Modulation of Suppressive Activity of Lipopolysaccharide-Induced Nitric Oxide Production by Glycosidation of Flavonoids

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Flavonoids have been demonstrated to exhibit a wide range of biological activities including anti-inflammatory and neuroprotective actions. Although a significant amount of flavonoids has been identified to be present as glycosides in medicinal plants, determinations of the biological activities of flavonoids were mainly carried out with aglycones of flavonoids. Therefore, the exact role of the glycosidation of flavonoid aglycones needs to be established. In an attempt to understand the possible role of glycosidation on the modulation of the biological activities of flavonoids, diverse glycosides of kaempferol, quercetin, and aromadendrin were examined in terms of their anti-inflammatory activity determined with the suppression of lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV2 microglial cells. The results indicated that glycosidation of aglycones attenuated the suppressive activity of aglycones on LPS-induced NO production. Although attenuated, some of glycosides, depending on the position and degree of glycosidation, maintained the inhibitory capability of LPS-induced NO production. These findings suggest that glycosidation of flavonoid aglycones should be considered as an important modulator of the biological activities of flavonoids.

Key words: Kaempferol, Quercetin, Aromadendrin, Glycoside, Aglycone, Nitric oxide, Microglia

INTRODUCTION

Flavonoids are a group of low molecular weight polyphenolic compounds of plant origin, exhibiting a variety of biological activities including anti-inflammatory, anti-oxidant, anti-viral, anti-tumor, and neuroprotective actions (Lee et al., 2003; Middleton et al., 2000). It has been previously demonstrated that a series of flavonoids inhibit the production of pro-inflammatory mediators such as TNF-α, nitric oxide (NO), and prostaglandin E2 (Kim et al., 1999). Especially, quercetin and kaempferol were reported to possess diverse biological activities, including anti-inflammatory (Aligiannis et al., 2001; Ha et al., 2003; Wattel et al., 2003). However, most of the studies that determined the diverse biological activities of flavonoids were carried out with aglycones of flavonoids (Aligiannis

et al., 2001; Ha et al., 2003; Kim et al., 1999), although a significant amount of flavonoids was present as glycosides in medicinal plants (Gudej, 2003; Markham et al., 1992; Masuoka et al., 2002). Further, it has been suggested that glycosidation of flavonoid aglycones differentially affects the biological activities of aglycones depending on the position and degree of gylcosidation (Hou et al., 2004). Kaempferol has been identified to be present as glucoside, galactoside, rhamnoside, and arabinopyranoside (Chang et al., 2004; Gudej, 2003; Markham et al., 1992; Won et al., 2004), quercetin as glucoside and galactopyranoside (Chang et al., 2004; Gudej, 2003), and aromadendrin as glucoside and glucopyranoside (Chang et al., 2004).

Pathological levels of NO, released from activated microglia during the pathogenesis of various neurological disorders, contribute to the aggravation of diseases (Liu et al., 2002). Therefore, drugs that inhibit NO production may have beneficial therapeutic effects in the treatment of diseases (Avontuur et al., 1999).

Given the fact that a significant amount of naturally occurring flavonoids in medicinal plants is present as

glycosides, the possible role of glycosidation on the biological activities of aglycones has not been clearly demonstrated. In the present study, the effects of various glycosides of three aglycones on anti-inflammatory activity, as determined with the suppression of lipopolysaccharide (LPS)-induced NO production, were examined in BV2 microglial cells.

MATERIALS AND METHODS

Isolation of flavonoid aglycones and glycosides

Kaempferol (K-0), aromadendrin (A-0), kaempferol-6-*C*-glucoside (K-1), aromadendrin-6-*C*-glucoside (A-1), taxifolin-6-*C*-glucoside (A-2), and quercetin-6-*C*-glucoside (Q-2) were isolated from *Hemiptelea davidii* (Chang *et al*, 2004); kaempferitrin (K-4), kaemferol-3-*O*-L-(3-O-acetyl)rhamnosyl-7-*O*-L-rhamnoside (K-8), sutchuenoside (K-9), and kaempferol-3-*O*-D-glucosyl-7-*O*-L-rhamnoside (K-3) from *Weigela subsessilis* (Won *et al*, 2004); kaempferol-3-*O*-D-glucoside (K-2), kaempferol-3-rhmanosyl(1→6)-glucoside (K-5), kaempferol-3-rhamnosyl(1→2)-glucoside (K-6), kaempferol-3-rhamnosyl(1→3)-rhamnosyl(1→6)-glucoside (K-7), and quercetin-*O*-D-glucoside (Q-1) from *Staphylea*

bumalda (manuscript in preparation); and orientin (Q-3) from *Digitaria sanguinalis*. Quercetin (Q-0) was purchased from Sigma (St. Louis, MO).

