, lower panel). When the nuclear fraction was assayed,

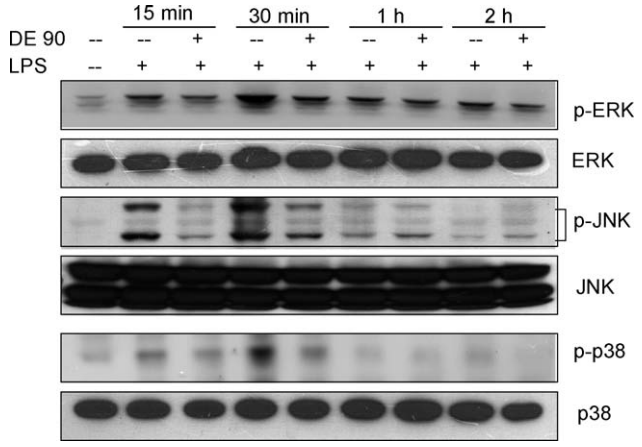

Fig. 5 – Effects of DE90 pectin on the phosphorylation of MAPKs in LPS-activated macrophages. RAW264.7 cells were treated with DE90 pectin (1 mg/ml) and then LPS (100 ng/ml) for various time periods as indicated. The protein levels of unphosphorylated and phosphorylated ERK, JNK, and p38 MAPKs were detected by Western blot analysis.

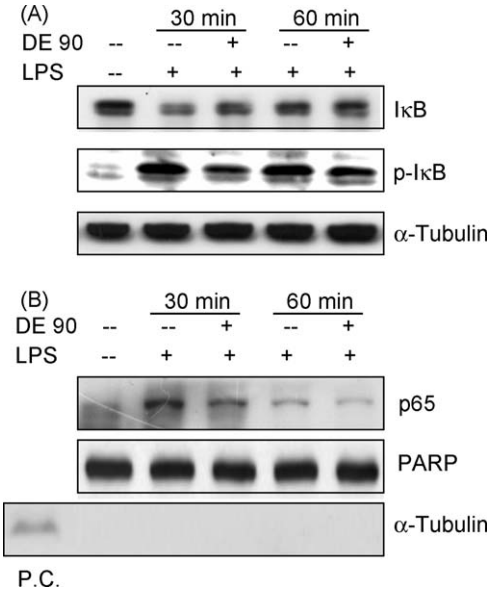

Fig. 6 – Effects of DE90 pectin on IκBα phosphorylation and degradation, and NF-κB translocation in LPS-activated macrophages. RAW264.7 cells were treated with DE90 pectin (1 mg/ml) and LPS (100 ng/ml) for various time periods as indicated. (A) Whole-cell lysates or (B) nucleic extracts were prepared, and immunoblotted with specific antibodies for IκBα, phosphorylated IκBα, p65, PARP, and α-tubulin were detected by Western blot analysis. P.C.: positive control.

DE90 pectin significantly blocked the translocation of nuclear factor-κB (p65) after 30 and 60 min of LPS treatment (Fig. 6B). Poly(ADP-ribose) polymerase (PARP, nucleic protein) and α-tubulin (cytoplasmic protein) were used as loading control and to verify the purity of nucleic proteins, respectively. Previous studies have indicated that iNOS and COX-2 promoters act as consensus sequences for NF-κB and AP-1 binding and activation. Next, we investigated the effect of DE90 pectin on AP-1 and NF-κB activities in LPS-activated macrophages. Cells were treated with DE90 pectin (1 mg/ml) and LPS (100 ng/ml) for 30, 60, 120, and 180 min. EMSA experiments indicated that LPS increased the binding activity of NF-κB and AP-1 after 30 min of treatment, and DE90 pectin significantly blocked the binding activity within 30 min of LPS treatment (Fig. 7A). Transient transfection was performed using NF-κB and AP-1 luciferase reporter constructs, and results showed that DE90 pectin inhibited both NF-κB and AP-1 activities in a dose-dependent manner (Fig. 7B). In addition, we investigated whether DE90 pectin can inhibit IKK activity. As shown in Fig. 7C, 1 mg/ml of DE90 pectin significantly inhibited the IKK activity induced by LPS. DE90 pectin had no effect on the level of IKK protein (data not shown). These results suggest that the inhibition of COX-2 and iNOS expressions by DE90 pectin might occur via: (1) suppression of IKK activity and the resultant prevention of NF-κB activation, and (2) inhibition of the phosphorylation of MAPK members (ERK, JNK, and p38) resulting in blockage of AP-1 activity.

