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ARTICLES

Inhibition of Nitric Oxide Synthase Inhibitors and Lipopolysaccharide Induced Inducible NOS and Cyclooxygenase-2 Gene Expressions by Rutin, Quercetin, and Quercetin Pentaacetate in RAW 264.7 Macrophages

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Abstract Several natural flavonoids have been demonstrated to perform some beneficial biological activities, however, higher-effective concentrations and poor-absorptive efficacy in body of flavonoids blocked their practical applications. In the present study, we provided evidences to demonstrate that flavonoids rutin, quercetin, and its acetylated product quercetin pentaacetate were able to be used with nitric oxide synthase (NOS) inhibitors (*N*-nitro-L-arginine (NLA) or *N*-nitro-L-arginine methyl ester (L-NAME)) in treatment of lipopolysaccharide (LPS) induced nitric oxide (NO) and prostaglandin E2 (PGE2) productions, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expressions in a mouse macrophage cell line (RAW 264.7). The results showed that rutin, quercetin, and quercetin pentaacetate-inhibited LPS-induced NO production in a concentration-dependent manner without obvious cytotoxic effect on cells by MTT assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide as an indicator. Decrease of NO production by flavonoids was consistent with the inhibition on LPS-induced iNOS gene expression by western blotting. However, these compounds were unable to block iNOS enzyme activity by direct and indirect measurement on iNOS enzyme activity. Quercetin pentaacetate showed the obvious inhibition on LPS-induced PGE2 production and COX-2 gene expression and the inhibition was not result of suppression on COX-2 enzyme activity. Previous study demonstrated that decrease of NO production by L-arginine analogs effectively stimulated LPS-induced iNOS gene expression, and proposed that stimulatory effects on iNOS protein by NOS inhibitors might be harmful in treating sepsis. In this study, NLA or L-NAME treatment stimulated significantly on LPS-induced iNOS (but not COX-2) protein in RAW 264.7 cells which was inhibited by these three compounds. Quercetin pentaacetate, but not quercetin and rutin, showed the strong inhibitory activity on PGE2 production and COX-2 protein expression in NLA/LPS or L-NAME/LPS co-treated RAW 264.7 cells. These results indicated that combinatorial treatment of L-arginine analogs and flavonoid derivates, such as quercetin pentaacetate, effectively inhibited LPS-induced NO and PGE2 productions, at the same time, inhibited enhanced expressions of iNOS and COX-2 genes. J. Cell. Biochem. 82: 537–548, 2001.

Key words: quercetin; rutin; quercetin pentaacetate; lipopolysaccharide; inducible nitric oxide synthase; cyclooxygenase 2

The pivotal role of nitric oxide (NO) as a messenger and effector molecule in a variety of tissues has been demonstrated in recent years [Palmer et al., 1988; Lowenstein et al., 1996]. NO has been identified as a neurotransmitter in central nervous system and a potent vasorelaxant in physiologically regulating the blood pressure through modulating muscular tone

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[Hibbs et al., 1987; Moncada et al., 1992]. NO also have been defined as an important molecule in inflammation and sepsis [Wheeler and Bernard, 1999]. Exposure to outer bacterial toxins such as lipopolysaccharide (LPS) or lipoteicholic acid (LTA) stimulated cellular inflammatory responses, and released some factors including NO, prostaglandin E2, cytokines, tumor necrosis factor-α and eicosanoid mediators to promote inflammatory responses. At least three types of nitric oxide synthase (NOS) isoforms have been identified in cells. Both endothelium nitric oxide synthase (eNOS) and neural nitric oxide synthase (nNOS) belong to constitutive NOS (cNOS), and NO was produced by cNOS as a factor in maintaining the normal vasoactivity in an active state of vasodilation through a Ca^{+2}-dependent pathway and as a neurotransmitter in neuron signal transmission. NOS in macrophages and hepatocytes is inducible (iNOS) and its activation is Ca^{+2} independent. After exposure to endogenous and exogenous stimulators, iNOS can be induced quantitatively in various cells such as macrophages, smooth muscle cells, and hepatocytes to trigger several disadvantage cellular responses and cause some diseases including inflammation, sepsis, and stroke [Marletta et al., 1988; Nathan, 1992; Marletta, 1993; Duval et al., 1996]. Therefore, NO production induced by iNOS may reflect the degree of inflammation and provide a measure to assess the effect of drugs on the inflammatory process.

Prostaglandin E2, as NO, is a pleiotropic mediator produced at inflammatory sites by inducible cyclooxygenase 2, an enzyme in the conversion of arachidonic acid to prostaglandin H2, the precursor of a wide group of biological active mediators such as PGE2, prostacyclin, and thromboxane A2 [Fu et al., 1990; Picot et al., 1994]. Cyclooxygenase 1 (COX-1), an isoform of COX-2, constitutively expressed in cells to perform several beneficial preventive effects including mucous and bicarbonate secretion. In contrast to COX-1, COX-2 is inducible and gives rise to pain, swelling, and stiffness [Vane et al., 1994; Smith et al., 1996]. Elevation of COX-2 protein has been demonstrated to be associated with the occurrence of some human diseases such as colon carcinoma, and COX-2 inhibitors effectively attenuated the symptoms of inflammation and reduced cancer occurring rate [Levy, 1997; Hartner et al., 1998; Wong et al., 1998]. Therefore, there is a great interest in studying the effects and usefulness of drugs on COX-2.

