Food Sci. Biotechnol. Vol. 14, No. 2, pp. 200 ~ 206 (2005)

Food Science and Biotechnology

Quercetin Ameliorates NO Production via Down-regulation of iNOS Expression, NFκB Activation and Oxidative Stress in LPS-Stimulated Macrophages

Hye-Yeon Cho, Ji-Young Park, Jong-Kyung Kim, Kyung-Hee Noh, Gap-Soon Moon, Jung-In Kim and Young-Sun Song*

School of Food and Life Science, Biohealth Products Research Center and Food Science Institute, Inje University, Obang-dong, 607, Kimhae, Korea, 621-749

Abstract Effect of quercetin on NO production and regulation mode of quercetin on oxidative stress, NFkB activation, and iNOS expression, possible mechanisms of NO suppression in LPS-stimulated macrophages were investigated. Treatment of RAW 264.7 cells with quercetin significantly reduced lipopolysaccharide (LPS)-stimulated nitric oxide (NO) production dose-dependently (IC_{50}, 9.2 μM). Expression of iNOS and specific DNA binding activities of nuclear factor kB (NFkB) were significanly suppressed by quercetin pretreatment. Quercetin reduced thiobarbituric acid-reactive substances (TBARS) accumulation, enhancing GSH level and antioxidant activities of enzymes, such as superoxide dismutase (SOD) and catalase. These results demonstrate quercetin may ameliorate inflammatory diseases by suppressing NO production through inhibition of iNOS expression, NFκB transactivation, and oxidative stress, which may be mediated partially by antioxidative effect of quercetin. Thus, quercetin appears to be used as a potential therapeutic agent for treating LPS-induced inflammatory processes.

Key words: quercetin, NO, NFκB, iNOS, oxidative stress

Introduction

Various studies implicated elevated nitric oxide (NO) production and oxidative stress in activated macrophages to inflammation and development of chronic diseases (1-2). NO is produced from L-arginine by constitutive and inducible nitric oxide synthase (iNOS) in various mammalian cells and tissues. iNOS, in turn, is induced by either bacterial lipopolysaccharide (LPS) or cytokines including tumor necrosis factor α and interferon-γ in macrophages (3-5). NO produced by iNOS reacts with superoxide and yields peroxynitrite, which contributes to etiology of cardiovascular disease and aging by promoting oxidative stress and inflammation processes (1,2,6). Expression of iNOS is closely related with the up-regulation of nuclear factor kappa B (NFκB), whose sites have been identified in the promoter region of iNOS gene (7-9). NFκB, an inducible transcription factor, is activated in response to various extracellular stimuli, including cytokines (10,11), LPS (12), and oxidative stress (13).

Quercetin (3,5,7,3′,4′-pentahydroxyflavone), a flavonoid, prevents oxidant injury by scavenging oxygen radicals and protecting lipid peroxidation (14-16). Recently, anti-inflammatory activity of quercetin has been proposed by several studies, in which flavonoids including quercetin were suggested to suppress NO production via down-regulation of iNOS expression (17, 18). However, little information is available on the effect of quercetin on NFκB, a ROS-sensitive transcriptional factor, which regulates the iNOS expression and subsequent NO production in LPS-stimulated murine macrophages. This study was designed to investigate the effect of quercetin on the NO production and how quercetin regulates oxidative stress, NFκB activation, and iNOS expression as its possible mechanisms of NO suppression in LPS-stimulated macrophage cell line.

*Corresponding author: Tel: 82-55-320-3235; Fax:
E-mail: fdsnsong@inje.ac.kr
Received August 13, 2004; accepted December 11, 2004

Materials and methods

Materials Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), and TRIzol® reagent were obtained from Gibco BRL (Gaithersburg, MD, USA). MMLV first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), Taq DNA polymerase and PCR reagents (Perkin-Elmer, Boston, MA, U.S.A), NFκB-specific oligonucleotide and T_4 polynucleotide kinase (Promega, Madison, WI, USA), and microspin G-25 column (Amersham Inc., Piscataway, NJ, USA) were used for reverse transcription-polymerase chain reaction (RT-PCR) and electrophoretic mobility shift assay (EMSA). All other reagents including lipopolysaccharide (LPS), glutamine, quercetin, DMSO, protease inhibitor cocktail, NADPH, and Griess reagent were obtained from Sigma Chemicals (St. Louis, MO, USA). The murine macrophage cell line RAW 264.7 (#TIB-71) was obtained from American Type Culture Collection (Rockville, MD, USA).