3.3. *DE90 pectin prevented LPS from binding to cells*

To test whether DE90 pectin can block LPS from binding to cells, RAW264.7 cells were pretreated with DE90 and then added to Alexa Fluor-LPS for 30 min. The unbound Alexa Fluor-LPS was removed by washing the cells with PBS, and the fluorescence of cell-associated Alexa Fluor-LPS was measured by fluorencence spectrophotometer. The baseline fluorescence of cells alone was 2896.5, and baseline fluorescence were 2812.3, 2894.8, 2931.5 and 3002.1 in the cells treated without Alexa Fluro-LPS and with 0.25, 0.5, 0.75 and 1 mg/ml of DE90, respectively. In Fig. 8A, the fluorescent intensities have been subtracted the baseline fluorescence (cells alone) from total fluorescence (cells treated with Alexa Fluor-LPS, column 1), or the baseline fluorescence (cells treated with DE90 alone) from total fluorescence (cells treated with both Alexa Fluor-LPS and DE90, columns 2–5). Therefore, the fluorescent intensity of cell-associated Alexa Fluor-LPS was 155.3 (column 1) in the absence of DE90, and 158.2, 176.6, 198.9 and 257.8 (columns 2–5) in the presence of 0.25, 0.5, 0.75, and 1 mg/ml of DE90, respectively. Interestingly, DE90 pectin markedly increased the amounts of cell-associated Alexa Fluor-LPS in a dose-dependent manner (Fig. 8A), with the binding increasing by approximately 75% in 1 mg/ml of DE90. However, the above results showed that DE90 significantly inhibited the downstream signals of LPS (Figs. 2–7). Why did DE90 inhibit the LPS signalings but increase the LPS binding to cells? To answer this question, we designed an experiment to test whether DE90 itself can bind with LPS in vitro. As described previously, pectin is composed of complexed polysaccharides and can be precipitated by the addition of enthanol. Alexa Fluor-LPS was incubated with various doses of DE90 pectin in a tube for 30 min. After incubation, ethanol was added to precipitate the DE90 pectin and Alexa Fluor-LPS if it had bound with DE90. The baseline fluorescence of distilled water was 3409.4, and baseline fluorescence were 3342.7, 3593.8, 3618.2 and 3901.3 in the distilled water without Alexa Fluro-LPS and with 0.25,