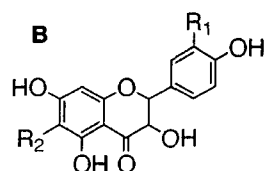### Measurement of DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was measured according to Hwang et al. (Hwang *et al.*, 2001). Briefly, 100 μL of 0.2 mM DPPH in methanol was added to 50 μL of each sample in methanol solution in a 96-well microtiter plate. After incubation at room temperature for 30 min, the optical density of each solution was determined at 517 nm using a microtiter plate reader (Bio Rad Laboratories Inc.). The parameter IC_{50} represents a concentration of each compound (μg/mL) exhibiting a 50% decrease of DPPH radicals.
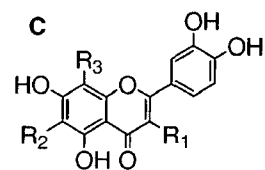
Materials and cell culture

LPS and other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise stated. The immortalized murine BV2 cell line that exhibits the phenotypic and functional properties of reactive microglial cells (Bocchini *et al.*, 1992) was obtained from M. McKinney (Mayo Clinic, Jacksonville, FL). The cells were grown and maintained in

A
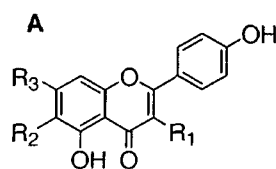
Kaempferol derivatives

K-0. R_1 = OH, R_2 = H, R_3 = OH
K-1. R_1 = OH, R_2 = *C*-glucose, R_3 = OH
K-2. R_1 = *O*-*D*-glucose, R_2 = H, R_3 = OH
K-3. R_1 = *O*-*D*-glucose, R_2 = H, R_3 = *O*-*L*-rhamnose
K-4. R_1 = *O*-*L*-rhamnose, R_2 = H, R_3 = *O*-*L*-rhamnose
K-5. R_1 = *O*-*L*-rhamnosyl(1 → 6)- *O*-*D*-glucoside, R_2 = H, R_3 = OH
K-6. R_1 = *O*-*L*-rhamnosyl(1 → 2)- *O*-*D*-glucoside, R_2 = H, R_3 = OH
K-7. R_1 = *O*-*L*-rhamnosyl(1 → 3)-*O*-*L*-rhamnosyl(1→ 6) - *O*-*D*-glucoside, R_2 = H, R_3 = OH
K-8. R_1 = *O*-*L*-(3-*O*-acetyl)rhamnose, R_2 = H, R_3 = *O*-*L*-rhamnose
K-9. R_1 = *O*-*L*-(4-*O*-acetyl)rhamnose, R_2 = H, R_3 = *O*-*L*-rhamnose

B

Aromadendrin derivatives

A-0. R_1 = H, R_2 =H
A-1. R_1 =H, R_2 = *C*-glucose
A-2. R_1 = OH, R_2 = *C*-glucose

C

Quercetin derivatives

Q-0. R_1 = OH, R_2 = H, R_3 = H
Q-1. R_1 = *O*-*D*-glucose, R_2 = H, R_3 = H
Q-2. R_1 = OH, R_2 = *C*-glucose, R_3 = H
Q-3. R_1 = H, R_2 = H, R_3 = *C*-glucose

Fig. 1. Structures of flavonoid aglycones and glycosides. A: kaempferol (K-0) and 9 glycosides (K-1, K-2, K-3, K-4, K-5, K-6, K-7, K-8, and K-9), B: aromadendrin (A-0) and 2 glycosides (A-1 and A-2), and C: quercetin (Q-0) and 3 glycosides (Q-1, Q-2, and Q-3). See Materials and Methods for the origins of these compounds.

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin and 10 U/mL penicillin at 37°C in a humidified incubator with 5% CO_2. All experiments were carried out on subconfluent cultures.

Measurement of nitrite release

Accumulated nitrite was measured in the cell supernatant by the Griess reaction (Green et al., 1982). The conditions of cell culture and treatment were the same as those in ELISA. In brief, 100 µL of Griess reagent (mixing equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) was added to 100 µL of each sample in a 96-well microtiter plate and absorbance was read at 540 nm using a plate reader. Sodium nitrite, diluted in culture media at concentrations ranging from 10 to 100 µM, was used to prepare a standard curve.

Statistics

Data were analyzed using the paired t-test, and values were considered significantly different when the two-tailed P value was <0.05. Results are expressed as mean±SEM values.

