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Effects of Anthocyanins and Other Phenolic Compounds on the Production of Tumor Necrosis Factor α in LPS/IFN-γ-Activated RAW 264.7 Macrophages

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Flavonoids have been reported to demonstrate their benefits in lowering oxidative stress and beneficial effects on cardiovascular and chronic inflammatory diseases. Common phenolic compounds, including phenolic acids, flavonols, isoflavones, and anthocyanins, present in fruits, vegetables, and grains were investigated for their effects on the production of tumor necrosis factor α (TNF-α) in LPS/IFN-γ-activated RAW 264.7 macrophages. Gallic acid and (+)-catechin showed small but significant effects, whereas chlorogenic acid had no effect on TNF-α production. The flavonol quercetin inhibited TNF-α production, but kaempferol and myricetin induced the secretion of TNF-α. The isoflavone genistein was an inhibitor of TNF-α, whereas daidzein induced TNF-α production. Glycosylation of genistein changed its inhibitory effects to TNF-α induction, and glycosylation of daidzein had no effect on its activity. Anthocyanidins/anthocyanins and anthocyanin-rich extracts induced TNF-α production and acted as modulators of the immune response in activated macrophages. This is the first study to report the effects of anthocyanins and berry extracts on TNF-α production.

KEYWORDS: TNF-α; RAW 264.7 macrophages; flavonols; isoflavones; anthocyanins; anti-inflammation; cytotoxicity; berries; functional foods; nutraceuticals

INTRODUCTION

Tumor necrosis factor α (TNF-α) is one of the most important regulatory cytokines and mediates a variety of cell functions, including stimulation of nitric oxide (NO) production, which has been related to oxidative stress and diseases such as arthritis, diabetes, stroke, and chronic inflammation (*1*, *2*). TNF-α is produced predominantly by macrophages but can also be expressed by a variety of other cells, including T cells, neutrophilis, NK cells, and synovial cells. Human TNF-α is synthesized as a 26 kDa protein that is cleaved by the metalloproteinase TNF-α converting enzyme to its mature 17 kDa secreted form (*3*). It has been shown that exposure of RAW 264.7 macrophages to lipopolysaccharides (LPS) leads to the secretion of large amounts of TNF-α, and exposure of the cells to interferon-γ (IFN-γ) induces smaller amounts of TNF-α in the absence of LPS; together these stimuli are reported to be synergistic (*4*).

Flavonoids including anthocyanins and other phenolic compounds (**Figure 1**) occur in a wide range of plants, with >8000 chemically distinct compounds identified (*5*). The average intake of flavonoids by humans has been estimated to range from 25 mg/day to 1 g/day (*6*, *7*). Several flavonoids have been shown to exhibit antioxidant, anti-inflammatory, anticarcinogenic, and estrogenic activities, to inhibit enzymes, and to prevent coronary heart disease (*6*, *8*−*10*).

The effects of selected flavonoids and flavonoid-rich extracts on TNF-α production in LPS/IFN-γ-activated RAW 264.7 macrophage cells have been reported. Published works have investigated extracts of *Ginkgo biloba* (*11*); quercetin and resveratrol from wine (*12*); extracts rich in procyanidins from pine bark (*13*); and the flavonoids myricetin, quercetin, catechin, hesperetin, genistein, and luteolin (*4*, *14*). These investigations have shown decreased as well as increased basal levels of TNF-α production. The phenolic compounds that have been found to possess TNF-α inhibitory activity are luteolin, genistein, and quercetin. One compound, resveratrol, has been found to stimulate TNF-α secretion in LPS-activated macrophages. The mechanism associated with the inhibitory and stimulatory activities of flavonoids on TNF-α production may result from both transcriptional and post-transcriptional events (*11*, *13*). For example, the suppression of LPS-induced TNF-α by quercetin has been attributed to the inhibition of gene transcription, and both quercetin and EGb 761 can also inhibit the activities of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK1/2), which are important in the post-transcriptional regulation of TNF-α mRNA (*11*).

The objectives of the present study were (1) to determine the effects of 23 commonly occurring phenolic compounds,