Flavonoids have been identified as either simple or complex glycosides in many plants [Kuhnau, 1976], and humans have been estimated to consume $\sim$1 g flavonoids/day. The most important groups of flavonoids are flavones, flavonols, flavanones, anthocyanins, and catechins. Several previous studies have demonstrated that flavonoids exhibit a wide variety of biological activities including antioxidant and free radical scavenging activities [Hodnick et al., 1990; Mora et al., 1990; Chen et al., 2000]. Quercetin is one of the most popular flavonoids in the diet and showed a wide range of biological activities including inhibition of Na+/K+ ATPase, protein kinase C, tyrosine kinase, etc [Kang and Liang, 1997; Kobuchi et al., 1999]. In the gastrointestinal tract, Quercetin was derived from rutin through hydrolization by the glucosidase activity [Manach et al., 1997]. Although, flavonoids have been studied extensively, application of flavonoids in treating diseases in vivo are still not common because of their higher effective concentration and poor absorptive activity in body. L-arginine analogs such as NLA or L-NAME showed effective inhibitory activities on LPS or IFN-γ induced nitric oxide production through directly blocking NOS enzyme activity. However, in vivo study it appeared that L-arginine analogs can not effectively inhibit LPS induced septic death in rat and Peng et al. [1998] reported that treatment of L-arginine analogs L-NAME stimulated LPS/mIFN-γ-induced iNOS expression. Our idea in this study is to demonstrate the combinatorial effects of flavonoids and NOS inhibitors on LPS induced NO and PGE2 productions. The results indicated three structure-related compounds rutin, quercetin, and the acetylated derivate quercetin pentaacetate showed the inhibitory effects on LPS-induced NO and PGE2 productions. Quercetin pentaacetate appeared to inhibit LPS-induced PGE2 production and COX-2 gene expression. Combination of indicated flavonoids and NOS inhibitors in treatment of LPS-induced NO and PGE2 production was performed in this study.

MATERIALS AND METHODS

Cells

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture

Collection. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco-BRL) and maintained at 37°C in a humidified incubator containing 5% CO_2.

Agents

Three structurally related flavonoids rutin, quercetin, and quercetin pentaacetate were used in the present study. Both rutin and quercetin were isolated from Chinese herbal plant *Sophorae Flos Immaturus* by the extraction and purification methods as described previously [Lozoya et al., 1994] and quercetin pentaacetate was derived from acetylation of quercetin. Purity test of rutin, quercetin, and quercetin pentaacetate were performed by high-performance liquid chromatography equipped with a 280 nm detector and LiChrospher 100 RP-18e column (4 mm i.d., ×125 mm). Purity of all compounds was more than 95%. Sulfanilamine, naphthylethylenediamine dihydrochloride, *N*-nitro-L-arginine, *N*-nitro-L-arginine methyl ester, indomethacin, lipopolysaccharide were obtained from Sigma (Sigma Chemical Co.).

Nitrite Assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [Kim et al., 1995]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrocholide in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories).

Measurement of iNOS Enzyme Activity

For the assay in intact cells, RAW 264.7 cells were plated in 100 mm tissue culture dishes (4×10^6 cells) and incubated with LPS (100 ng/mL) for 12 h. The cells were washed twice with PBS. Cells were harvested and plated into a 24 well plate (2×10^5 cells/well) and incubated in the absence or presence of tested compounds for further 12 h without LPS in medium. The supernatants were removed, and the Griess reaction was performed as above. For the assay in cell lysates, RAW 264.7 cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500*g* for 10 min at 4°C. The cell pellet was resuspended in 0.5 mL 40 mM Tris–buffer (pH 8.0) containing 5 mg/mL pepstain A, 1 μg/mL chymostain, 5 μg/mL aprotinin, and 100 μM phenylmethylsulfonyl fluoride, and lysed by three freeze-thaw cycles. Aliquots of the lysate were used for Bradford protein assay. INOS enzyme activity was measured as described [Vodovotz et al., 1993]. Briefly, 50 mg of cell lysate protein were incubated in 20 mM Tris–HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-arginine and NADPH. The reaction was carried out in duplicate for 180 min at 37°C in 96-well plates. Residual NADPH was oxidized enzymatically as described previously, and the Griess reaction was performed as above.

Cell Viability Assay

RAW 264.7 macrophages were plated at a density of 10^4 cells/well into 96-well plates. After overnight growth, cells were treated with a different concentration of flavonoids described above for 24 h. At the end of treatment, 20 ml of combined solution of a tetrazolium compound MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyMethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium; inner salt) and an electron coupling reagent, phenazine methosulfate, were added to each well. After incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere, absorbance at wavelength 600 was recorded using an ELISA plate reader [Chen et al., 1999a].

Western Blots

Total cellular extracts were prepared according to our previous papers [Chen et al., 1999b] separated on 8% SDS–polyacrylamide minigels, and transferred to Immobilon polyvinylidenedifluoride membranes (Millipore). The membrane was incubated overnight at 4°C with 1% bovine serum albumin and then incubated with anti-iNOS, anti-COX-2 or anti-α tubulin monoclonal antibodies (Transduction Laboratories). The expression of protein was detected by NBT and BCIP staining (Sigma Chemical Co.)

Measurement of COX-2 Enzyme Activity

RAW 264.7 cells were plated at 2×10^5 cells/well in a 24 well plate and incubated for 6 h with LPS. The cell supernatants were removed and cells in each well were washed twice with fresh

culture medium and allowed to equilibrate in the absence or presence of tested compounds for 30 min. The cells were further incubated with 100 μM arachidonic acid for 15 min without LPS in medium. The supernatants were removed and assayed for PGE2.