Cell culture and treatment The murine macrophage cell line RAW 264.7 was cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. Cells in 100-mm dishes (5×10^6 cells/dish) or 24-well plates (4×10^5 cells/well) were pre-incubated with and without quercetin (10, 20, and 50 μM) for 2 hr, then further incubated with LPS (2 μg/ml) for 20 hr at 37°C in a humidified atmosphere containing 5% CO_2. Untreated and control indicate

negative control without LPS and positive control treated with LPS, respectively.

Cell viability Cell viability was assessed by measuring the uptake of the supravital dye neutral red by viable cells according to the procedure of Fautz *et al.* (19). After culturing the cells as described previously, the medium was removed and replaced with 0.5 ml fresh medium containing 1.14 mmol/L neutral red. After incubation for 3 hr, the medium was removed, and the cells were washed twice with phosphate buffered saline solution (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 ml cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the amount of dye taken up, the cell lysis products were centrifuged, and absorbance of the supernatant was measured spectrophotometrically at 540 nm.

NO production The nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction (20). One hundred microliters of each medium supernatant was mixed with 50 μl of 1% sulphanilamide (in 5% phosphoric acid) and 50 μl of 0.1% naphthylenediamine dihydrochloride, and incubated at room temperature for 10 min. The absorbance at 550 nm was measured using the $NaNO_2$ serial dilution standard curve, and nitrite production was determined.

Glutathione (GSH) concentration GSH was measured by an enzymatic recycling procedure of Tietze (21), in which GSH is sequentially oxidized by 5,5″-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase. The medium was removed from the cultured RAW cells, and the cells were washed twice with PBS, added with 1 ml PBS, and scraped. Cell suspensions were sonicated three times on ice for 5 sec each time, and the cell sonicates were centrifuged at 2,300 × g for 10 min. Four hundred microliters cell supernatant was mixed with 100 μl of 5% sulfosalicylic acid and centrifuged at 2,300 × g for 10 min. Fifty microliters supernatant was mixed with 100 ml reaction mixture [100 mM sodium phosphate buffer with 1 mM EDTA (pH 7.5), 1 mM dithiolnitirobezene, 1 mM NADPH, 1.6 U GSH reductase]. The rate of formation of 2-nitro-5-thio-benzoic acid was monitored spectrophotometrically at 412 nm. GSH content was determined by comparing the rate to a standard curve generated with known amount of GSH.

Antioxidant enzyme assays Cells (5×10^6 cells/dish) in 100-mm dishes were preincubated with and without quercetin for 2 hr, then further incubated with LPS (2 μg/ml) for 20 hr. The medium was removed, and the cells were washed twice with PBS, added with 1 ml of 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.0), and scraped. Cell suspensions were sonicated three times on ice for 5 sec each, and the cell sonicates were centrifuged at 10,000 × g for 20 min at 4°C. Cell supernatants were used for antioxidant enzyme activities. The protein concentration was measured using the method of Bradford assay (22) with bovine serum albumin as the standard. Superoxide dismutase (SOD) activity was determined by monitoring the auto-oxidation of pyrogallol according to the method of Marklund *et al.* (23). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol. The reduction of the catalase activity by 50% was measured according to the method of Aebi based on the decrease in absorbance of H_2O_2 (24). The decrease in absorbance at 240 nm was measured for 2 min. Standards containing 0, 0.2, 0.5, 1, and 2 mM H_2O_2 were used to construct a standard curve. Glutathione peroxidase (GSH-px) activity was assayed by the method of Paglia and Valentine (25). A unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmole NADPH consumed per min. GSH reductase activity was measured based on the oxidation of NADPH according to the method of Inger and Bengt (26). One unit of GSH reductase was defined as the amount of enzyme that catalyzes the reduction of 1 nmole NADPH per min.

Expressions of iNOS by RT-PCR Total RNA was isolated using Trizol-reagent and quantitated spectrophotometrically at 260 nm. Total RNA (2 μg) was used to produce the first strand cDNA using MMLV first strand cDNA synthesis kit. PCR was carried out in 50 μl reaction mixture containing the first strand cDNA, 10 × PCR buffer, 2.5 mm dNTP, 20 pM each primer, and Taq DNA polymerase. PCR was performed for 27 cycles. The expressed house-keeping gene GAPDH served as the control. Primer sequences and PCR product size of iNOS and GAPDH are shown in Table 1. The PCR products specific for each cDNA were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The intensity of the bands were densitometrically measured using the Gel Doc EQ System (Bio-Rad, Hercules, CA, USA).