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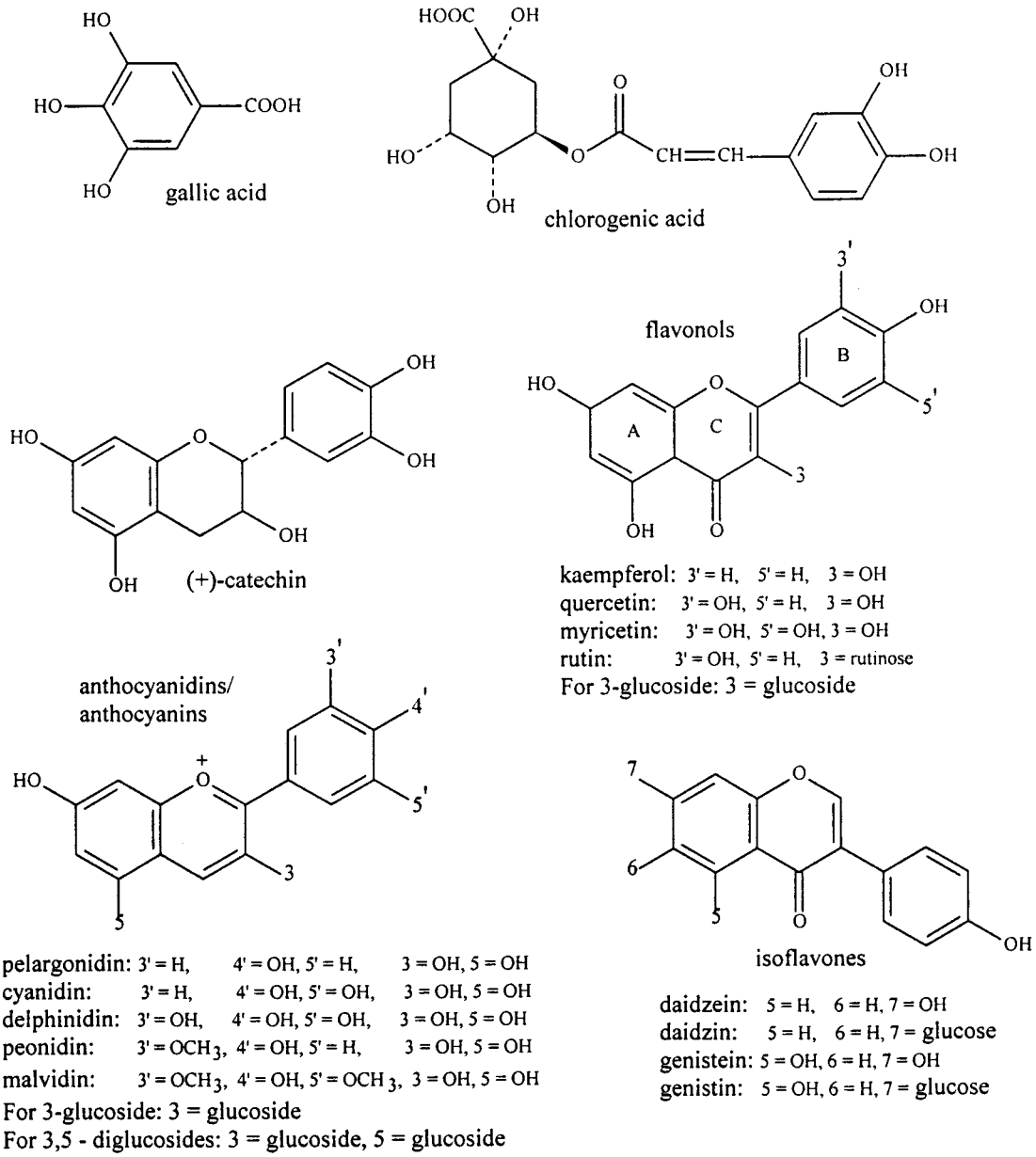

Figure 1. Structures of phenolic compounds.

including 9 anthocyanidins/anthocyanins, 7 flavonols, 4 isoflavones, and anthocyanin-rich crude extracts and their concentrates from four types of berries on TNF-α production in LPS/IFN-γ-activated RAW 264.7 macrophages and (2) to gain new knowledge on the potential uses of anthocyanin-rich extracts as functional foods and nutraceuticals that may reduce oxidative stress and provide protective effects against cardiovascular and chronic inflammatory diseases.

MATERIALS AND METHODS

Materials and Reagents. Recombinant mouse IFN-γ was obtained from BD PharMingen (San Diego, CA). Lipopolysaccharides (LPS; by phenolic extraction from *Salmonella enteritidis*), (+)-catechin, sodium nitrite, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, phosphoric acid, myricetin, dimethyl sulfoxide (DMSO), chlorogenic acid, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) were from Sigma Chemical Co. (St. Louis, MO). Gallic acid and rutin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dulbecco's modified Eagle medium (DMEM), penicillin−streptomycin, glutamine, sodium pyruvate, and fetal bovine serum (FBS) were obtained from Life Technologies (Gaithersburg, MD). Quercetin, quercetin 3-glucoside (isoquercitrin), kaempferol, kaempferol 3-glucoside, kaempferol 3-rutinoside, daidzein, daidzin, genistein, genistin, cyanidin chloride, cyanidin 3-glucoside (kuromanin) chloride, cyanidin 3,5-diglucoside (cyanin) chloride, delphinidin chloride, malvidin chloride, malvidin 3-glucoside (oenin) chloride, malvidin 3,5-diglucoside (malvin) chloride, pelargonidin chloride, and peonidin chloride were from Extrasynthese (Genay, France). Crude extracts and concentrates were prepared from frozen Saskatoon berries (*Amelanchier alnifolia* Nutt.), blueberries, blackberries, and black currants as described previously (*15*).

Cell Culture. The mouse monocyte/macrophage cell line RAW 264.7 [American Type Culture Collection (ATCC), Manassas, VA] was cultured in DMEM (phenol red free) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, streptomycin (100 μg/mL), and penicillin (100 units/mL) (*16*). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell number was assessed by trypan blue dye exclusion on a Neubauer hemacytometer. Cells were grown to 90% confluence in sterile cell culture flasks and gently detached using a scraper (Fisher Scientific, Pittsburgh, PA). For phenolic compound treatment tests, cells were cultured in triplicate in Costar flat-bottom cell culture plates (Corning Inc., Corning, NY). Cells were plated at a density of 6×10^5 cells/well in 24-well cell culture plates and grown for ~1 h to allow

them to attach to the plate. Compounds to be tested were initially dissolved in 10 μL of DMSO, and then DMEM was added to make solutions in a series of concentrations with a dilution factor of 2. The final concentrations of test compound that cells received were 16, 31, 63, 125, 250, and 500 μM, respectively. For berry crude extracts and concentrates, the final concentrations were 16, 31, 63, 125, 250, and 500 μg of product/mL, respectively. Cells were supplemented with the test compounds for 1 h before stimulation with 10 ng/mL LPS and 10−50 units/mL IFN-γ. The activated cells were further incubated for 24 h (*13*, *16*). Supernatants were then collected to determine nitrite concentration as previously described (*15*) and/or stored at −80 °C for a few days prior to TNF-α quantification. Control cells were grown under identical conditions but were not exposed to the test compounds and/or LPS/IFN-γ.

