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Anti-inflammatory and anti-apoptotic effects of strawberry and mulberry fruit polysaccharides on lipopolysaccharide-stimulated macrophages through modulating pro-/anti-inflammatory cytokines secretion and Bcl-2/Bak protein ratio

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ABSTRACT

This study is the first to isolate strawberry (SP) and mulberry fruit polysaccharides (MP) and assess their anti-inflammatory and anti-apoptotic activities using lipopolysaccharide (LPS)-stimulated mouse primary macrophages. Pro-/anti-inflammatory cytokine levels secreted by LPS-stimulated macrophages cultured with SP and MP for 48 h were determined using ELISA method to evaluate anti-inflammatory effects of SP and MP. The Bcl-2/Bak (anti-/pro-apoptotic) protein levels in the cells were determined using Western blotting method to evaluate anti-apoptotic effects of SP and MP. The results showed that the maximum absorption peak of SP and MP appeared at 240 nm with a small shoulder around 280 ~ 310 nm, suggesting that SP and MP might be glycoproteins. SP- and MP-treatment significantly ($P < 0.05$) decreased pro-inflammatory cytokines including interleukin (IL)-1β and IL-6, whereas the anti-inflammatory cytokine IL-10 was markedly increased, suggesting that SP and MP have anti-inflammation potential via modulating pro-/anti-inflammatory cytokine secretion profiles. Both SP and MP modulated Bak and Bcl-2 protein levels in the cells, suggesting that the SP and MP protected LPS-stimulated macrophages from apoptotic cell death. A negative correlation between cytokine secretion levels and Bcl-2 protein levels suggested that pro-inflammatory IL-1β and IL-6 cytokines decreased Bcl-2 levels in the LPS-stimulated macrophages.

1. Introduction

Cytokines are small proteins (about 25 kDa) secreted by different cells and play an important role as hormonal mediators for host defense, growth and repair processes within injured tissues (Hopkins, 2003). Among cytokines, interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor (TNF) can roughly be classified as pro-inflammatory cytokines because they highlight the way and initiate local inflammation within injured tissues (Hopkins, 2003), although some cytokines exert both pro- and anti-inflammatory effects depending upon the present immune cells and their state of responsiveness to the cytokine (Borish and Steinke, 2003). In contrast, IL-10 is recognized as an anti-inflammatory cytokine for it produced by T helper type 2 (Th2) lymphocytes, T regulatory cells (Th3 cells), macrophages and some B cells to inhibit the synthesis of other cytokines and macrophage functions during the late inflammation phase (Lin and Li, 2010). Uncontrolled or excess inflammation may induce apoptosis of immune cells and subsequently cause chronic degenerative diseases. Therefore, appropriate regulation of inflammation through modulating pro- and anti-inflammatory cytokine expression in immune cells by potential food components may avoid immune disorder diseases.

Apoptosis, namely programmed cell death, is a complicated process by which cells undergo a form of non-necrotic cellular suicide through the immuno–pharmaco–redox signaling pathways (Haddad, 2004). Among apoptotic signaling, B-cell lymphoma 2 (Bcl-2) family members that are comprised of three subgroups: the anti-apoptotic family members (Bcl-2, Bcl-xL, Mcl-1, A1, Bcl-b and Bcl-w), the pro-apoptotic BH3-only proteins (Bid, Bad, Bmf, Bik, Noxa, Puma and Hrk) and the pro-apoptotic Bax/Bak sub-family, play a vital role in the apoptosis progression in different cell types (Sheridan and Martin, 2010; Yokoyama et al., 2002). Bax, namely Bcl-2–associated X protein, and Bak, namely a Bcl-2 homologous antagonist/killer, are critical in programmed cell death regulation. It is found that healthy cells have high relative amounts of free anti-apoptotic Bcl-2 family members to bind and sequester pro-apoptotic Bax and Bak (Sheridan and Martin, 2010); freeing Bax and Bak, a requisite gateway to mitochondrial dysfunction and cell death in response to diverse stimuli, enhance mitochondrial cytochrome c release and induce caspase cascade activation (Wei et al., 2001). Under cellular stress, BH3-only proteins are activated via transcriptional regulation or post-translational modification, and subsequently combined with the

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anti-apoptotic Bcl-2 family members (Sheridan and Martin, 2010). Recently, it is found that Bax and Bak initiate apoptosis in higher eukaryotes (Westphal et al., 2011). Apoptosis related protein levels in cells may dramatically change depending upon apoptotic status. Therefore, apoptotic status in cells may be delineated according to intracellular anti-apoptotic Bcl-2 and pro-apoptotic Bak protein levels. Apoptosis resulting from internal or external stimuli is generally harmless. However, uncontrolled or unexpected apoptotic cell death causes many degenerative diseases, including neurodegenerative diseases, Alzheimers and Parkinsons (Jacobson, 1998), and neuromuscular diseases (Miller and Girgenrath, 2006). Anti-apoptotic therapy to avoid uncontrolled or unexpected apoptotic cell death in normal cells may be beneficial for the corresponding human diseases (Jacobson, 1998; Miller and Girgenrath, 2006).