Measurement of PGE2 Production

RAW 264.7 cells were subcultured in six-well plates and were incubated with indicated compounds for 12 h. One hundred microliters of supernatant of culture medium was collected for the determination of PGE2 concentration by ELISA (Cayman Enzyme Immunoassay kit)

Statistics

The values are expressed as means±SE. The significance of difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. P value <0.05 was regarded as indicating significant differences.

RESULTS

Rutin, Quercetin, and Quercetin Pentaacetate Inhibit LPS-Induced NO Production in RAW 264.7 Macrophages

The chemical structures of rutin, quercetin, and quercetin pentaacetate are shown in Figure 1. These three compounds are flavonoids, and rutin and quercetin were the major components of Chinese herbs *Sophorae Flos Immaturus* and have been shown to perform several biological functions. Quercetin pentaacetate is derived from acetylation of quercetin and replace the OH groups in 3,5,7,3′,4′ sites with acetyl groups and the purity of quercetin pentaacetate is more than 95% analyzed by HPLC. In the present study, effects of rutin, quercetin, and quercetin pentaacetate on LPS-induced NO production in RAW 264.7 macrophages were investigated. Nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO synthesis from the cells. Each of compounds, at concentration of 40 μM, did not interfere with the reaction between nitrite and Griess reagents (data not shown). After 24 h incubation, unstimulated macrophages produced background level of nitrite in medium about 5 μM in culture medium (Fig. 2). When the cells incubated with rutin, quercetin, and quercetin pentaacetate alone, the amount of nitrite in medium was maintained at a background level similar to that in the unstimulated samples (data not shown). After treatment with LPS (100 ng/mL) for 24 h, nitrite concentration in medium increased remarkable, about 10-fold (~30 μM). When RAW 264.7 macrophages were treated with different concentration of indicated compounds together with LPS (100 ng/mL) for 24 h, significant concentration dependent inhibition of nitrite production was detected in the presence of rutin, quercetin, and quercetin pentaacetate (Fig. 2). The IC50 values of rutin, quercetin, and quercetin pentaacetate on LPS-induced NO production were 41.5±1.8, 17.1±0.9, 8.7±1.1 μM, respectively. Examination of cytotoxicity of rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages by MTT assay indicated that all

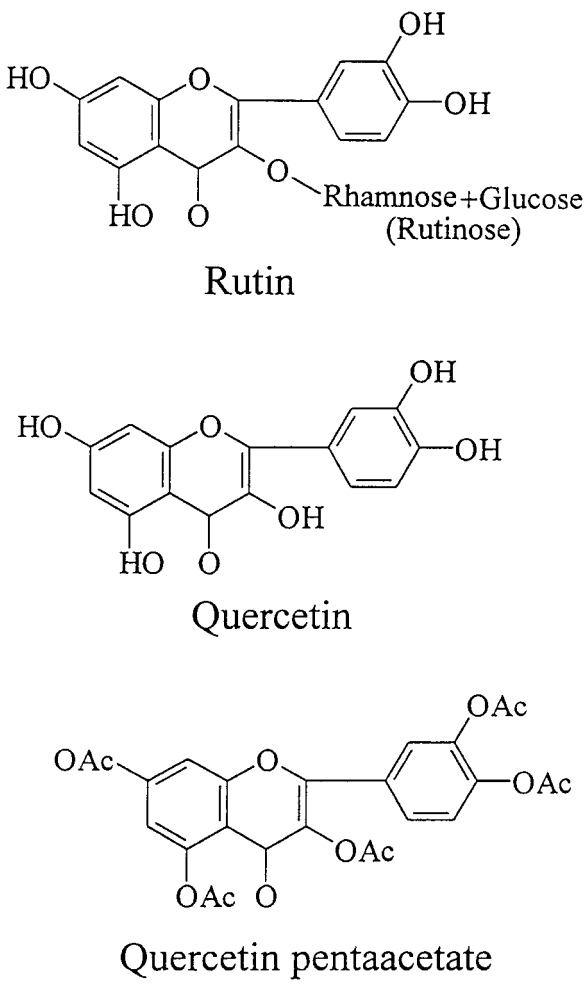


Fig. 1. Chemical structures of rutin, quercetin, and quercetin pentaacetate examined in the present study.

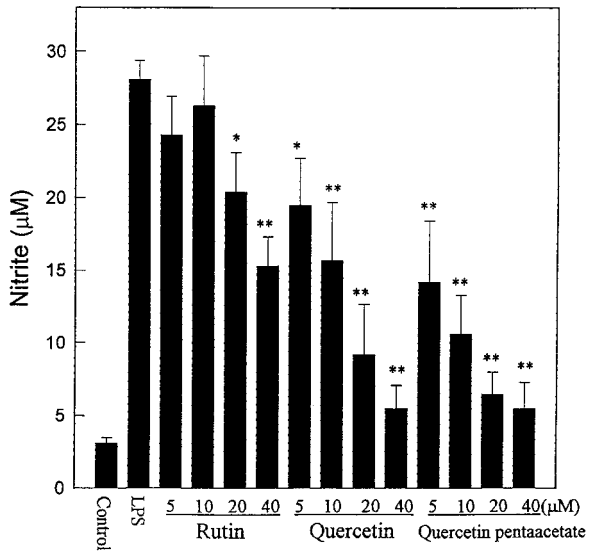


Fig. 2. Effects of rutin, quercetin, and quercetin pentaacetate on LPS-induced nitrite production in RAW 264.7 macrophages. The cells were treated with 100 ng/ml of LPS only (LPS) or LPS plus different concentration (5, 10, 20, 40 μM) of rutin, quercetin, and quercetin pentaacetate at 37°C for 24 h. At the end of incubation, 100 μl of the medium was removed for measuring nitrite production. Control values were obtained in the absence of LPS or flavonoids. Data were derived from three independent experiments and expressed as means±SE. $*P<0.05$ and $**P<0.01$ indicate significant differences from the LPS-treated group.

three compounds, even at the concentration of 40 μM, did not decrease cell viability in RAW 264.7 cells (data not shown). Therefore, inhibition of LPS-induced nitrite production by rutin, quercetin, and quercetin pentaacetate was not the result of their cytotoxicity on cells.