TNF-α Quantification. TNF-α in cell supernatants was determined using an OptEIA Set mouse TNF-α (mono/poly) from BD PharMingen. The enzyme-linked immunosorbent assay (ELISA) was carried out as specified by the manufacture (*17*). Cell supernatants were diluted 121 times before the assay. For the assay, a 96-well plate (Nunc-Immuno plate, Nalge Nunc International, Roskilde, Denmark) was coated using 100 μL per well of capture antibody (1:250 dilution from stock solution in coating buffer/0.1 M sodium carbonate, pH 9.5) and incubated overnight at 4 °C. The wells were then incubated with 200 μL/well of assay diluent [10% (v/v) FBS in a pH 7 phosphate-buffered saline (PBS); PBS was prepared by mixing 8.0 g of NaCl, 1.16 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and 0.2 g of KCl in 1 L of water] at room temperature for 1 h to block nonspecific protein binding sites. After washing with wash buffer (PBS with 0.05% Tween-20), standard recombinant murine TNF-α (in a range of 15.6−1000 pg/mL) or sample dilutions (100 μL) were added to appropriate wells and incubated at room temperature for 2 h. Plates were washed with wash buffer, and then biotinylated anti-mouse TNF-α detection antibody (100 μL) (1:500 dilution from stock solution in assay diluent) was added and incubated at room temperature for 1 h. Enzyme reagent (100 μL) (avidin/horseradish peroxidase conjugate, 1:250 dilution in assay diluent) was then added to wells and incubated at room temperature for 30 min. Wells were washed one more time prior to the addition of a mixture (100 μL) of tetramethylbenzidine (TMB) and hydrogen peroxide (ParMingen's TMB substrate reagent set). After 30 min at room temperature, 50 μL of stop solution (1 M H_3PO_4) was added to stop the reaction. Absorbance was measured at 450 nm within 30 min of stopping reaction on a microplate reader (Molecular Devices, Spectramax Plus 384, Sunnyvale, CA).

Nitric Oxide Determination. NO, measured as nitrite, was determined according to the Griess reaction (*18*). Quantification of nitrite in supernatants containing anthocyanins was conducted as described by Wang and Mazza (*19*).

Cell Viability. Cell viability was determined by using the MTT assay (*20*) and/or the Resazurin-based in vitro toxicology assay kit, TOX-8 (Sigma Chemical Co.). MTT assay was used for compounds having no interference at 570 nm while detecting the converted dye, formazan. The Resazurin-based in vitro toxicology assay kit, or resazurin assay, was used for cells treated with anthocyanins, which interfere with the detection of formazan (*15*). The viability of cells activated only with LPS/IFN-γ was arbitrarily set as 100, and all other viabilities, that is, cells received different treatments but within the same 24-well plate, were normalized to that of the LPS/IFN-γ-activated control cells.

Statistics. Means and standard deviations were calculated using Statistical Analysis System (SAS for Windows V8). Mean comparisons were made with the least significant difference (LSD) test at a 0.05 significant level.

RESULTS AND DISCUSSION

When LPS/IFN-γ was administered to RAW 264.7 macrophages, the NO and TNF-α production increased dramatically. Phenolic compounds, including flavonols quercetin and myricetin, the isoflavone daidzein, and the anthocyanins/anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides, and anthocyanin-rich berry extracts from four types of berries, showed inhibitory effects on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages (*15*). **Figure 2** shows the effects of representative flavonoids and anthocyanin-rich extract, that is, quercetin, genistein, cyanidin, and blueberry concentrate on the production of NO and TNF-α in macrophages and cell viability.

Gallic Acid, Chlorogenic Acid, and (+)-Catechin. Gallic acid may induce TNF-α production in LPS/IFN-γ-activated macrophages in the concentration range of 16−250 μM compared with the control group of LPS/IFN-γ (**Table 1**). The significant decrease in TNF-α production at 500 μM gallic acid reflected the decrease in cell viability; that is, at this treatment level, gallic acid displayed significant cytotoxicity toward the cells (**Table 1**). Chlorogenic acid, although a very strong antioxidant in vitro (*21*), showed no inhibitory effect on RAW 264.7 cells (**Table 1**). (+)-Catechin displayed a small but significant effect on LPS/IFN-γ-activated macrophages (**Table 1**). First, it inhibited TNF-α production at a concentration of 31 μM. Then, at the concentration range of 31−500 μM, it slightly induced TNF-α production, and compared with the LPS/IFN-γ control, it showed no inhibitory effects at 125−500 μM. However, Park et al. (*13*) reported that monomeric flavonoids, such as catechin at a concentration of 344 μM, decreased the secretion of TNF-α in IFN-γ-activated cells. This apparent lack of agreement in the effect of catechin may be attributed to the difference in the activation of macrophages with only IFN-γ by Park et al. (*13*) and with LPS/IFN-γ in our experiments.