EMSA Nuclear protein was extracted by the slightly modified method of Dignam *et al.* (27). Cells in 100-mm dishes were lysed with a buffer containing 0.6% igepal, 0.15 M NaCl, 10 mM Tris (pH 7.9), 1 mM EDTA, and 0.1% protease inhibitor cocktail, vortexed, kept on ice for 5 min, and centrifuged at 500 × g for 5 min at 4°C. Pelleted nuclei were resuspended in 50 μl extraction buffer

Table 1. List of primer sequence and product size

Name	Product Size (bp)	Sequence (5′- 3′)	Annealing temperature (°C)
iNOS	920	F 5′-GCC TTC AAC ACC AAG GTT GTC TGC A-3′ R 5′-TCA TTG TAC TCT GAG GGC TGA CAC A-3′	59
GAPDH	375	F 5′-CAA TGC CAA GTA TGA TGA CAT-3′ R 5′-CCT GTT ATT ATG GGG GTC TG-3′	49

[10 mM Hepes, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM DTT, and 0.33% protease inhibitor cocktail]. The samples were kept on ice for 20 min with gentle mixing, and were centrifuged at 500 × g for 5 min at 4°C. The supernatant fraction was transferred into new tubes and stored at -70 °C. Protein concentration was determined by Bradford assay (22). NFκB-specific oligonucleotide was end-labeled with [γ-^{32}P]-ATP using T_4 polynucleotide kinase, and purified using microspin G-25 column for EMSA, which was performed according to the instruction manual of Promega. Nuclear protein (5 μg), binding buffer, ^{32}P-labeled NFκB, and loading buffer were incubated for 30 min at room temperature. DNA-protein complexes were separated from the unbound DNA probe by 4% polyacrylamide gel electrophoresis using 0.5X Tris-Borate EDTA buffer as the running buffer. The gels were exposed to a phospho screen (Perkin-Elmer, Boston, MA, USA) for 2 hr at -20°C, and the bands were quantitated by a phospho imager (Packard, Meriden, CT, USA).

Statistical analysis All data are expressed as means ± S.D. Statistical analyses were performed using an SPSS program. One-way ANOVA and Duncan's multiple range tests were used to examine the difference between groups. P values <0.05 were considered significant, unless otherwise stated.

Results and Discussion

Cell viability and NO production The cytotoxic effect of quercetin was examined in the presence of LPS using the neutral red assay. Cell viability was >97% at the concentrations treated (data not shown). LPS induced nitrite production, and this induction was suppressed by quercetin treatment. Quercetin pretreatment was found to suppress NO production in a dose-dependent manner ($p < 0.05$) with an IC_{50} of 9.2 μM, and quercetin treatment at 50 μM suppressed NO production to the level of LPS-untreated control (Fig. 1), as confirmed by several studies (28-30). These results indicate suppressive effect of quercetin on NO production is not due to the cell death by quercetin treatment.

iNOS mRNA expression and NFκB activity RT-PCR was performed to examine whether the inhibition of NO production by quercetin was involved in the iNOS mRNA expression. iNOS mRNA expression was highly induced in LPS-stimulated macrophages, whereas it was not detected under normal condition. However, pretreatment of quercetin with LPS suppressed the expression of iNOS mRNA significantly ($p<0.05$) (Fig. 2). EMSA was performed to determine whether quercetin interferes with the binding of NFκB, a transcriptional factor regulating several genes including iNOS, to DNA. Specific DNA binding of NFκB showed that LPS treatment to RAW 264.7 cells enhanced NFκB activation. However, this enhancement in NFκB activity by LPS was inhibited by quercetin treatments at 10-50 μM ($p<0.05$) (Fig. 3).

Quercetin pretreatment suppressed NO production, iNOS mRNA expression, and NFκB activation. Several previous studies implicated quercetin in the suppression of

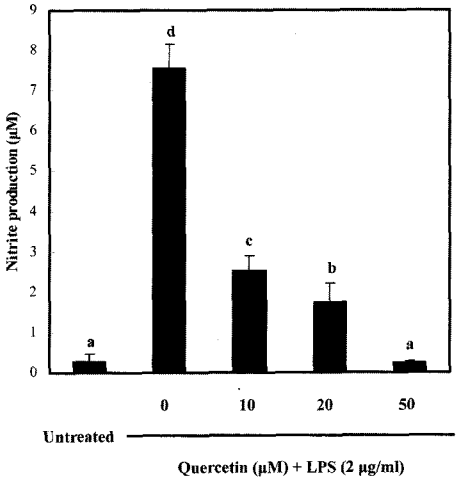

Fig. 1. Inhibitory effect of quercetin on NO production in LPS-stimulated RAW 264.7 cells. Cells (4×10^5 /plate) in 24-well plates were first incubated with and without indicated concentrations of quercetin for 2 hr, and then incubated with LPS (2 μg/ml) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means ± S.D. of triplicate experiments. Values sharing same superscript are not significantly different at $p<0.05$.