Rutin, Quercetin, and Quercetin Pentaacetate Inhibit LPS-Induced iNOS Gene Expression by Western Blot Analysis

RAW 264.7 cells did not express detectable iNOS protein when incubated in the medium without LPS for 24 h and the basal level of iNOS protein was not affected when incubated with rutin, quercetin, and quercetin pentaacetate (data not shown). Upon LPS (100 ng/mL) treatment for 24 h, iNOS protein dramatically increased in cells, and co-treatment of cells with LPS (100 ng/mL) and different concentration (20, 40 μM) of indicated compounds for 24 h significantly inhibited iNOS protein induction in RAW 264.7 macrophages (Fig. 3). The amount of α-tubulin protein as an internal control remained unchanged.

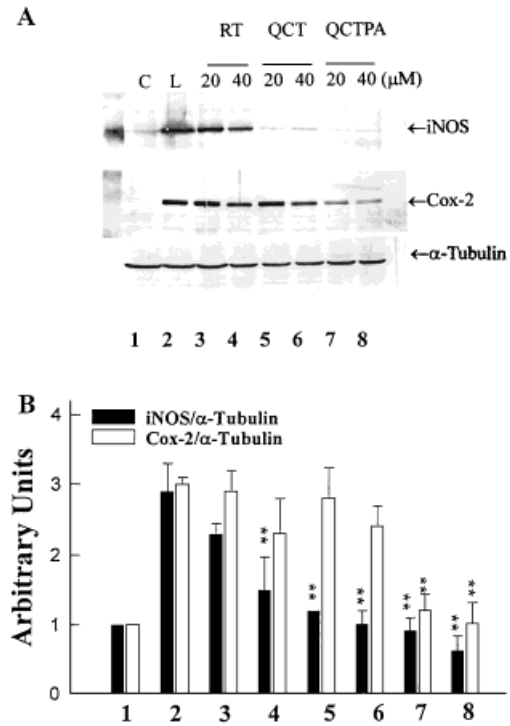


Fig. 3. Inhibition of LPS-induced iNOS and COX-2 proteins in RAW 264.7 macrophages by rutin (RT), quercetin (QCT), and quercetin pentaacetate (QCTPA). **A:** The cells were treated as described in Figure 3. Equal amounts of total proteins (50 μg/lane) were subjected into 10% SDS–PAGE, and expression of iNOS, COX-2, and α-tubulin protein was detected by western blotting using specific antibodies. α-Tubulin protein here was used as an internal control. C: control; L: LPS-treated. **B:** Quantification of band intensities in (A) from three independent experimental results by densitometry (IS-1000 Digital Imaging System). Data was described as means±SEM of iNOS/α-tubulin or Cox-2/α-tubulin. $*P<0.05$ and $**P<0.01$ indicate statistically significant differences from the LPS-treated group.

Quercetin Pentaacetate Significant Inhibition of LPS-Induced PGE2 Production and COX-2 Gene Expression

Activation of COX-2 gene expression and PGE2 production has been demonstrated in the process of LPS treatment. Therefore, we investigated the effects of rutin, quercetin, and quercetin pentaacetate on LPS-induced PGE2 production and COX-2 gene expression. Unstimulated RAW 264.7 macrophages in culture medium for 24 h produced basal amount of PGE2 (1.5 ng/mL) in the medium. After treatment with LPS (100 ng/mL) for 24 h, the amount of PGE2 elevated apparently to 7 ng/mL in medium and co-treatment of cells with LPS and different concentration of quercetin pentaacetate was able to strongly suppress

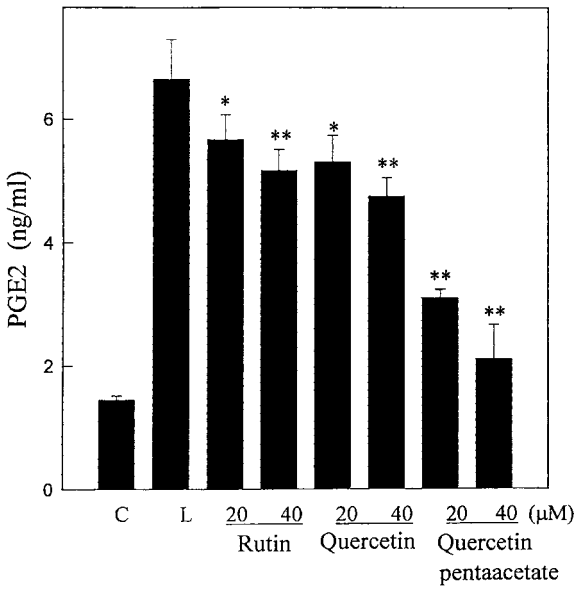


Fig. 4. Inhibition of LPS-induced PGE2 production by rutin, quercetin, and quercetin pentaacetate. RAW 264.7 macrophages were treated with LPS (100 ng/ml) in association with different concentrations (20 or 40 μM) of indicated compounds and incubated at 37°C for 24 h. The amount of PGE2 in medium was measured as described in Materials and Methods.