Flavonols. The effect of seven flavonols on TNF-α production in LPS/IFN-γ-activated macrophages is shown in **Table 1**. As can be observed, different compounds behaved differently. Kaempferol induced TNF-α production at low concentrations of 16 and 31 μM. Although TNF-α production decreased with increased kaempferol concentration, this effect clearly resulted from its marked cytotoxicity toward cells, which was observed by both the MTT and resazurin assays. Quercetin was able to inhibit TNF-α production in a dose-dependent manner at concentrations of 125, 250, and 500 μM without significant cytotoxic effect (**Table 1** and **Figure 1**), and the inhibitory effect was in good agreement with the literature, although RAW 264.7 macrophages were only activated using LPS in previous studies (*11*, *12*). However, it seems that at low concentrations, 16 and 31 μM, quercetin induced TNF-α production. The suppression of TNF-α by quercetin might result from the inhibition of gene transcriptional and post-transcriptional levels of TNF-α production in RAW 264.7 macrophages (*11*). It is worth noting that the effects of quercetin on both NO and TNF-α production showed the same trend (**Figure 2**). Myricetin did not inhibit the production of TNF-α; instead, it induced its secretion at 250 and 500 μM in LPS/IFN-γ-activated cells. It is not clear that additional hydroxyl groups on the B ring of these flavonols affect their activities on TNF-α production as shown for their antioxidant activities (*21*). However, glycosylation of flavonols significantly decreased their effects on TNF-α production and cell viability (**Table 1**). The decreased effects of glycosylated compounds, that is, glucosides and/or rutinosides of quercetin and/or kaempferol, may be attributed to the increased hydrophilicity and/or steric hindrance by additional sugar moieties, which may decrease the absorption of glycosylated compounds into cells (*22*). In addition, the observed cytotoxicity of flavonol aglycons appears to decrease with increasing hydroxylation of the B ring (**Table 1**).

Isoflavones. The effects of the isoflavones daidzein, genistein, daidzin, and genistin on TNF-α production in LPS/IFN-γ-activated macrophages are presented in **Table 1**. Daidzin and genistin are the glycosylated forms of daidzein and genistein, respectively (**Figure 1**). The effects of isoflavones were dose-dependent. Daidzein and genistein, that is, aglycons, displayed

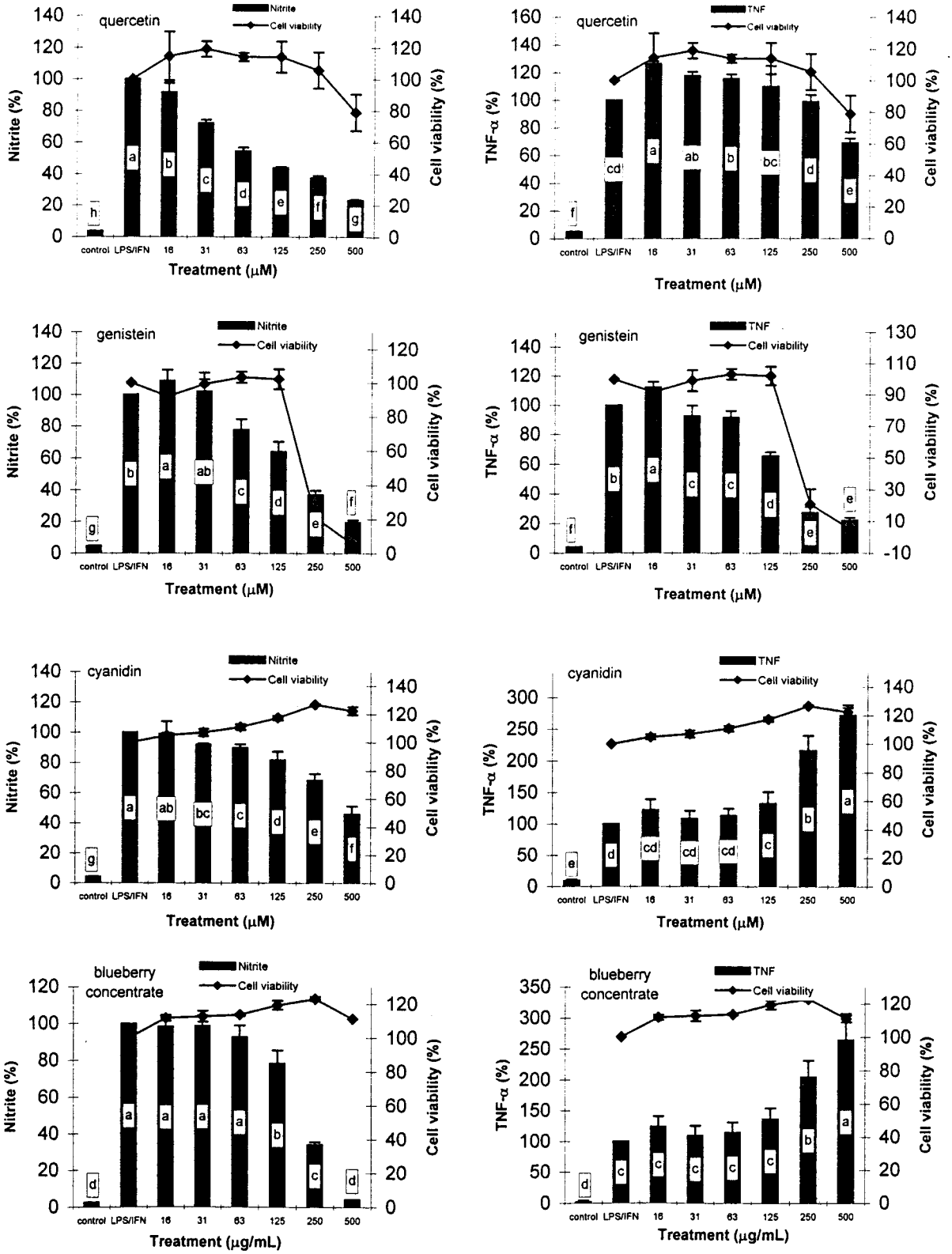

Figure 2. Effects of quercetin, genistein, cyanidin, and blueberry concentrate on NO and TNF-α production in LPS/IFN-γ-activated RAW 264.7 macrophages. Nitrite or TNF-α content of LPS/IFN-γ was arbitrarily set as 100, and all other values were normalized to this LPS/IFN-γ control value, respectively. Each column represents the mean of triplicates. Error bars indicate standard deviations ($n = 3$). Columns with different letters denote the significant difference in nitrite and/or TNF-α levels ($p < 0.05$).