Recently, macromolecules, such as polysaccharides or glycoproteins in selected fruits, vegetables and herbal plants are screened for their anti-apoptotic and anti-inflammatory activities. A novel immune-stimulating protein with a molecular weight of 313 kDa from *Amaranthus spinosus* (a wild vegetable) water extract was found to directly stimulate proliferation of B lymphocytes *in vitro* (Lin et al., 2005). *Typha latifolia* L. fruit polysaccharides induced differentiation and stimulated the proliferation of human keratinocytes *in vitro* (Gescher and Deters, 2011). Polysaccharides from the leaves, roots and fruits of *Panax ginseng* C.A. Meyer show different biological activities, such as antitumor, immuno-regulatory, anti-adhesive, anti-oxidant, anti-ulcer, anti-radiation, anti-septicaemic, hepatoprotective, anti-asthmatic, anti-depressant, qi-invigorating, anti-fatigue and anti-viral activity (Sun, 2011). These studies suggest future physiological potential of fruit polysaccharides.

Among numerous fruits, strawberry and mulberry fruits were found to have many physiological activities (Lin and Tang, 2007a, 2008a,b; Butt et al., 2008). Recently, number of polysaccharides was isolated from the leaves of *Morus alba* (mulberry) (Tang et al., 2011). However, polysaccharides from mulberry and strawberry fruits on immunomodulation are still scarcely reported. The aim of the study was to determine the anti-inflammatory and anti-apoptotic effects of strawberry and mulberry polysaccharides using primary peritoneal macrophages from female BALB/c mice. Pro-/anti-inflammatory cytokine secretion profiles and intracellular Bcl-2/Bak (anti-/pro-apoptotic) protein levels in the macrophages were determined using enzyme-linked immunosorbent assay (ELISA) and Western blotting protein assay to judge their inflammation and apoptotic status affected by isolated strawberry and mulberry polysaccharides.

2. Material and methods

2.1. Isolation of strawberry and mulberry fruit polysaccharides

Strawberry (*Fragaria ananassa*) and mulberry (*M. alba*) fruits were purchased from a local supermarket in Taichung, Taiwan. The edible portions were weighed, washed and chopped to squeeze fruit juice using a manual stainless screw squeezer (Vegetable & Fruit Grinder, manual type, Mei-Er-Then Co., Ltd., Taipei, Taiwan, ROC). The juice was centrifuged at 10,000*g* (4 °C) for 30 min, and then the supernatant was collected using suction filtration through filter papers (Toyo No. 5B). The filtrate was measured, lyophilized and stored at -30 °C for future use (Lin and Tang, 2008a).

Polysaccharides are ethanol-insoluble compounds. To isolate polysaccharides, an aliquot of 1 gram of the lyophilized fruit juice was added with 12 mL of deionized water to dissolve the juice sample. The juice was added with three volumes of 95% ethyl alcohol to achieve a mixture with a final concentration of 70% ethyl alcohol (Liao et al., 2011). The mixture was allowed to stand with slow stirring at 4 °C for 48 h to precipitate ethanol-insoluble polysaccharides. The resultant mixture was centrifuged at room temperature, 5,500*g* for 20 min to isolate ethanol-insoluble polysaccharides from the supernatant. The insoluble pellets (polysaccharides) were isolated and the solvent removed using nitrogen gas, lyophilized and stored at −30 °C until use. The yields of isolated strawberry fruit polysaccharide (SP) and mulberry fruit polysaccharide (MP) from the lyophilized powder of strawberry and mulberry fruit juice were 14.0 ± 6.3% and 10.6 ± 0.7%, respectively. The protein and carbohydrate contents of the isolated polysaccharide samples were further analyzed. The protein contents of the polysaccharide samples were analyzed using the BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL), according to the manufacturer's proptocol, using a 96-well microtitre plate (Lin and Li, 2010). The carbohydrate content in the SP and MP samples was analyzed using the phenol–sulfuric acid method; the basic protocol of Dubois et al. was followed (Dubois et al., 1956), with the micro-plate format modifications (Cuesta et al., 2003; Masuko et al., 2005). The ratios between carbohydrate and protein contents in SP and MP were 43%:57% and 28.4%:71.6%, respectively, indicating that both SP and MP may be glycoproteins. The UV–Vis absorption spectra of SP and MP were further recorded (Trabelsi et al., 2009).