RESULTS

Determination of flavonoid aglycones and glycosides

Spectrums from UV, IR, Mass, ^1H-NMR, and ^{13}C-NMR were thoroughly examined to determine the position and number of glycosidation. Structures of the 3 flavonoid aglycones and 14 glycosides are illustrated in Fig. 1.

In vitro antioxidant activities of flavonoid aglycones and glycosides

To determine the in vitro antioxidant activities of flavonoid aglycones and glycosides, a DPPH radical scavenging activity assay was carried out. As shown in Table I, kaempferol aglycone (K-0) and C-glycoside at R2 position of kaempferol (K-1) showed significant antioxidant activities among the kaempferol glycosides. Glycosides, K-6 and K-7, having O-sugars at R1 position, exhibited mild antioxidant activities whereas other kaempferol glycosides possessed low antioxidant activities (Table I).

Aromadendrin (A-0) showed negligible in vitro antioxidant potential in DPPH radical scavenging activity assay. However, compound A-2 carrying C-glycoside at R2 position and a hydroxyl group at R1 position showed significantly strong antioxidant activity compared with that of aromadendrin aglycone (A-0), whereas compound A-1, carrying C-glycoside at R2 position but no hydroxyl group, showed no significant antioxidant activity. The antioxidant

potential of compound A-2 might be due to the hydroxyl substitution at R1 position, rather than the glycosidation, given that both A-1 and A-2 compounds have C-glucoside at R2 position.

Quercetin aglycone (Q-0) and the three quercetin glycosides, Q-1, Q-2, and Q-3, showed approximately the same in vitro antioxidant activities (Table I). Quercetin glycosides are all glycosidated with glucose, although the substitution positions are different. Compound Q-2 was glycosidated with C-glucose at R2 position, compound Q-1 was with O-glucose at R1 position, and compound Q-3 was with C-glucose at R3 position.

Suppressive effects of flavonoid aglycones and glycosides on LPS-induced NO production in BV2 microglial cells

To determine the anti-inflammatory property of flavonoid aglycones and glycosides, the inhibitory effects of these compounds on LPS-induced NO production in BV-2 microglial cells were investigated. The cells were challenged with LPS in the absence or presence of pretreatment of each compound. When BV2 cells were stimulated with LPS (200 ng/mL) for 16 h, the accumulation of nitrite, a stable oxidized product of NO, was significantly increased in the culture medium.

Table I. DPPH radical scavenging activity of tested compounds

Tested compounds	IC_{50} $(\mu M)^{a)}$
Kaempferol (K-0)	53.9
Kaempferol-6-C-glucoside (K-1)	60.3
Kaempferol-3-O-D-glucoside (K-2)	>1,000
Kaempferol-3-O-D-glucosyl-7-O-L-rhamnoside (K-3)	>1,000
Kaempferitrin (K-4)	>1,000
Kaempferol-3-rhmanosyl(1→6)-glucoside (K-5)	>1,000
Kaempferol-3-rhamnosyl(1→2)-glucoside (K-6)	548.2
Kaempferol-3-rhamnosyl(1→3)-rhamnosyl(1→6)-glucoside (K-7)	637.6
Kaemferol-3-O-L-(3-O-acetyl)rhamnosyl-7-O-L-rhamnoside (K-8)	>1,000
Sutchuenoside (K-9)	>1,000
Aromadendrin (A-0)	>1,000
Aromadendrin-6-C-glucoside (A-1)	>1,000
Taxifolin-6-C-glucoside (A-2)	31.1
Quercetin (Q-0)	23.0
Quercetin-3-O-D-glucoside (Q-1)	21.9
Quercetin-6-C-glucoside (Q-2)	31.3
Orientin (Q-3)	18.5
BHA[*]	79.5
Ascorbic acid[**]	36.9

[*] and [**]: positive controls
[a)] Concentration giving a 50% decrease of DPPH radicals.
The values are the means of triplicate experiments.