cytotoxicity at high concentrations (250 or 500 μM) (**Table 1**). Daidzein, daidzin, and genistin induced TNF-α production. Genistein demonstrated significant inhibitory effect at 31, 61, and 125 μM without significant cytotoxic effects (**Figure 2** and **Table 1**). Glycosylation of isoflavones, unlike flavonols, induced TNF-α production activities in macrophages (**Table 1**). The inhibitory effect of genistein was in good agreement with previous results (*4*). Like quercetin, genistein showed the same effects on both NO and TNF-α production in LPS/IFN-γ-activated macrophages (**Figure 2**).

Table 1. Effects of Phenolic Acids, Catechins, Flavonols, and Isoflavones on TNF-α Production in LPS/IFN-γ-Activated RAW 264.7 Macrophages

concn (μM)	compound	TNF-α[a] (%)	cell viability[b] (%)	compound	TNF-α (%)	cell viability (%)	compound	TNF-α[a] (%)	cell viability[b] (%)
control[c]	gallic	4.9f (1.1)		chlorogenic	4.9d (1.1)		(+)-catechin	3.8e (1.0)	
LPS/IFN-γ[d]	acid	100.0d (0.0)	100 (0)	acid	100.0c (0.0)	100 (0)		100.0ab (0.0)	100 (0)
16[e]		122.0abc (4.7)	110 (11)		123.3a (8.9)	117 (10)		100.7ab (6.5)	98 (10)
31		112.1c (8.8)	108 (12)		104.0bc (9.3)	114 (16)		89.3d (1.2)	107 (12)
63		117.4bc (6.6)	108 (16)		104.8bc (10.2)	111 (6)		91.8cd (1.4)	118 (5)
125		123.8ab (12.9)	97 (4)		109.4abc (10.5)	113 (15)		96.8abc (3.4)	102 (5)
250		130.6a (4.5)	108 (15)		107.4bc (8.9	117 (13)		94.8bcd (3.5)	108 (10)
500		48.0e (5.0)	20 (1)		115.5ab (7.6)	100 (6)		101.5a (6.4)	126 (11)
control	kaempferol	3.5f (2.0)		kaempferol	3.5e (2.0)		kaempferol	5.6d (1.4)	
LPS/IFN-γ		100.0d (0.0)	100 (0)	3-glucoside	100.0b (0.0)	100 (0)	3-rutinoside	100.0b (0.0)	100 (0)
16		221.8a (2.3)	117 (11)		110.1a (2.5)	111 (16)		108.7a (5.4)	114 (4)
31		165.0b (0.9)	89 (9)		84.2d (0.8)	108 (20)		91.0c (4.1)	112 (13)
63		120.3c (5.5)	53 (15)		84.0d (4.4)	107 (12)		88.4c (8.3)	108 (2)
125		103.9d (1.9)	27 (4)		86.0cd (4.4)	98 (9)		92.5cb (3.6)	109 (11)
250		107.0d (9.3)	19 (5)		91.2c (4.1)	95 (10)		96.2cb (3.7)	111 (11)
500		85.2e (5.5)	18 (7)		86.5cd (4.8)	101 (16)		92.0cb (6.4)	85 (10)
control	quercetin	5.0f (0.3)		quercetin	6.9d (3.2)		quercetin	6.9c (3.2)	
LPS/IFN-γ		100.0cd (0.0)	100 (0)	3-glucoside	100.0bc (0.0)	100 (0)	3-rutinoside	100.0ab (0.0)	100 (0)
16		126.3a (2.0)	114 (15)		111.0a (4.8)	117 (6)		103.4a (12.4)	107 (8)
31		117.8ab (2.9)	119 (5)		94.4c (3.1)	111 (13)		91.1ab (10.3)	112 (7)
63		115.7b (3.1)	114 (3)		105.1ab (13.0)	113 (10)		94.4ab (11.3)	99 (11)
125		110.0bc (14.9)	114 (10)		98.7bc (5.0)	113 (8)		83.6b (7.7)	109 (8)
250		98.9d (5.0)	106 (11)		96.8bc (2.9)	111 (11)		89.9ab (12.1)	100 (6)
500		69.3e (3.4)	79 (12)		104.5abc (5.5)	111 (8)		92.8ab (10.9)	89 (14)
control	daidzein	2.0f (1.3)		daidzin	2.0e (1.3)		myricetin	6.7f (0.9)	
LPS/IFN-γ		100.0e (0.0)	100 (0)		100.0c (0.0)	100 (0)		100.0e (0.0)	100 (0)
16		111.3cd (8.0)	98 (11)		93.8cd (7.9)	113 (10)		118.6bcd (5.9)	107 (8)
31		103.4de (6.5)	108 (2)		84.9d (2.8)	104 (4)		115.0cd (7.6)	104 (11)
63		114.5c (7.1)	114 (1)		89.8cd (2.5)	108 (17)		110.0de (2.5)	100 (10)
125		133.4b (8.4)	107 (3)		130.8b (8.6)	101 (8)		129.3bc (7.1)	102 (13)
250		149.8a (8.0)	76 (6)		161.8a (9.3)	52 (4)		152.2a (6.8)	118 (9)
500		131.0b (1.8)	33 (7)		162.8a (15.7)	62 (7)		132.8b (19.5)	74 (9)
control	genistein	4.1f (0.0)		genistin	4.1e (0.0)				
LPS/IFN-γ		100.0b (0.0)	100 (0)		100.0d (0.0)	100 (0)			
16		112.4a (3.7)	92 (0)		106.0d (11.7)	103 (21)			
31		92.7c (7.1)	99 (7)		101.7d (4.5)	88 (12)			
63		91.7c (4.4)	103 (3)		111.7d (12.4)	108 (6)			
125		65.6d (2.6)	102 (6)		134.2c (12.7)	116 (16)			
250		27.3e (3.9)	21 (10)		188.5b (13.6)	130 (6)			
500		22.2e (2.0)	5 (1)		224.8a (13.8)	142 (8)			