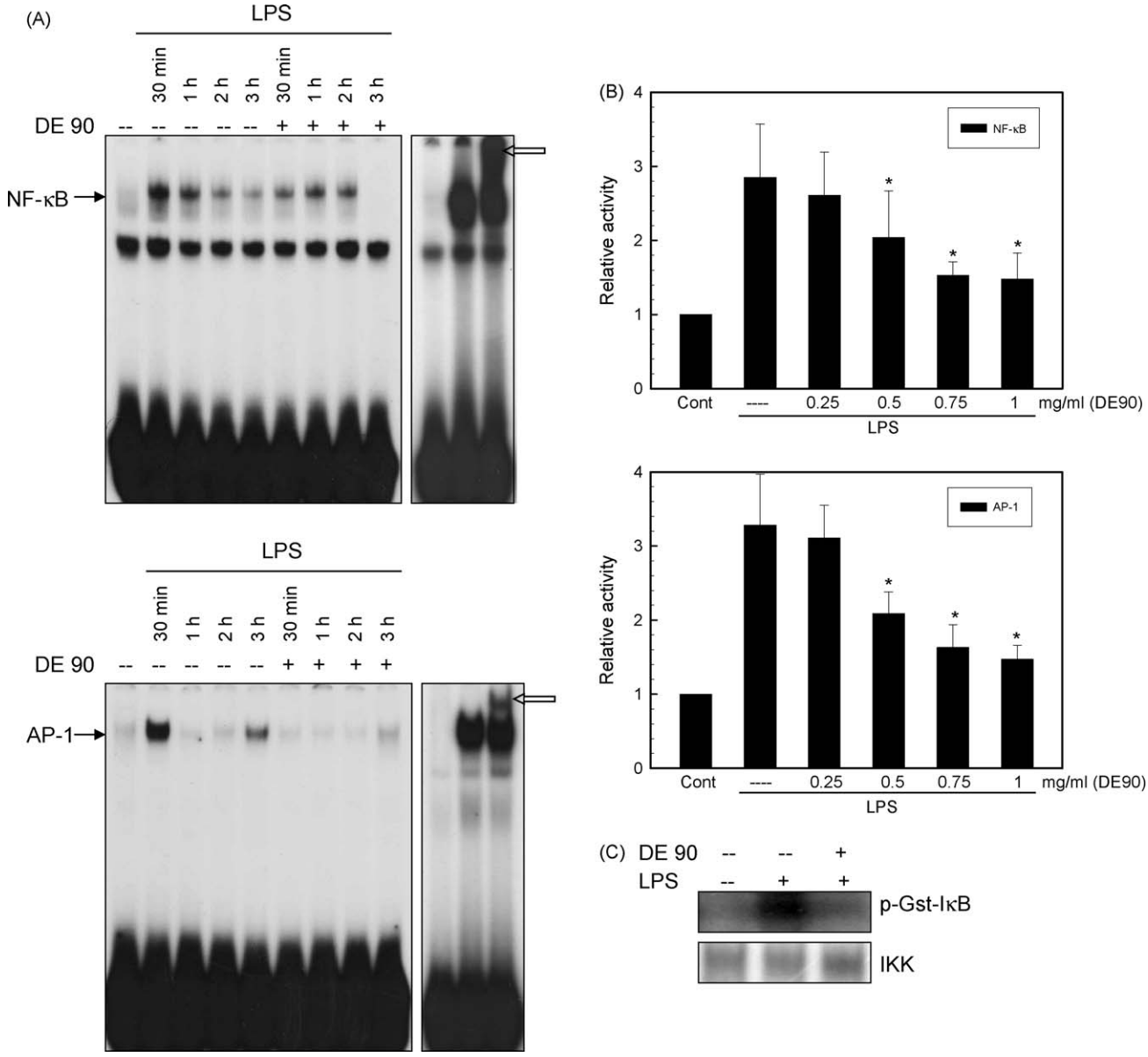

Fig. 7 – Effects of DE90 pectin on AP-1 and NF-κB activities determined by (A) an electrophoretic mobility shift assay and (B) reporter plasmid, and (C) LPS-induced IκB kinase activity. (A) RAW264.7 cells were treated with DE90 pectin (1 mg/ml) and LPS (100 ng/ml) for various time periods as indicated. Nucleic extracts were prepared, and electrophoretic mobility shift assays were carried out as described in Section 2. The specificity of AP-1 and NF-κB complex formation is shown. Open arrow indicates the supershift band. (B) RAW264.7 cells were transfected with AP-1 or NF-κB reporter plasmids, and the phRL-TK plasmid was used as an internal control. Transfected cells were treated with various concentrations of DE90 pectin and LPS (100 ng/ml) for 8 h. Cells were harvested, and the levels of luciferase activities were determined as described in Section 2. Values are expressed as the mean ± S.E. of triplicate tests. $^{*}p < 0.01$ vs. LPS treatment. (C) RAW264.7 cells were treated with DE90 pectin (1 mg/ml) and LPS (100 ng/ml) for 30 min. Whole-cell lysates (200 μg) were used for immunoprecipitation, and IκB kinase activity was assayed with Gst-IκB as the substrate. The experiments were performed as described in Section 2, and ^{32}P-labeled Gst-IκB is shown.