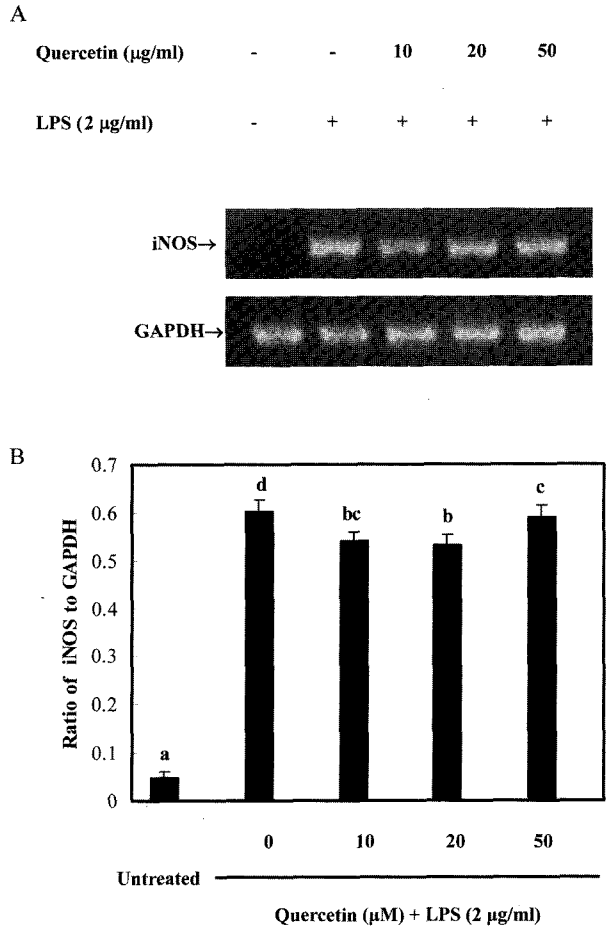

Fig. 2. Effect of quercetin on iNOS gene expression in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 100 mm dishes were first incubated with and without indicated concentrations of quercetin for 2 hr, and then incubated with LPS (2 μg/ml) for 20 hr. Untreated is negative control without LPS treatment. Levels of iNOS mRNA were determined by RT-PCR analysis. GAPDH was used as an internal control.

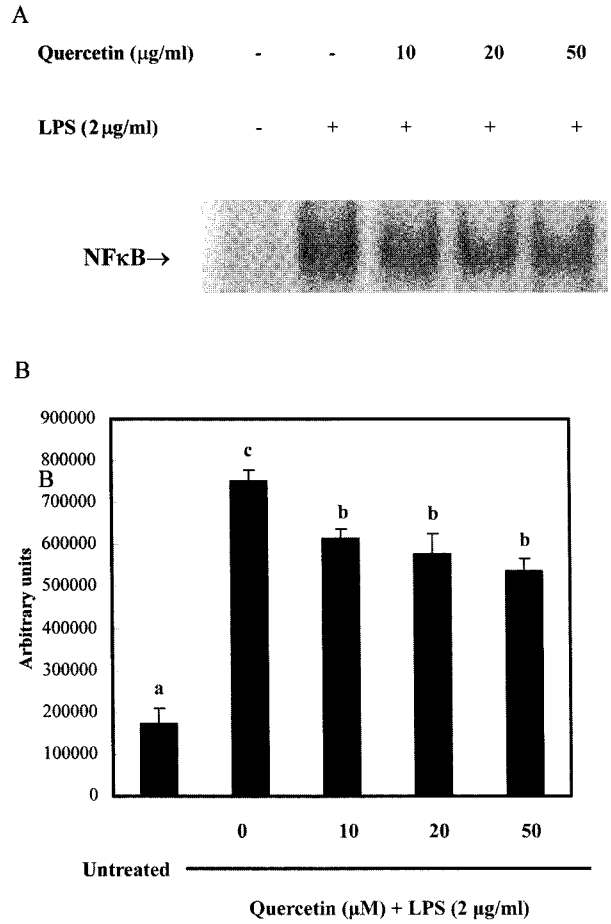

Fig. 3. Effect of quercetin on NFκB activity in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 100 mm dishes were first incubated with and without indicated concentrations of quercetin for 2 hr, and then incubated with LPS (2 μg/ml) for 20 hr. Untreated is negative control without LPS treatment. (A) DNA binding activity of NFκB was performed by EMSA. (B) Values are expressed as relative intensity of radioactivity. Data represent the means ± S.D. of triplicate experiments. Values sharing the same superscript are not significantly different at $p<0.05$.