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). Data for individual compounds with different letters denote significant difference in TNF-α (%) ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. TNF-α content of LPS/IFN-γ was arbitrarily set as 100, and all other values were normalized to this LPS/IFN-γ control value. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

Anthocyanins. Anthocyanins are a group of bioactive flavonoids that have shown significant inhibitory effect on NO production in LPS/IFN-γ-activated macrophages (*15*). The effects of anthocyanins on TNF-α production are shown in **Table 2**. Unlike their significant inhibitory effects on NO production, all anthocyanins tested induced TNF-α production in a dose-dependent manner as can be seen for cyanidin in **Figure 2**. At concentrations of 250 and 500 μM, most anthocyanidins/anthocyanins significantly induced TNF-α production; cells that were treated with cyanidin, pelargonidin, delphinidin, or peonidin produced ⩾2 times the concentration of TNF-α compared to the LPS/IFN-γ control cells. The reduced cell viability in treatments with malvidin and delphinidin at 500 μM might account for the decreased TNF-α in cell supernatants. Glycosylation of cyanidin and malvidin reduced the activities of their aglycons, but they still induced TNF-α production (**Table 2**).

Berry Extracts and Concentrates. The berry crude extracts, from 80% ethanol extraction of berries, contained 2.6−7.6% total phenolic compounds and 0.9−1.8% anthocyanins. When the crude extracts of berries were further purified, their relative phenolic content increased severalfold. Concentrates contained 46.6−85.1% total phenolic compounds and 17.4−32.7% anthocyanins. The effects on TNF-α production by berry crude extracts and their concentrates are shown in **Figure 2** and **Table 3**. The crude extracts exhibited little effect on TNF-α production, likely because of their low concentration of phenolics. At high concentration, 500 μg/mL, crude extracts of blackberries and black currants moderately induced TNF-α production. Crude extracts of Saskatoon berries and blueberries, however, had no effect on TNF-α production (**Table 3**).

The concentrates of these berries demonstrated considerable effects on TNF-α induction compared to the crude extracts (**Table 3**). Previous studies clearly indicated that inhibitory effects of phenolic compounds on NO production in LPS/IFN-γ-activated macrophages were significantly correlated with the content of individual categories of phenolic compounds present in berry crude extracts and concentrates (*15*). However, there was no quantitative correlation between phenolic content and their inducible effects on TNF-α production, yet the TNF-α induction of berry anthocyanin-rich extracts in activated macrophages was observed at high-concentration treatments, for example, at 250 or 500 μg/mL.

It has been reported that some phenolic compounds may

Table 2. Effects of Anthocyanins on TNF-α Production in LPS/IFN-γ-Activated RAW 264.7 Macrophages

concn (μM)	compound	TNF-α[a] (%)	cell viability[b] (%)	compound	TNF-α (%)	cell viability (%)	compound	TNF-α[a] (%)	cell viability[b] (%)
control[c]	cyanidin	9.2e (2.6)		cyanidin	5.0f (1.4)		cyanidin	4.0f (0.2)	
LPS/IFN-γ[d]		100.0d (0.0)	100 (0)	3-glucoside	100.0e (0.0)	100 (0)	3,5-diglucosides	100.0bc (0.0)	100 (0)
16[e]		122.0cd (17.0)	105 (2)		116.4d (3.8)	111 (1)		106.8a (1.3)	112 (1)
31		108.4cd (12.6)	107 (2)		116.5d (7.2)	110 (4)		94.9d (2.9)	107 (1)
63		113.2cd (11.4)	111 (2)		109.7d (7.1)	111 (2)		101.9b (0.9)	108 (1)
125		132.7c (18.6)	117 (2)		125.9c (5.1)	107 (2)		96.5cd (3.2)	104 (1)
250		216.2b (23.9)	127 (1)		135.9b (2.7)	112 (2)		95.6d (1.9)	109 (2)
500		272.3a (16.6)	122 (3)		167.7a (7.9)	113 (2)		90.0e (4.2)	110 (3)
control	malvidin	6.0e (0.4)		malvidin	6.0c (0.4)		malvidin	5.8d (0.6)	
LPS/IFN-γ		100.0d (0.0)	100 (0)	3-glucoside	100.0b (0.0)	100 (0)	3,5-diglucosides	100.0bc (0.0)	100 (0)
16		104.3d (1.8)	114 (2)		97.6b (11.6)	117 (3)		102.8bc (16.7)	107 (2)
31		107.7cd (4.5)	115 (2)		95.6b (7.7)	110 (2)		100.1bc (5.8)	105 (2)
63		104.0d (3.2)	117 (0)		89.9b (7.4)	112 (3)		99.8bc (11.4)	102 (3)
125		124.3b (5.6)	117 (1)		90.7b (6.4)	112 (2)		90.3c (2.3)	103 (2)
250		168.0a (6.2)	116 (1)		98.6b (3.8)	118 (0)		112.8b (4.9)	107 (1)
500		115.0c (9.2)	71 (2)		157.4a (16.5)	95 (8)		154.3a (6.3)	93 (3)
control	pelargonidin	7.3d (1.2)		delphinidin	5.0g (3.5)		peonidin	8.9d (2.4)	
LPS/IFN-γ		100.0c (0.0)	100 (0)		100.0f (0.0)	100 (0)		100.0c (0.0)	100 (0)
16		107.6c (10.1)	100 (2)		150.8de (18.3)	106 (1)		102.5c (15.7)	106 (3)
31		101.2c (12.3)	99 (3)		118.2ef (7.1)	108 (1)		92.8c (13.9)	107 (2)
63		109.2c (12.2)	96 (4)		168.6cd (21.3)	114 (1)		97.3c (16.9)	103 (1)
125		142.3b (25.1)	97 (2)		328.2b (22.0)	113 (0)		106.1c (17.5)	108 (2)
250		148.6b (21.6)	97 (6)		463.4a (35.5)	111 (4)		156.3b (10.1)	115 (0)
500		247.7a (12.0)	111 (5)		187.3c (21.5)	67 (2)		254.3a (37.3)	113 (1)

