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Effects of Welsh onion on oxidation of low-density lipoprotein and nitric oxide production in macrophage cell line RAW 264.7

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Abstract

The effects of aqueous extracts of Welsh onion green leaves (WOE) on oxidation of low-density lipoproteins (LDL) and production of nitric oxide (NO) in macrophage were investigated. The results showed that WOE in the range of 0.1–1.0 mg/ml inhibited LDL oxidation and scavenged $ABTS^{\cdot+}$ radical in an acellular system. Moreover, the antioxidant activity of WOE correlated well with the total polyphenolic content ($r = 0.968$). In addition, WOE in the range of 0.1–1.0 mg/ml inhibited lipopolysaccharide (LPS)-induced NO production in a concentration-dependent manner. The induction of iNOS and COX-2 proteins in RAW 264.7 cells was markedly suppressed by WOE. WOE at 1 mg/ml significantly inhibited iNOS mRNA expression, as determined by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, LPS-induced nuclear factor-kappa B (NF-κB) activation, through IκB-α degradation, was reduced by WOE. These results suggest that WOE could attenuate excessive NO and prostaglandin generation in macrophages and lipoprotein oxidation in vitro.

Keywords: Welsh onion; Low-density lipoprotein; Polyphenolic content; Nitric oxide; iNOS; COX-2

1. Introduction

There is enough evidence to indicate that oxidative free radicals cause oxidative damage to carbohydrates, proteins, lipids and DNA (Burke & Fitzgerald, 2003; Nakao, Iwai, Kalil, & Augusto, 2003). These potential harmful effects have been proposed to be involved in the pathogenesis of various diseases, including coronary artery diseases and cancers (Oikawa, Murakami, & Kawanishi, 2003; Weinbrenner et al., 2003). In recent years, oxidative modification of low-density lipoproteins (LDL) has been considered to play an important role in the initiation and progression of atherosclerosis and cardiovascular diseases (Heinecke, 1998). Therefore, it is urgent to decrease oxidation modification of LDL if we are to avoid the crises of coronary artery diseases. Many reports have suggested that dietary antioxidants (e.g. polyphenol and flavonoid) have some protective effects against LDL oxidation (Fuhrman & Aviram, 2001) and atherosclerosis progression (Gottstein et al., 2003). Furthermore, numerous studies have indicated that garlic and onion, members of the *Allium* family, may be used as anti-atherosclerosis (Campbell, Efendy, Smith, & Campbell, 2001), anti-thrombotic (Ali and Mohammed, 1986; Jung et al., 2002) and anti-hyperlipemic agents (Liu & Yeh, 2001; Yeh & Liu, 2001). Welsh onion (*Allium fistulosum* L., Alliaceae) is reported to lower blood pressure (Chen, Chen, Tsai, & Jen, 2000a,

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2000b), modulate aortic vascular tone (Chen, Tsai, & Chen, 1999) and inhibit platelet aggregation (Chen et al., 2000a, 2000b). Although some of the biological functions of Welsh onion have been demonstrated, data on LDL oxidation have been few. Therefore, investigation of the antioxidant activity against human LDL oxidation is needed.

Except for LDL oxidation, many studies have shown that reactive nitrogen species (e.g. nitric oxide; NO) are generated during the initiation of atherosclerosis, particularly by macrophages (Alfon, Guasch, Berrozpe, & Badimon, 1999; Aliev et al., 2001; Behr-Roussel et al., 2000; De Meyer et al., 2002). NO is formed by nitric oxide synthase (NOS). In contrast to the constituted neuronal NOS (nNOS) and endothelial NOS (eNOS), activated inflammatory cells produce nitric oxide through inducible isoform NOS (iNOS). At low concentrations, NO plays a vital role in the regulation of the vascular and immune system. However, excessive levels of NO generated can be toxic, causing either circulatory shock (Md, Moochhala, & Siew-Yang, 2003) or oxidative damage of DNA (Inano & Onoda, 2003). Thus, the inhibition of NO overproduction represents an important goal. In addition, the iNOS gene from macrophages has been cloned and characterized (Chartrain et al., 1994). A sequence for the transcription factor NF-κB/Rel binding side has been recognized as the promoter of the gene encoding iNOS (Xie, Kashiwabara, & Nathan, 1994). In the cytoplasm, masking NF-κB activation occurs, as a result of an inhibitory molecule of the IκB family bound to NF-κB (Karin & Ben-Neriah, 2000). Upon stimulation, dissociation of IκB from the NF-κB complex and a rapid degradation of IκB, caused by protease, results in NF-κB activation. Previous studies have shown that some natural antioxidants, such as flavonoids in fruits and vegetables, contribute in part to the inhibition of NO production (Kim, Cheon, Kim, Kim, & Kim, 1999). Kobuchi, Droy-Lefaix, Christen, and Packer (1997) reported that Ginhgo biloba extract acts as a potent inhibitor of NO production. However, whether Welsh onion could affect NO production in activated macrophages is still unclear. Thus, this study aimed to explore the effects of aqueous extract of Welsh onion green leaves (WOE) on oxidation of LDL and production of NO in macrophages.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, *Escherichia coli* 0127:B8), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, (3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyl-tetrazolium bromide) and 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trolox was purchased from Aldrich Chemical Co. (St. Louis, MO, USA). Trizol reagent was obtained from Life Technologies (California, USA).