0.5, 0.75 and 1 mg/ml of DE90, respectively. In Fig. 8B, the fluorescent intensities have been subtracted the baseline fluorescence (distilled water alone) from total fluorescence (distilled water with Alexa-Fluro-LPS without DE90, column 2), or the baseline fluorescence (distilled water with various dose of DE90) from total fluorescence (distilled water with both Alexa Fluor-LPS and various dose of DE90, columns 3–6). Therefore, the fluorescent intensity of DE90 pectin-associated Alexa Fluor-LPS was 533.8 (column 2) in the absence of DE90, and 553.8, 1032.9, 1251.7, and 1843.9 (columns 3–6) in the presence of 0.25, 0.5, 0.75, and 1 mg/ml of DE90, respectively. The results indicated that Alexa Fluor-LPS was present in DE90 pectin precipitate, and the increase of DE90 pectin-associated Alexa Fluor-LPS by DE90 pectin occurred in a dose-dependent

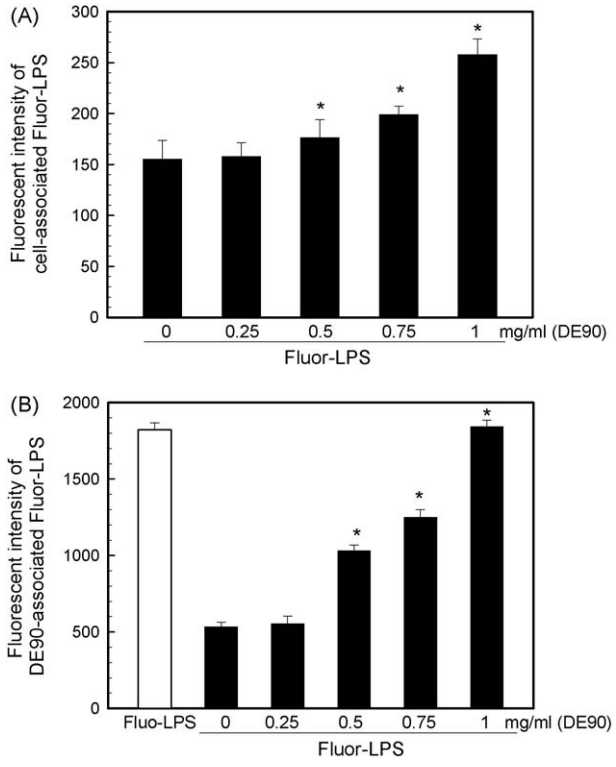

Fig. 8 – Effects of DE90 on the LPS binding to cells. (A) RAW264.7 cells were treated with various concentrations of DE90 and 100 ng/ml Alexa Fluor-LPS for 30 min at 37 °C. The cells-associated Alexa Fluor-LPS were determinated by fluorescence spectrophotometer as described in Section 2. Values are expressed as the mean ± S.E. of triplicate tests. *$p < 0.01$ vs. Alexa Fluor-LPS alone. (B) Various concentrations of DE90 were incubated with 200 ng/ml Alexa Fluor-LPS for 30 min at 37 °C. The DE90-associated Alexa Fluor-LPS were determinated by fluorescence spectrophotometer as described in Section 2. Values are expressed as the mean ± S.E. of triplicate tests. *$p < 0.01$ vs. Alexa Fluor-LPS alone. The fluorescent intensity of open bar has been subtracted baseline fluorescence (distilled water alone) from directly determined the fluorescent intensity of a distilled water solution contained with 200 ng/ml of Alexa Fluor-LPS as reference.

manner. The results suggest that DE90 pectin was able to bind with LPS and might prevent it from binding to cells.

4. Discussion

Pectin is abundant in plant-based diets and especially in citrus fruits. Pectin is a biochemically active compound with known anti-inflammatory, anticarcinogenic, and free radical-scavenging properties. However, the anti-inflammatory functions of pectin are not well established. In this study, we demonstrated that pectin significantly inhibited the expressions of iNOS and COX-2 in LPS-activated macrophages. These findings provide a significant molecular basis for understanding how dietary pectin acts to prevent inflammation and cancer.