In accordance with previous reports that kaempferol and quercetin aglycones exhibited anti-inflammatory activities (Kim *et al.*, 1999; Lin *et al.*, 2003), LPS-induced NO production was significantly suppressed in a concentration-dependent manner by kaempferol (K-0) and quercetin (Q-0) aglycones in LPS-challenged BV2 microglial cells (Fig. 2A and 2B). However, cytotoxicity was observed at concentrations over 100 mM with kaempferol and quercetin aglycones (data not shown). Aromadendrin

aglycone (A-0) showed no significant suppression of LPS-induced NO production (Fig. 2C). Glycosylation of kaempferol and quercetin aglycones significantly attenuated the suppressive activity of aglycones on LPS-induced NO production in BV2 microglial cells (Fig. 2A and 2B). Although significantly decreased, some glycosides still exhibited suppressive activities on LPS-induced NO production at relatively higher concentration (Fig. 2A and 2B). Among kaempferol glycosides, compounds K-1,
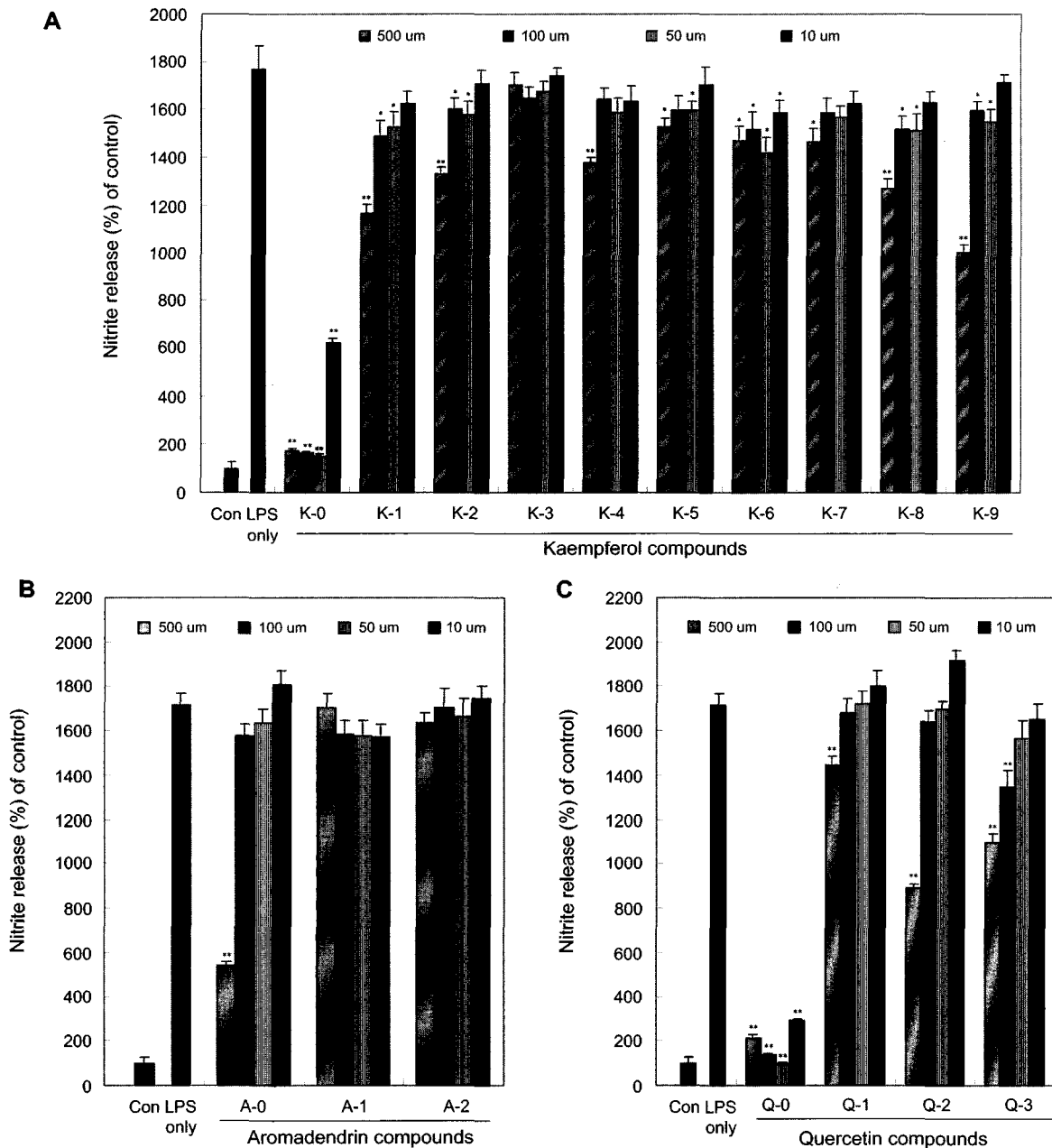
Fig. 2. Effects of glycosidation of kaempferol (A), aromadendrin (B), and quercetin (C), on the nitrite production of LPS-activated BV2 microglial cells. BV2 microglial cells were challenged with LPS (200 ng/mL) in the presence or absence of pretreatment of each compound. Released nitrite in culture medium was measured. Data are presented as a percentage of the control value. Data represent three independent experiments and are expressed as mean±SEM. *p<0.05 and **p<0.01 indicate statistically significant differences from the LPS alone group.