LPS-induced amount of PGE2 (Fig. 4). However, both rutin and quercetin showed slight but significant inhibitory activity on LPS-induced PGE2 production. Western blot analysis was performed in the following experiment to demonstrate the effect of these compounds on COX-2 gene expression. RAW 264.7 macrophages expressed only little amount of COX-2 protein in unstimulated cells, and rutin, quercetin, and quercetin pentaacetate treatment alone showed no alternation on basal COX-2 expression. Upon LPS (100 ng/mL) treatment for 24 h, COX-2 protein dramatically increased in cells, and cotreatment of cells with LPS (100 ng/mL) and different concentration (20, 40 μM) of quercetin pentaacetate for 24 h dose dependently inhibited COX-2 protein induction in RAW 264.7 cells (Fig. 3). In contrast, rutin and quercetin only, at the high concentration of 40 μM showed a slight decrease on LPS-induced COX-2 protein.

Rutin, Quercetin, and Quercetin Pentaacetate do not Inhibit Intrinsic NOS and Cyclooxygenase Enzyme Activities in RAW 264.7 Macrophages

In order to identify whether the inhibitory effect of rutin, quercetin, and quercetin pentaacetate on inducible NO and PGE2 production through a direct effect on the intrinsic enzyme activity of iNOS and COX-2 direct and indirect enzyme activity assays for iNOS and COX-2 were performed in the following experiments. *N*-nitro-L-arginine (NLA) and *N*-nitro-L-arginine methyl ester (L-NAME) are known to

TABLE I. Effect of Added Compounds After LPS Induction of iNOS Enzyme in RAW 264.7 Macrophages

LPS pretreatment of cells	Addition to LPS-treated RAW 264.7 cells[a]	NO in medium (μM/ 6×10^5 cells)[b]	INOS specific activity: NO formation (μM/ 200ug protein)
None	DMSO control	0.0±0.0	1.7±0.4
LPS (100 ng/ml), 12 h	Control	17.3±0.9	9.1±1.7
	Rutin		
	20 μM	17.1±1.1	8.2±2.7
	40 μM	16.2±0.9	8.9±1.6
	Quercetin		
	20 μM	14.5±0.6	8.9±2.5
	40 μM	13.2±1.1	9.1±1.4
	Quercetin pentaacetate		
	20 M	14.7±1.3	9.6±1.8
	40 M	13.1±1.9	10.1±2.7
	N-nitro-L-arginine		
	2 mM	0.5±0.1**	9.4±1.1
	N-nitro-L-arginine methyl ester		
	2 mM	0.7±0.3**	9.3±2.1

[a]RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 12 h and cells were washed twice with PBS to remove LPS. Raw cells were then scraped and placed in a 24-well plate and indicated compounds were added and incubated at 37°C incubator for further 12 h.
[b]The amount of NO accumulated in the medium and iNOS enzyme activity in cell lystes are described in Materials and Methods. Data are mean±SE from three independent experiments.
*$P < 0.01$, **$P < 0.05$, significantly different from LPS alone, analyzed by Student's *t*-test.

inhibit iNOS enzyme activity as positive control here. Table I shows that the addition of different concentration (20 or 40 μM) of rutin, quercetin, and quercetin pentaacetate to RAW 264.7 macrophages, which had been pretreated with LPS to induce NOS, do not affect enzyme activity in intact cells by measuring the amount of nitrite production in medium. In the same part of the experiment, NOS inhibitors NLA and L-NAME effectively decreased the nitrite production in medium as positive controls, but did not alter the iNOS enzyme activity in the cell lysates by direct NOS enzyme activity assays in vitro. Further confirmation of lack of direct enzyme inhibition of rutin, quercetin, and quercetin pentaacetate was obtained in the experiment in which different concentration (20, 40 μM) of rutin, quercetin, and quercetin pentaacetate were added into the lysates of RAW 264.7 macrophages that had been pretreated with LPS (100 ng/mL) to induce NOS followed by an enzyme assay for iNOS on the lysates using arginine as an added substrate (Table II). There was no inhibition of NO production by rutin, quercetin, and quercetin pentaacetate. Both NLA and L-NAME treatment showed the significant inhibition on NO production in a direct enzyme assay as positive controls. Thus, rutin, quercetin, and quercetin pentaacetate inhibit NO production induced by LPS by a mechanism other than direct enzyme inhibition. As shown above, the inhibition of quercetin pentaacetate on PGE2 production is not a result of direct inhibition on cyclooxygenase enzyme. When different concentration (20 or 40 μM) of rutin, quercetin, and quercetin pentaacetate were added to RAW 264.7 macrophages in which COX-2 proteins has already been induced by LPS, there was no decrease in PGE2 production using added arachidonic acid as a substrate (Table III). Both NLA and L-NAME, NOS enzyme inhibitors, did not inhibit PGE2 production, whereas a cyclooxygenase enzyme inhibitor indomethacin decrease the PGE2 production in this assay as a positive control.