The COX enzyme possesses both cyclooxygenase and peroxidase functions. Prostaglandins formed by COX impair immune surveillance and modulate proliferation in a variety of cell types [3]. Overexpression of COX-2 or an increase of COX activity is involved in the pathogenesis of colon cancer [25], and COX inhibitors, such as aspirin, exhibit preventive effects on colon carcinogenesis. The beneficial effects on human health of citrus pectin have been investigated both *in vitro* and *in vivo*. Previous studies demonstrated that dietary pectin may confer protection against colon carcinogenesis in part by blocking carcinogen-induced PKC expression [26]. In this study, we demonstrated that pectin is a potential inhibitor of COX-2 expression in macrophages, and it might have the same inhibitory effect on colon epithelial cells in humans and prevent colon carcinogenesis. The inhibitory mechanisms of pectin for the production of iNOS and COX-2 were investigated. We found that pectin blocked the two transcription factors NF-κB and AP-1 essential for iNOS [27] and COX-2 [7] expression. Further examination demonstrated that pectin inhibited activation of IκB kinase by LPS, prevented the phosphorylation and degradation of IκB, and finally blocked NF-κB translocation to the nucleus. Activation of MAPKs has been implicated in the activation of AP-1 and NF-κB in LPS-treated cells. Our findings indicated that pectin inhibited the LPS-induced phosphorylation of JNK, ERK, and p38, suggesting that the inhibition of AP-1 and NF-κB activation by pectin may be due to inhibition of phosphorylation of MAPKs.

Pectin with different degrees of esterification was able to inhibit iNOS and COX-2 expressions, but with different potencies. Among the three types of pectin tested, 90% esterified pectin (DE90) was a more potent inhibitor than the other two pectins (DE30 and DE60). These phenomena suggest that inhibitory capacity might depend on the structure of the pectin. Recent studies also suggest that the beneficial effects of pectin are closely related to its structural characteristics. It was suggested that pectin with lower methoxyl content and lower molecular weight (<10,000 kDa) may be more efficient for cancer metastasis prevention while pectin with a greater methoxyl content and higher molecular weight may be a better cholesterol-lowering agent [28,29].

Galectin-3 belongs to the galectin superfamily of proteins and contains a conserved sequence of the carbohydrate-binding domain and an affinity to β-galactosides [30]. Previous studies have demonstrated that galectin-3 levels are involved in neoplastic transformation and cancer progression. Mock galectin-3 by monoclonal antibody strongly inhibited experimental lung metastasis of a melanoma [31]. Recently, Nangia-Makker et al. showed that modified citrus pectin was able to inhibit the galectin-3-mediated functions of angiogenesis, tumor growth, and metastasis *in vivo* and *in vitro* [19]. Toll-like receptor 4 (TLR4) was potentially identified as the LPS receptor, and is known to be required for cells to effectively respond to LPS. These findings reveal the activation of downstream pathways of LPS to be dependent on LPS binding to its receptor, TLR4. Our results showed that the highly methyl-esterified pectin, DE90 pectin, had a higher potential to inhibit the downstream signals of LPS, and the inhibition was not a result of DE90 directly inhibiting the activities of signal proteins. Since IκB expression and the phosphorylation levels of ERK, JNK and p38 did not change in cells treated with DE90 alone (data not

shown), it is possible the highly methyl-esterified pectin (DE90) had a higher affinity to both cell surface carbohydrates and LPS and therefore blocked LPS from binding to its receptor (Fig. 8). Two possibilities present themselves: (a) DE90 pectin binds to multiple sites on the cell surface and, consequently, results in shadow TLR4 or a conformation change to TLR4, which blocks it from binding to LPS; or (b) DE90 pectin binds directly to the LPS-binding site of TLR4, blocking the binding of LPS. Considering the biological nature of pectin presented in this and previous studies, it is quite possible that pectin contributes to the preventive effects on inflammation and carcinogenesis attributed to a vegetarian diet. The possible relationships between the structural properties of pectin and their anti-inflammatory and anticarcinogenic activities deserve further investigation.

Acknowledgments

This work was supported by the National Science Council, Republic of China (NSC94-2311-B-038-002). We are greatly indebted to Prof. Charles J. Lowenstein for providing the pGL-iNOS plasmid.

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