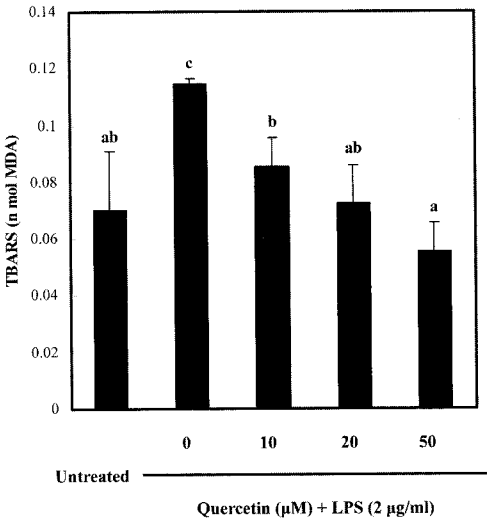

Fig. 4. Inhibitory effect of quercetin on TBARS generation in LPS-stimulated RAW 264.7 cells. Cells (4×10^5 cells) in 24-well plates were first incubated with and without indicated concentrations of quercetin for 2 hr, and then incubated with LPS (2 μg/ml) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means ± S.D. of triplicate experiments. Values sharing same superscript are not significantly different at $p<0.05$.

NO production via down-regulation of iNOS pathway (17, 18). The finding that LPS-stimulated NO production, iNOS mRNA expression, and NFκB activation were suppressed by quercetin suggests that inhibition of LPS-stimulated NO production by quercetin might be mediated through the down-regulation of NFκB, because the activation of NFκB is critical for the induction of iNOS gene expression in macrophages stimulated with LPS (8). In addition, the promoter region of the iNOS gene contains several consensus sequences for the binding of NFκB (9), as supported by the results of our study and those of Cho *et al.* (29), in which the amelioration of LPS-stimulated NFκB activity by quercetin treatments was observed; however, Wadsworth and Koop (28) reported that quercetin had no effect on LPS-induced activation of NFκB. This discrepancy observed might have been caused by the differences in the incubation period and the concentration of quercetin used, because NFκB activity has been reported to be rapid and transient (31).

Early works implicated reactive oxygen species (ROS) in LPS-stimulated NFκB activation, which was largely based on the suppression of oxidative stress and NFκB activation by antioxidants. Indeed, antioxidants such as organosulfur compounds (32), anthocyanins (33), selenium (7), and carnosol in rosemary (8) have been reported to suppress NO production in macrophages, and their inhibition mechanisms are based on their ability to inhibit the activation of NFκB. Furthermore, quercetin is a well known antioxidant. These evidences suggest a role of quercetin as a ameliorator of oxidative stress and NFκB activation. However, quercetin treatment did not show dose-dependent suppressive effects on iNOS expression and NFκB activity, while NO production was suppressed in a dose-dependent manner, which implies that suppression of NO production by quercetin involves different mechanisms as well as down-regulations of iNOS expression and NFκB activation.

Status of oxidative stress and antioxidative enzyme activities To assess the defensive function of quercetin against oxidative stress, markers of oxidative stress status, such as levels of TBARS, GSH, and antioxidative enzyme activities, were investigated. Results show treatment with LPS elevated TBARS level, an indicative of lipid peroxidation (Fig. 4). Pre-incubation with quercetin significantly decreased TBARS ($p<0.05$) in a dose-dependent manner, suggesting that lipid peroxidation was attenuated in LPS-stimulated macrophages by quercetin treatment. Exposure of RAW 264.7 cells to LPS depleted GSH level, whereas GSH levels were enhanced in LPS-treated RAW 264.7 cells pre-incubated with quercetin (Fig. 5). The effects of quercetin on the specific activities of antioxidative enzymes in LPS-stimulated cells are shown in Table 2. Exposure of cells to quercetin before LPS treatment resulted in different behavior to that of the antioxdative enzyme activity. Indeed, activities of catalase and SOD increased dose-dependently, while those of