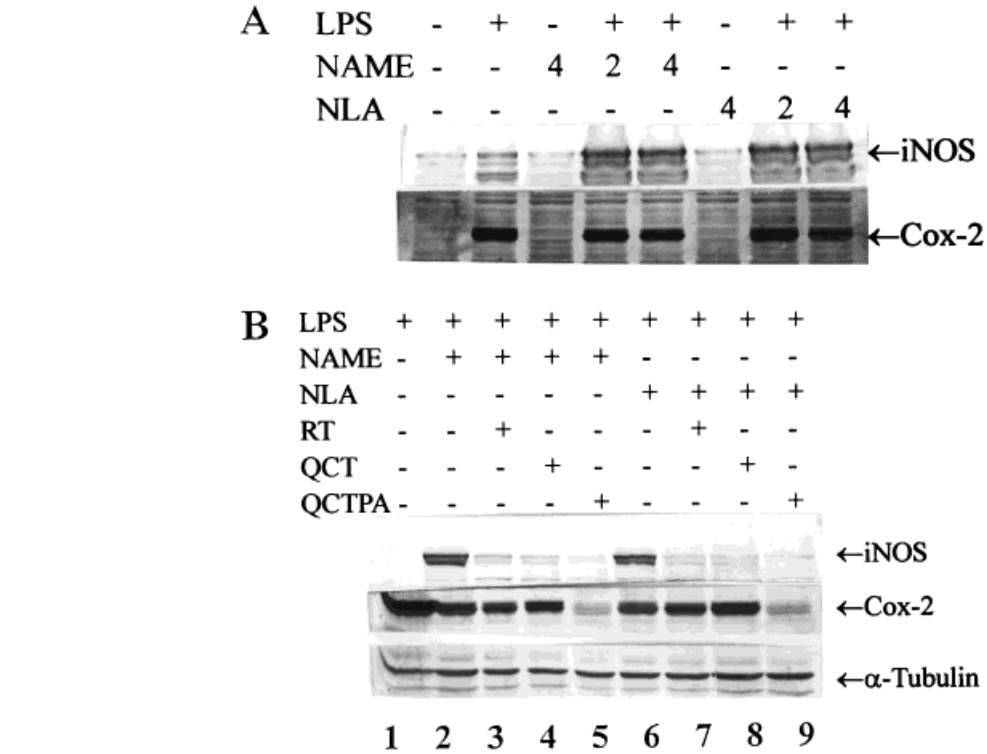
Effects of Indicated Compounds and NOS Inhibitors on LPS-Induced iNOS and COX-2 Gene Expressions

Peng et al. [1998] proposed that NO might be a factor to terminate the inflammation through an autoregulatory feedback inhibition in LPS or cytokines treated cells. Cells treated with NOS enzyme inhibitors such as NLA or L-NAME effectively inhibited the NO production, but stimulated iNOS gene expression. In the present study, NLA and L-NAME significantly inhibited LPS-induced NO (not PGE2) production (Fig. 6), but apparently stimulated LPS (100 ng/mL)-induced iNOS protein, compared with LPS-treated group ($P < 0.01$) (Fig. 5A). Treatment of cells with different concentration (20 or 40 μM) of rutin, quercetin, and quercetin pentaacetate inhibited NLA or L-NAME stimulated iNOS protein, and quercetin pentaacetate showed the obvious inhibition on COX-2

TABLE II. Effects of Rutin, Quercetin, and Quercetin Pentaacetate on iNOS Activity by Direct Enzyme Activity Assay in RAW 264.7 Cell Lysates

Pretreatment of cells before lysis	Addition to lysate	INOS specific activity: NO formation (μM/200 μg total protein)[a]
None	DMSO	0.9±0.3
LPS (100 ng/ml), 12 h	DMSO	7.3±0.8
	Rutin	
	20 μM	7.4±0.7
	40 μM	7.2±0.1
	Quercetin	
	20 μM	8.7±1.3
	40 μM	7.9±0.4
	Quercetin pentaacetate	
	20 μM	6.8±0.2
	40 μM	6.9±0.6
	N-nitro-L-arginine	
	4 mM	4.6±0.4**
	N-nitro-L-arginine Methyl ester	
	4 mM	4.3±0.3**

[a]The values were obtained from three separate experiments and described as mean±SE. Lysate preparation and iNOS activity assay were described in the section of Materials and Methods. Each indicated compound was added into lysates (200 μg) from LPS-treated RAW 264.7 macrophages and iNOS activity was measured.
**$P < 0.01$, significantly different from LPS alone, analyzed by Student's *t*-test.



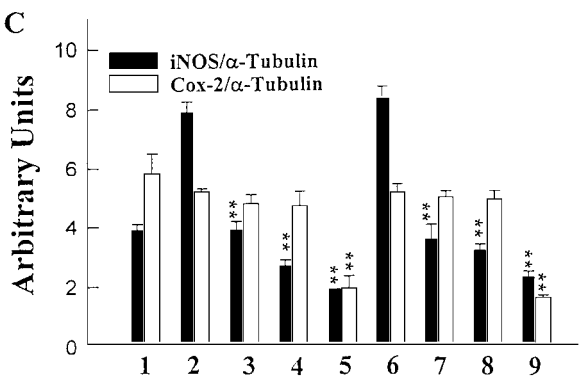


Fig. 5. Inhibition of NLA/LPS or NAME/LPS induced iNOS and COX-2 proteins by rutin (RT), quercetin (QCT), and quercetin pentaacetate (QCTPA) in RAW 264.7 macrophages. **A:** The cells were treated with LPS (100 ng/mL) in combination with NLA or NAME (2 or 4 mM) at 37°C for 24 h. The expression of iNOS, COX-2, and α-tubulin was determined by western blotting. **B:** Adding rutin (RT), quercetin (QCT), and quercetin pentaacetate (QCTPA) (40 μM) into NLA/LPS or NAME / LPS-treated RAW 264.7 macrophages at 37°C for 24 h. The expression iNOS, COX-2, and α-tubulin protein was detected by western blotting using specific antibody. **C:** Quantification of band intensities in (B) from three independent experiments by densitometry (IS-1000 Digital Imaging System). Data were described as means±SEM of iNOS/α-tubulin or Cox-2/α-tubulin. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences from the LPS+NOS inhibitor (NLA or L-NAME)-treated group.