2.2. Source of mouse primary peritoneal macrophages

Female BALB/cByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC, and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet, Diet MF 18, Oriental Yeast Co., Ltd., Osaka, Japan). The animal room was kept on a 12-h-light and 12-h-dark cycle. Constant temperature (25 ± 2 °C) and relative humidity (50–75%) were maintained. After the mice were acclimatized for 4 weeks, they were sacrificed to obtain peritoneal macrophages. BALB/c strain mice weighting 20 ~ 25 g were used throughout the experiment. The animal use protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC.

The primary peritoneal macrophages from mice were collected according to the method described by Lin et al. (Lin and Tang, 2008a). Briefly, the adult female BALB/c mice were anesthetized with diethyl ether, bled using a retro-orbital venous plexus puncture to collect blood and immediately euthanized by CO_2 inhalation. Peritoneal macrophages were prepared by lavaging the peritoneal cavity with 2 aliquots of 5 mL sterile Hank's balanced salts solution (HBSS) (50 mL of 10× HBSS (Hyclone Laboratories Inc., Logan, UT), 2.5 mL of antibiotic–antimycotic solution (100× PSA) containing 10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per mL in 0.85% saline (Atlanta Biologicals Inc., Norcross, GA), 20 mL of 3% bovine serum albumin (BSA, Sigma–Aldrich Co., St. Louis, MO) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.2 μm filtered), 2.5 mL of 7.5% $NaHCO_3$ (Wako, Osaka, Japan), 425 mL sterile water) for a total of 10 mL through peritoneum. The peritoneal lavage fluid was centrifuged at 400*g* for 10 min at 4 °C. The cell pellets were collected and re-suspended in tissue culture medium (TCM, a serum replacement; Celox Laboratories Inc., Lake Zurich, IL), suspended in a medium consisting of 10 mL TCM, 500 mL Roswell Park Memorial Institute (RPMI) 1640 medium (Atlanta Biologicals Inc., Norcross, GA), and 2.5 mL of antibiotic–antimycotic solution (100× PSA). The peritoneal adherent cells (>90% of macrophages) from each animal were adjusted to 2×10^6 cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method.

2.3. Cultures of mouse peritoneal macrophages with SP and MP

We found that appropriate doses of food components used at mouse primary splenocytes and peritoneal macrophages were quite similar to each other (Lin and Tang, 2007a, 2008a). To avoid excessive cytotoxicity at high doses, appropriate concentrations of SP and MP were used to assess their effects on the cell viability of primary splenocytes. The remaining cell viability showed that SP treatment does not have cytotoxicity on the splenocytes even at 4.0 mg/mL and MP treatment lower than 2.0 mg/mL did not have cytotoxicity on the splenocytes. Lipopolysaccharide (LPS, Sigma–Aldrich Co.) at 5 μg/mL also significantly ($P < 0.05$) increased the cell viability of primary splenocytes (data not shown). To avoid unpredictable cytotoxicities at high doses, appropriate concentrations of SP (0, 250, 500, and 1000 μg/mL) and MP (0, 250, 750, and 1250 μg/mL) in the absence or presence of LPS (5 μg/mL) were used for the following mouse primary splenocytes and macrophages experiments. LPS, an endotoxin, was selected to induce inflammation *in vitro* in this study. The peritoneal macrophages, respectively, in the absence or presence of stimulus LPS (5 μg/mL) and SP (0, 250, 500, and 1000 μg/mL) or MP (0, 250, 750, and 1250 μg/mL) were co-plated in 48-well plates. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for up to 48 h. The plates were then centrifuged at 400*g* for 10 min to obtain the cell culture supernatants and cell pellets. The cell culture supernatants were collected for cytokine assay using ELISA. The cell pellets were collected and stored at −80 °C for intracellular protein level assay using Western blotting assay method.

2.4. Measurement of pro-inflammatory and anti-inflammatory cytokine levels secreted by macrophage cultures using an enzyme-linked immunosorbent assay (ELISA)

The culture supernatants of six biological replicates in each individual treatment were collected to measure cytokine levels using sandwich ELISA kits, respectively. The concentrations of cytokines, including IL-1β, IL-6, IL-12, and TNF-α (pro-inflammatory cytokines), and IL-10 (anti-inflammatory cytokine), were assayed