K-2, K-8, and K-9, showed a significant inhibition on LPS-induced NO production in a concentration-dependent manner (Fig. 2A). Quercetin glycosides, compounds Q-1, Q-2, and Q-3, also showed suppressive activities on LPS-induced NO production in a concentration-dependent manner, although the suppression was attenuated compared with that of quercetin aglycone (17) (Fig. 2C). Aromadendrin aglycone (A-0) exhibited considerably weak inhibitory activity on LPS-induced NO production compared to that of kaempferol and quercetin aglycones, showing a significant suppression of LPS-induced NO production at higher concentration (Fig. 2B). However, the two glycosides of aromadendrin, compounds A-1 and A-2, were proven to be inactive in the suppression of the LPS-induced NO production in BV2 microglial cells.

DISCUSSION

Flavonoids exhibit a variety of biological activities and their cellular action mechanisms for pharmacological activities have been attributed to their antioxidant nature (Havsteen, 1983). The present study demonstrated that glycosidation modulates the biological activity of their parent flavonoid aglycones as measured with the inhibition of LPS-induced NO production in BV2 microglial cells. Flavonoids have been reported to inhibit NO production in cell models (Kim et al., 2001; Kim et al., 1999). Although NO has been recognized to be an important mediator of cellular communication, a pathologically large amount of NO has been reported to be produced during the pathogenesis of neurodegenerative diseases like cerebral ischemia, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and many inflammatory conditions (Liu et al., 2002; Murphy, 1999). It has been previously reported that LPS treatment (200 ng/ml) resulted in the production of a large amount of NO in BV2 microglial cell through the induction of inducible NO synthase (iNOS) (Kim et al., 2004).

It has been previously demonstrated that aglycones of quercetin and kaempferol exhibited more potent free radical scavenging activity than their glycosides (Hou et al., 2004). However, the effects of glycosidation in terms of the position and degree of sugar substitution were not fully addressed in their study. The present study therefore examined the effects of various glycosides and found that glycosidation generally attenuated the biological activity of parent flavonoid aglycones. The attenuated biological activities of glycosides might be attributed to the decreased intracellular transport of flavonoids due to the increased hydrophilicity by sugar residues. However, some glycosides maintained significant levels of biological activity even though the hydrophilicity was considerably decreased compared to that of parent aglycones. Further,

it has been reported that types of sugar moiety determine the bioavailability of glycosides (Arts et al., 2004) and that deglycosylation by β-glucosidases plays an important role in the absorption of glycosides (Nemeth et al., 2003).

Interestingly, some glycosides showed significant in vitro antioxidant potentials as determined with DPPH radical scavenging activity assay. For example, compound K-1, having C-glucose at R1 position of kaempferol, showed an antioxidant potential as strong as that of kaempferol aglycone (K-0). Similar results were also observed with quercetin glycosides. Compounds Q-1, Q-2, and Q-3 possessed approximately the same in vitro antioxidant potential as quercetin aglycone (Q-0). These results implied that some glycosides still maintain biologically important functional residues because sugar substitutions were made to biologically unimportant positions. However, these glycosides exhibited significantly attenuated in vivo activity of NO suppression in BV2 microglial cells, suggesting that the introduction of hydrophilic sugar moieties resulted in decreased bioavailability although biologically active residues were intact.

Previously, the possible relationship between flavonoid structure and its activity was suggested with a study of various aglycones, but not glycosides (Kim et al., 1999). According to their study, 5,7-dihydroxyl groups in the A-ring were important for the biological activities and the 8-methoxy group in the A-ring and 4- or 3,4-vicinal substitutions in the B-ring might have been favorable, whereas 2,4-hydroxyl substitution in the B-ring abolished the activity. However, the modulation by sugar substitutions that are dominantly present in medicinal plants has not been examined.

In conclusion, the present study demonstrated that glycosidation of flavonoid aglycones considerably attenuated the biological activity of their parent aglycones in BV2 microglial cells. However, some glycosides exerted a biological activity depending on the position and degree of glycosidation. The discrepancy in antioxidant potentials of some of the glycosides between in vitro and in situ environments might be due to the capability for intracellular uptake of the glycosides. These findings suggest that the glycosidation of flavonoid aglycones should be considered as an important modulator of biological activities of flavonoids. However, further studies are necessary to determine the role of glycosidation in other biological activities, besides anti-inflammatory action, in order to establish a generalized role of glycosidation of flavonoid aglycones.

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