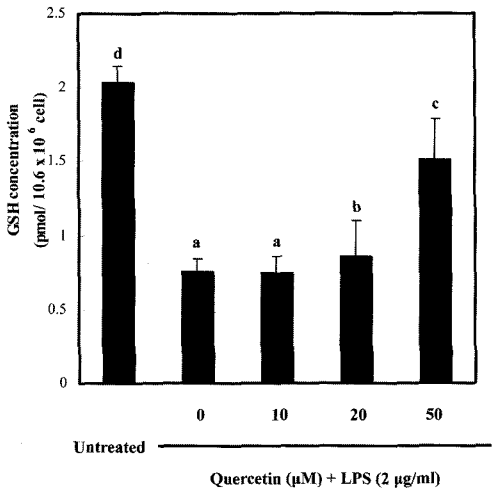

Fig. 5. Effect of quercetin on GSH concentration in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 100 mm dishes were first incubated with and without indicated concentrations of quercetin for 2 hr, and then incubated with LPS (2 μg/ml) for 20 hr. Untreated is negative control without LPS treatment. Data represent the means ± S.D. of triplicate experiments. Values sharing same superscript are not significantly different at $p<0.05$.

GSH-px and GSH-reductase slightly decreased at 10 and 20 mM then increased at 50 mM quercetin. Similar result was obtained from Molina *et al.* (34), who reported that quercetin pretreatment prevented and protected against ethanol-induced oxidative stress in mouse liver. However, the effect of antioxidants on antioxidative enzyme has not yet been fully elucidated. Kameoka *et al.* (35) reported that dietary flavonoids did not affect the antioxidant protein expression in human intestinal Caco-2 cells, while Rohrdanz *et al.* (36) reported that quercetin treatment increased catalase mRNA expression and decreased SOD and GSH-px expressions in hepatome cells.

Our results showed that quercetin pretreatment in LPS-stimulated macrophages suppressed oxidative stress by enhancing GSH and antioxidative enzyme activities and suppressing TBARS and NO production. It was proposed that high NO level along with decreased SOD activity leads to peroxynitrite formation, while the degradation of hydrogen peroxide by catalase and GSH-px ameliorates cytotoxic effects of ROS (37). Furthermore, NO was found to interact with thiol-containing GSH. Depletion of GSH increased the sensitivity of cells to the toxic effects of NO (38). Furthermore, suppression of NO production by flavonoids may be attributed to direct NO- or ROS-scavenging activity (14-17). These findings support antioxidative and free radical-scavenging activities of quercetin.

Based on the findings of this study, the inhibitory effect of quercetin on NFkB activation induced by LPS in RAW 264.7 cells can be explained by the attenuated intracellular oxidative stress in the signaling pathway of LPS to NFκB activation. Indeed, suppressive effect of quercetin on NFκB activation coincides with attenuated NO, TBARS, elevated GSH generation, and enhanced antioxidative enzyme activities. These evidences suggest that suppressed NO production via down-regulation of NFκB activation by quercetin treatments might be attributed in part to antioxidant properties of quercetin. On the other hand, suppressed NO production through the suppressed iNOS expression and NFκB activity by quercetin may contribute to the attenuation of intracellular oxidative stress in reverse by ameliorating peroxynitrite formation of NO. Thus, suppressed NO production and ameliorated oxidative stress by quercetin treatment support that dietary quercetin could prevent damages of many biological molecules including DNA (40) and NO-related inflammatory diseases (1, 2).

In summary, quercetin may attenuate intracellular oxidative stress by delaying the consumption of cellular GSH and enhancing antioxidative enzyme activities, which result in the suppression of NFκB activation, iNOS expression, and NO production by LPS. These results demonstrate that quercetin may ameliorate inflammatory diseases by suppressing NO production through the inhibition of iNOS expression and NFκB transactivation, which may be mediated by antioxidative effect of quercetin. Thus, quercetin appears to be a potential therapeutic agent for treating LPS-induced inflammatory processes.

Acknowledgments

This study was funded by the Research Institute of Inje University (2004), Ministry of Science and Technology (MOST), and the Korea Science and Engineering Foundation (KOSEF) through the Biohealth Products Research Center (BPRC), Inje University, Korea.