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). Data for individual compounds with different letters denote significant difference in TNF-α (%) ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. TNF-α content of LPS/IFN-γ was arbitrarily set as 100, and all other values were normalized to this LPS/IFN-γ control value. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

Table 3. Effects of Berry Crude Extracts and Concentrates on TNF-α Production in LPS/IFN-γ-Activated RAW 264.7 Macrophages

	crude extracts		concentrates		crude extracts		concentrates	
concn (mg/μL)	TNF-α[a] (%)	cell viability[b] (%)	TNF-α (%)	cell viability (%)	TNF-α (%)	cell viability (%)	TNF-α (%)	cell viability (%)
	Saskatoon Berry				Blackberry			
control[c]	13.9f (0.9)		1.8d (2.4)		8.1f (0.9)		0.2f (0.0)	
LPS/IFN-γ[d]	100.0e (0.0)	100 (0)	100.0c (0.0)	100 (0)	100.0e (0.0)	100 (0)	100.0de (0.0)	100 (0)
16[e]	105.3d (4.3)	105 (1)	119.3b (8.1)	112 (1)	122.8b (8.0)	104 (1)	114.8b (5.2)	114 (6)
31	109.9bc (2.4)	104 (2)	104.2bc (8.6)	111 (2)	105.0de (2.3)	105 (2)	92.5e (6.4)	111 (3)
63	113.7ab (1.8)	104 (1)	104.3bc (9.0)	117 (0)	105.8de (1.3)	105 (1)	107.0cd (4.6)	111 (1)
125	114.5a (3.4)	106 (2)	115.7bc (12.5)	123 (1)	107.4d (3.6)	108 (1)	111.3bc (5.1)	122 (2)
250	110.1bc (1.0)	108 (2)	115.4bc (2.3)	127 (1)	116.2c (1.4)	111 (1)	118.8b (5.7)	129 (1)
500	107.5cd (1.1)	112 (2)	137.4a (18.9)	128 (0)	139.9a (4.1)	113 (1)	136.8a (3.7)	133 (0)
	Blueberry				Black Currant			
control	13.9d (0.9)		1.8d (2.4)		8.1d (0.9)		4.5f (1.2)	
LPS/IFN-γ	100.0bc (0.0)	100 (0)	100.0c (0.0)	100 (0)	100.0c(0.0)	100 (0)	100.0e (0.0)	100 (0)
16	122.1a (14.5)	101 (2)	124.4c (16.6)	112 (2)	116.4ab (18.9)	101 (1)	118.0b (6.0)	103 (5)
31	107.1bc (13.7)	99 (3)	109.7c (16.1)	113 (3)	103.4bc (7.6)	100 (2)	104.8de (5.0)	104 (2)
63	95.0c (4.8)	101 (4)	114.6c (17.1)	114 (1)	98.6c (10.7)	103 (1)	110.0bcd (3.7)	113 (5)
125	109.3ab (1.7)	100 (2)	136.9c (17.8)	119 (3)	100.0c (5.9)	106 (1)	106.9cde (5.9)	113 (1)
250	100.7bc (2.7)	103 (3)	205.0b (27.1)	123 (1)	105.5bc (9.9)	107 (3)	114.2bc (5.1)	123 (2)
500	96.4bc (10.1)	109 (3)	264.9a (42.8)	111 (2)	125.4a (1.9)	113 (3)	131.7a (8.3)	132 (1)

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). Data for individual compounds with different letters denote significant difference in TNF-α (%) ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. TNF-α content of LPS/IFN-γ was arbitrarily set as 100, and all other values were normalized to this LPS/IFN-γ control value. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

induce TNF-α production. Park et al. (*13*) reported that procyanidin C2 and pycnogenol (pine bark extract) substantially elevated TNF-α release and NO production in both inactivated and activated macrophages by IFN-γ compared to control cells. The red wine polyphenol resveratrol post-transcriptionally decreased NO production but significantly increased basal TNF-α mRNA expression with a concomitant increase in TNF-α secretion in LPS-activated RAW 264.7 macrophages (*12*). Resveratrol is a well-known anticancer compound (*23*); it may be possible that increased production of TNF-α in LPS and/or IFN-γ-activated macrophages treated with resveratrol is linked to the resveratrol cancer chemopreventive activity, because it has been reported that the TNF-α from activated macrophages can lead to cytostatic and cytotoxic activities on malignant cells (*24*), and resveratrol-mediated release of TNF-α may contribute to its antitumorigenic properties (*12*). However, TNF-α production or release has deleterious effects upon the organism and, therefore, its synthesis must be tightly governed (*25*). Although

the production of TNF-α is crucial for the synergistic induction of NO synthesis in LPS- and/or IFN-γ-activated macrophages (*13*), and anthocyanins or anthocyanin-rich extracts induced TNF-α production, our findings show that anthocyanins and anthocyanin-rich extracts can significantly reduce NO production so as to reduce the oxidative stress in anti-inflammatory process. Many studies have shown that a diet rich in fruits and vegetables is associated with reduced risk of some types of cancers (*26*). Thus, it is conceivable that the observed activities of anthocyanins to reduce the production of NO and to increase the production of TNF-α may indicate that these compounds also have anticancer properties similar to those of resveratrol.