protein and PGE2 production in NLA/LPS or L-NAME/LPS co-treated RAW 264.7 macrophages (Fig. 5B)

DISCUSSION

Results of the present study indicated that flavonoids rutin, quercetin, and its derivates quercetin pentaacetate effectively inhibited LPS-induced NO production and iNOS gene expression in RAW 264.7 macrophages, but no inhibition on iNOS enzyme activity. Quercetin pentaacetate showed the obvious inhibitory effect on LPS-induced PGE2 production through blocking COX-2 gene expression. Co-treatment of LPS-treated RAW 264.7 cells with L-arginine analogs (NLA or L-NAME) and rutin, quercetin, or quercetin pentaacetate decrease NO production effectively and block NLA or L-NAME stimulated iNOS gene expression. In addition to firstly identifying the inhibitory activity of these flavonoids on LPS-induced NO and PGE2 productions, we proposed a possible idea that flavonoids can be used with NOS enzyme inhibitors in treatment of LPS-induced responses.

Polyphenols are popular components which exist in the natural plants. Several biological

TABLE III. Effects of Added Rutin, Quercetin, and Quercetin Pentaacetate or Indicated Compounds After LPS Induction of COX-2 Enzyme in RAW 264.7 Cells

LPS treatment of cells[a]	Addition to LPS-treated RAW 264.7 cells	PGE2(ng/ml)[b]
None	DMSO	0.21±0.0
LPS	DMSO	6.24±0.29
	Rutin	
	20 μM	5.83±0.11
	40 μM	5.77±0.53
	Quercetin	
	20 μM	5.66±0.22
	40 μM	5.85±0.35
	Quercetin pentaacetate	
	20 μM	5.63±0.49
	40 μM	5.51±0.37
	Indomethacin	
	20 μM	1.92±0.47**
	N-nitro-L-arginine	
	2 mM	5.81±0.23
	N-nitro-L-arginine methyl ester	
	2 mM	6.21±0.31

[a]RAW 264.7 cells were stimulated with LPS (100 ng/ml) for 6 h, and cells were washed twice with fresh medium. Rutin, quercetin or other indicated compounds were then added and incubated at 37°C for 30 min. The cells were further incubated with arachidonic acid (100 μM) for 15 min.
[b]The amount of PGE2 in the supernatant was assayed as described in Materials and Methods. Data are means±SE of three samples from two independent experiments. In each experiment, duplicate determinations were made for each experiment.
**$P < 0.01$, significantly different from LPS alone, analyzed by Student's *t*-test.

activities of polyphenols have been studied extensively including inhibiting TPA-induced PKC activation, blocking EGF-induced signal transduction and inhibiting LPS-induced responses [Lin et al., 1999; Ray et al., 1999]. *Sophorae Flos Immaturus* has been used in Chinese medicine as a remedy for treating hypertension and uterine bleeding, however, the active principle in *Sophorae Flos Immaturus* has not been determined fully. Rutin is one of the major flavonoids in *Sophorae Flos Immaturus*, but the biological activity of rutin is still unclear. Quercetin is a prototypical polyphenolic plant flavonoid and can be derived from rutin through hydrolization by glucosidase. Quercetin has potent antioxidant and anti-inflammatory effects. It prevent cisplatin-induced cytotoxicity in LLC-PK1 cells in vitro and tubular injury induced by acute renal ischemia in vivo and inhibiting LPS induced NO production [Kuhlmann et al., 1998; Middleton, 1998]. Rangan et al. [1999] reported that quercetin inhibited LPS induced cytokines such as IL-1β, TNF-α productions through blocking NF*k*B activation . In addition to above beneficial effects, quercetin has also been implicated as a strong mutagen without microsomal activation and the mutagenic activity of quercetin was increased significantly after microsomal activation [Bjeldanes, 1977]. Quercetin pentaacetate was derived from quercetin through in vitro acetylation and biological function of quercetin pentaacetate was still unclear. Results of present study showed that rutin, quercetin, and quercetin pentaacetate inhibited the LPS-induced iNOS gene expression in macrophages and the inhibitory potency is quercetin pentaacetate > quercetin > rutin. Among them, quercetin pentaacetate, not rutin and quercetin, showed significant inhibition on LPS-induced PGE2 production and COX-2 expression. These data indicated that acetylation of quercetin might be able to stimulate the inhibitory effects of flavonoid on PGE2 and NO production induced by LPS.

Large amount of NO production induced by bacterial lipopolysaccharide or cytokines plays an important role in endotoxaemia and inflammatory conditions [Gidday et al., 1998; Stoclet et al., 1998]. Therefore, drugs that inhibit NO production through inhibiting iNOS gene expression or its enzyme activity have beneficial therapeutic effects in treatment of sepsis [Suh et al., 1998]. Several natural polyphenolic compounds including curcumin, oroxylin A and wogonin have been demonstrated to inhibit LPS-induced NO production by blocking iNOS gene expression [Chen et al., 2000]. In this study, we found rutin, quercetin, and quercetin pentaacetate were capable of inhibiting the production of NO when co-incubated with LPS in a time- and concentration-dependent man-