References

1. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL,

Table 2. Effect of quercetin on antioxidative enzyme activities in LPS-stimulated RAW 264.7 cells

	untreated	Quercetin (μM) + LPS [5](2 μg/ml)			
		0	10	20	50
Catalase (μmole/ mg protein/min)	0.12±0.00[a1]	0.13±0.01[b]	0.14±0.01[c]	0.15±0.00[c]	0.24±0.00[d]
SOD[2] (unit/ mg protein)	25.6±2.7[a]	26.4±3.0[a]	28.1±0.0[a]	29.0±1.3[a]	82.6±4.3[b]
GSH-px[3] (unit/ mg protein)	4.26±0.51[a]	3.43±0.56[a]	3.47±0.50[a]	3.50±0.00[a]	6.86±2.12[b]
GSH-reductase[4] (unit/ mg protein)	100.5±1.1[b]	77.6±1.3[a]	99.9±1.8[b]	112.1±5.3[c]	114.1±5.3[c]

[1]Data represent the means ± S.D. of triplicate experiments. A value sharing same superscript is not significantly different at $p<0.05$
[2]Superoxide dismutase
[3]Glutathione peroxidase
[4]Glutathione reductase
[5]Lipopolysaccharide

Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: Basic Mechanisms: Oxidation, Inflammation, and Genetics. Circulation 91: 2488-2496 (1995)
2. Albrecht EW, Stegeman CA, Tiebosch AT, Tegzess AM, Van Goor H. Expression of inducible and endothelial nitric oxide synthases, formation of peroxynitrite and reactive oxygen species in human chronic renal transplant failure. Am. J. Transplant. 2: 448-453 (2002)
3. Shen SC, Lee WR, Lin HY, Huang HC, Ko CH, Yang LL, Chen YC. In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E(2) production. Eur. J. Pharmacol. 446: 187-194 (2002)
4. Lee SK, Hong CH, Huh SK, Kim SS, Oh OJ, Min HY, Park KK, Chung WY, Hwang JK. Suppressive effect of natural sesquiterpenoids on inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) activity in mouse macrophage cells. J. Environ. Pathol. Toxicol. Oncol. 21: 141-148 (2002)
5. Stolz DB, Zamora R, Vodovotz Y, Loughran PA, Billiar TR, Kim YM, Simmons RL, Watkins SC. Peroxisomal localization of inducible nitric oxide synthase in hepatocytes. Hepatology 36: 81-93 (2002)
6. Hu HL, Forsey RJ, Blades TJ, Barratt ME, Parmar P, Powell JR. Antioxidants may contribute in the fight against ageing: an in vtro model. Mech. Ageing Dev. 121: 217-230 (2000)
7. Prabhu KS, Zamamiri-Davis F, Stewart JB, Thomson JT, Sordillo LM, Reddy CC. Selenium deficiency increases the expression of inducible nitric oxide synthase in RAW 264.7 macrophage: role of nuclear factor-κB in up-regulation. Biochem. J. 366: 203-209 (2002)
8. Lo AH, Liang YC, Lin-Shiau SY, Ho CT, Lin JK. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. Carcinogenesis 23: 983-991 (2000)
9. Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-α and COX-2 expression by suchinone effects on I-κBα phosphorylation, C/EBP and AP-1 activation. British J. Phamacol. 139: 11-20 (2003)
10. Bowie AG, Moynagh PN, O'Neill LA. Lipid peroxidation is involved in the activation of NF-κB by tumor necrosis factor but interleukin-1 in the human endothelial cell line ECV304. J. Biol. Chem. 272: 25941-25950 (1997)
11. Manna SK, Zhang HJ, Yant T, Oberley LW, Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-κB and activated protein-1. J. Biol. Chem. 273: 13245-13254 (1998)
12. Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inbibitors of nuclear factor kappa B activation in intact cells. J. Exp. Med. 175: 1181-1194 (1992)
13. Bonizzi G, Piette J, Schoonbroodt S, Greimers R, Havard L, Merville MP, Bours V. Reactive oxygen intermediate-dependent NF-κB activation by interleukin-1β, requires 5-lipoxygenase or NADPH oxidase activity. Mol. Cell Biol. 19: 1950-1960 (1999)
14. Chen YT, Zheng RL, Jia ZJ, Ju Y. Flavonoids as superoxide scavengers and antioxidants. Free Radic. Bio. Med. 9: 19-21 (1990)
15. Hirano R, Sasamoto W, Matsumoto A, Itakura H, Igarash O, Kondo K. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. J. Nutr. Sci. Vitaminol. (Tokyo). 47: 357-362 (2001)
16. Filipe P, Lanca V, Silva JN, Morliere P, Santus R, Fernandes A. Flavonoids and urate antioxidant interplay in plasma oxidative stress. Mol. Cell Biochem. 221: 79-87 (2001)
17. Matsuda H, Morikawa T, Ando S, Toguchida I and Yoshikawa M. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. Bioorganic Med. Chem. 11: 1995-2000 (2003)