In general, gallic acid, chlorogenic acid, and (+)-catechin showed few effects on TNF-α production in LPS/IFN-γ-activated RAW 264.7 macrophages. The flavonol quercetin inhibited TNF-α production, but kaempferol and myricetin induced the secretion of TNF-α, and glycosylation of flavonols decreased their potent effects; the isoflavone genistein was an inhibitor of TNF-α, but daidzein induced TNF-α production. Glycosylation of genistein changed the inhibitory effect to induction of TNF-α, whereas glycosylation of daidzein had no effect on its activity. All anthocyanidins/anthocyanins examined and anthocyanin-rich extracts induced TNF-α production in LPS/IFN-γ-activated RAW 264.7 macrophages. The underlying mechanisms by which different flavonoids affect TNF-α secretion and/or NO production have not yet been clarified.

Although flavonols quercetin and myricetin; the isoflavone daidzein; and the anthocyanins/anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides showed significant inhibitory effects on NO production in LPS/IFN-γ-activated macrophages, their effects on TNF-α production and/or induction reflected a view that NO and TNF-α production by murine macrophages are regulated differently. This research provides further evidence that phenolic compounds are able to reduce the oxidative stress from NO, which increases the protective effects against cardiovascular and chronic inflammatory diseases, and anthocyanins and anthocyanin-rich fruit extracts can act as modulators of the immune response in macrophages.

LITERATURE CITED

(1) Moncada, S.; Palmer, R. M.; Higgs, E. A. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109−142.

(2) Akaike, T.; Fujii, S.; Kato, A.; Yoshitake, J.; Miyamoto, Y.; Sawa, T.; Okamoto, S.; Suga, M.; Asakawa, M.; Nagai, Y.; Maeda, H. Viral mutation accelerated by nitric oxide production during infection in vivo. *FASEB J.* **2000**, *14*, 1447−1454.

(3) Standiford, T. Anti-inflammatory cytokines and cytokine antagonists. *Curr. Pharm. Design* **2000**, *6*, 633−649.

(4) Orlicek, S. L.; Meals, E.; English, B. K. Differential effects of tyrosine kinase inhibitors on tumor necrosis factor and nitric oxide production by murine macrophages. *J. Infect. Dis.* **1996**, *174*, 638−642.

(5) Pietta, P. G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035−1042.

(6) Hollman, P. C. H.; Katan, M. B. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem. Toxicol.* **1999**, *37*, 937−942.

(7) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S−2085S.

(8) Peterson, J.; Dwyer, J. Flavonoids: Dietary occurrence and biochemical activity. *Nutr. Res.* **1998**, *18*, 1995−2018.

(9) Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401−436.

(10) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.

(11) Wadsworth, T. L.; McDonald, T. L.; Koop, D. R. Effects of *Ginkgo biloba* extract (EGb 761) and quercetin on lipopolysaccharide-induced signaling pathways involved in the release of tumor necrosis factor-α. *Biochem. Pharmacol.* **2001**, *62*, 963−974.

(12) Wadsworth, T.; Koop, D. R. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem. Pharmacol.* **1999**, *57*, 941−949.

(13) Park, Y. C.; Rimbach, G.; Saliou, C.; Valacchi, G.; Packer, L. Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-α secretion, and NF-κB-dependent gene expression in RAW 264.7 macrophages. *FEBS Lett.* **2000**, *464*, 93−97.

(14) Xagorari, A.; Papapetropoulos, A.; Mauromatis, A.; Economou, M.; Fotsis, T.; Roussos, C. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 181−187.

(15) Wang, J.; Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-γ-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 850−857.

(16) Brouet, I.; Ohshima, H. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 533−540.

(17) BD PharMingen. Instruction/analysis certificate. OptEIA set: mouse TNF-α (mono/poly). Catalog no. 558874. 10975 Torreyana Rd., San Diego, CA, 2001; 3 pp.

(18) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [^{15}N]nitriate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131−138.

(19) Wang, J.; Mazza, G. Quantification of nitrite in the presence of anthocyanins using Griess and GC/MS assays. *J. Food Biochem.* **2002**, in press.

(20) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.

(21) Fukumoto, L. R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 3597−3604.

(22) Kim, H. K.; Cheon, B. S.; Kim, Y. H.; Kim, S. Y.; Kim, H. P. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure−activity relationships. *Biochem. Pharmacol.* **1999**, *58*, 759−765.

(23) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.

(24) Camussi, G.; Albano, E., Tetta, C.; Bussolino, F. The molecular action of tumor necrosis factor-α. *Eur. J. Biochem.* **1991**, *202*, 3−14.

(25) Beutler, B.; Cerami, A. The biology of cachectin/TNF-α primary mediator of the host response. *Annu. Rev. Immunol.* **1989**, *78*, 919−925.

(26) Steinmetz, K. A.; Potter, J. D. Vegetable, fruit, and cancer prevention: A review. *J. Am. Diet. Assoc.* **1996**, *96*, 1027−1039.

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