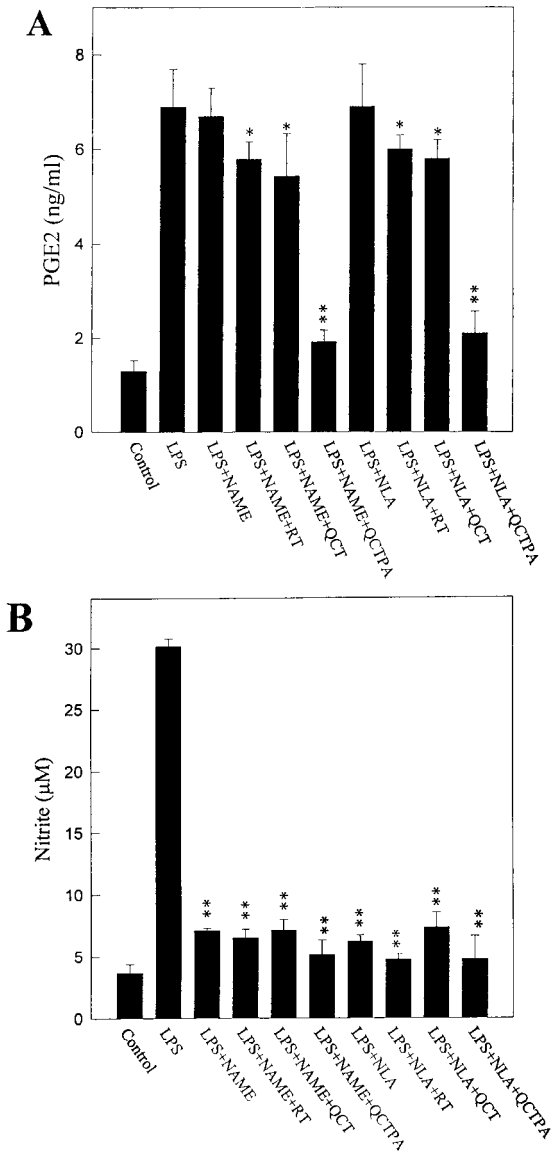


Fig. 6. Inhibitory effects of rutin (RT), quercetin (QCT), and quercetin pentaacetate (QCTPA) on L-NAME/LPS or NLA/LPS induced NO and PGE2 productions in RAW 264.7 macrophages. **A:** The cells were treated with LPS (100 ng/ml) in combination with L-NAME or NLA (4 mM) and indicated compounds (40 μM) at 37°C for 24 h. The amount of PGE2 (A) and nitrite (**B**) in the culture medium was measured by the method as described in Materials and Methods. Each value was derived from three independent experiments and described as mean±SE. $^{*}P < 0.05$; $^{**}P < 0.01$.

ners without obvious cytotoxicity. The inhibitory effect of these compounds on LPS-induced NO production was ineffective when iNOS was expressed by pre-activation with LPS, and an enzyme assay for iNOS using L-arginine as a substrate was performed to further demonstrate that these three compounds did not directly inhibit NOS enzyme activity. By western blotting analysis, iNOS protein in RAW 264.7 macrophages treated with LPS could be suppressed by rutin, quercetin, and quercetin pentaacetate in a concentration dependent manner. These results indicated that inhibition of LPS-induced NO production by rutin, quercetin, and quercetin pentaacetate was through inhibition of iNOS gene expression rather than the activity of NO synthase. This action profile is similar to that of glucocorticoids and polyphenolic compound oroxylin A that inhibit NO production induced by LPS by blocking iNOS gene expression [Joly et al., 1997].

NOS enzyme inhibitors such as *N*-nitro-L-arginine (NLA) and *N*-nitro-L-arginine methyl ester (L-NAME) are well known nonspecific NOS inhibitors that effectively attenuate NO production induced by LPS and cytokines. Several in vitro and in vivo studies indicated that NO may also function as an anti-inflammatory mediator. For instance, release of NO by adding exogenous NO donors decrease cytokine-induced endothelial cell activation, inhibit endothelial-leukocyte interaction, and attenuate vascular inflammation [De Caterina et al., 1995; Khan et al., 1996]. Peng et al. [1998] recently reported that the treatment of L-arginine analogs L-NAME in combination with LPS/mIFN-γ decreases stimulated iNO production, but augment LPS/mIFN-γ-induced iNOS expression by 2.5-fold. Therefore, agents blocked NOS inhibitors stimulated iNOS protein might be useful in treatment of LPS-mediated responses with NOS inhibitors. Data of this study provided evidences that flavonoids in combination with NOS inhibitors decreased iNO production and iNOS protein expression induced by LPS. Quercetin pentaacetate showed the significant inhibitory effect on PGE2 production and COX-2 gene expression in NLA/LPS or L-NAME/LPS-co-treated RAW 264.7 macrophages. These results provided an idea that flavonoids such as quercetin pentaacetate can be used with L-arginine analogs including NLA and L-NAME in the treatment of LPS-induced responses and suppressed NLA and L-NAME stimulated adverse effects including stimulated iNOS gene expression in LPS-treated RAW 264.7 macrophages.

In summary, this study provided evidences that flavonoid can be used as a complement agent with NOS inhibitors in treatment of LPS-mediated responses. As we know, flavonoids

have several beneficial functions such as antioxidative activities, inhibiting iNOS gene expressions, but poor absorption and higher IC50 are their disadvantages in practical application. This study provided evidence to demonstrate combinatorial treatment with functional flavonoid derivates and NOS inhibitors that could block NOS inhibitors induced side effects such as stimulated iNOS protein here, and also performed inhibition on deleterious gene expression which could not be achieved by NOS inhibitors such as COX-2 protein and PGE2 production and deserved for further in vivo study.

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