18. Chan MM, Mattiacci JA, Hwang HS, Shah A, Fong D. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol in the inhibition of the inducible nitric oxide synthase pathway. Biochem. Pharmacol. 60: 1539-1548 (2000)
19. Fautz R, Husen B, Hechenberger C. Application of the neutral red assay(NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test(UDS). Mutat. Res. 253: 173-179 (1991)
20. D'Agostino P, Ferlazzo V, Milano S, La Rosa M, Di Bella G, Caruso R, Barbera C, Grimaudo S, Tolomeo M, Feo S, Cillari E. Anti-inflammatory effects of chemically modified tetracyclines by the inhibition of nitric oxide and interleukin-12 synthesis in J774 cell line. Inter. Immunopharmacol. 1: 1765-1776 (2001)
21. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidaized glutathione: applications to mammalian blood and other tissue. Anal. Biochem. 27: 502-522 (1969)
22. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Ann. Biochem. 72: 248-254 (1976)
23. Marklund S, Marklund G. Involvement of the superoxide anion radical in antioxidant of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47: 469-474 (1974)
24. Abei H. Catalase Vol. II, pp. 165-166. In: Methods of Enzymatic Analysis. Bergmeyer HU (ed.) Academic Press, New York, NJ., USA (1983)
25. Paglia ED, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxidases. J. Lab. Clin. Med. 70: 158-169 (1967)
26. Inger C, Bengt M. Methods in enzymology. Academic press Inc 113: 484-490 (1985)
27. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract isolated from mammalian nuclei. Nucleic acids Res. 11: 1475-1489 (1983)
28. Wadsworth TL, Koop DR. Effects of Ginkgo biloba extract (GBb 761) and quercetin on lipolysaccharide-induced release of nitric oxide. Chemico-Biologicla Interactions 137: 43-58 (2001)
29. Cho SY, Park SH, Kwon MJ, Jeong TS, Bok SH, Choi WY, Jeong WI, Ryu SY, Do SH, Lee CS, Song JC, Jeong KS. Quercetin suppressed proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipolysaccharide-stimulated macrophage. Mole. Cell Biochem. 243: 153-160 (2003)
30. Olszanecki R, Gebska A, Kozlovski VI, Gryglewski RJ. Flavonoids and nitric oxide synthase. J. Physiol. Pharmacol. 53: 571-584 (2002)
31. Caivano M, Gorgoni B, Cohen P, Poli V. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein β (C/EBPβ) and C/EBPδ transcription factors. J. Biol. Chem. 276: 48693-48701 (2001)
32. Ippouchi K, Itou H, Azuma K, Higashio H. Effect of naturally occurring organosulfur compounds on nitric oxide production in lipopolysaccharide-activated macrophages. Life Sci. 71: 411-419 (2002)
33. Wang J, Mazza G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-γ-activated RAW264.7 macrophages. J. Agric. Food Chem. 50: 850-857 (2002)
34. Molina MF, Sanchez-Reus I, Iglesias I, Benedi J. Quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse liver. Biol. Pharm. Bull. 26: 1398-1402 (2003)
35. Kameoka S, Leavitt P, Chang C, Kuo SM. Expression of antioxidant proteins in human intestinal Caco-2 cells treated with dietary flavonoids. Cancer Lett. 146: 161-167 (1999)
36. Rohrdanz E, Bittner A, Kahl QTR. The effects of quercetin on the mRNA expression of different antioxidant enzymes in hepatoma cells. Arch. Toxicol. 77: 506-510 (2003)
37. Iqbal M, Cohen RI, Marzouk K, Liu SF. Time course of nitric oxide, peroxynitrite, and antioxidants in the endotoxemic heart. Crit. Care. Med. 30: 1291-1296 (2002)
38. Berendji D, Kolb-Bachofen V, Meyer KL, Kroncke KD. Influence of nitric oxide on the intracellular reduced glutathione pool: Different cellular capacities and strategies to encounter nitric oxide-mediated stress. Free Radic. Biol. Med. 27: 773-780 (1999)
39. Lee JC, Kim J, Park JK, Chung GH and Jang YS. The anti-

oxidant, rather than prooxidant, activities of quercetin on normal cells: quercetin protects mouse thymocytes from glucose oxidase-mediated apoptosis. Exp. Cell Res. 291: 386-397 (2003)
40. Yen GC, Lai HH. Inhibitory effects of isoflavones on nitric oxide-or peroxynitrite-mediated DNA damage in RAW 264.7 cells and ΦX174DNA. Food Chem. Toxicol. 40: 1433-1440 (2002)