2.2. Cell culture

RAW 264.7 cells, an Abelson virus-transformed murine macrophage cell line (American Type Culture Collection), was cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 0.002 M L-glutamine, 0.001 M pyruvate and maintained in humidified 5% CO_2/95% air at 37 °C. For immunoblot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, cells were grown in a 6-well plate (1×10^6 cells/well) in 2 ml of growth medium for 18 h to allow the cell number to approximately double. Then, the growth medium was replaced and WOE was added with or without the presence of LPS.

2.3. Preparation of Welsh onion green leaves extracts

Briefly, mature Welsh onions were used. The green leaves of Welsh onion were squeezed and filtered by a food processor (National, Model MJ-C85; Tokyo, Japan; pore size of the filter $r=0.2$ mm), 10 times, to obtain the juice. After centrifugation for 30 min at 10,000*g*, the supernatant was filtered, frozen at −80 °C, and then lyophilized for 24 h. This dehydrated powder was then dissolved in phosphate buffer saline. 28 batches of Welsh onion were mixed well, and one batch of these mixture samples was used for the entire set of experiments (Chen et al., 2000a, 2000b).

2.4. Low-density lipoproteins – preparation and oxidative modification

Human LDL ($d=1.02-1.06$ g/ml) was prepared from fasting plasma, routinely pooled from 5 healthy normolipemia individuals. Lipoproteins were isolated by sequential preparative ultra-centrifugation, as previously described, and oxidized (Viana et al., 1996). Prior to the oxidation experiments, LDL was dialyzed overnight against phosphate buffer saline, pH 7.4, containing 100 μM ethylenediaminetetraacetic acid. Freshly prepared native LDL (0.1 mg/ml) was treated with 10 μM $CuSO_4$ for up to 24 h at 37 °C and oxidation was stopped by addition of butylated hydroxytoluene. The degree of lipoprotein oxidation was determined in the presence or in the absence of different concentrations (0.1–1.0 mg/ml) of WOE and trolox for comparison. Lipid peroxidation was analyzed by thiobarbituric acid-reactive substance assay as previously described, by measuring absorbance at 532 nm

(Halliwell & Chirico, 1993). The calibration was done with a malondialdehyde standard solution prepared from 1,1,3,3-tetramethoxypropane. The results were expressed as percentage inhibition of LDL oxidation as determined by the following formula:

$$\%\text{Inhibition} = [(B - W)/B] \times 100,$$

where B is the MDA produced by $CuSO_4$ treatment alone, and W is the MDA produced with WOE or trolox addition.

2.5. Determination of the trolox equivalent antioxidant capacity

This method is based on the capacity of WOE to scavenge the 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation compared with trolox in a concentration-dependent response. The determination of $ABTS^{\cdot+}$ radical-scavenging was carried out as previously described (Arnao, Cano, & Acosta, 2001). The $ABTS^{\cdot+}$ radical was generated by reacting 0.001 M ABTS, 500 μM hydrogen peroxide and 10 units/ml horseradish peroxidase in the dark for 2 h at 30 °C. After 1 ml $ABTS^{\cdot+}$ was added to WOE or trolox standards, the absorbance at 734 nm was recorded after 10 min. The radical-scavenging capacity was plotted as a function of concentration and the trolox equivalent antioxidant capacity (TEAC) was calculated against a trolox calibration curve.

2.6. Total polyphenolics assay

Total polyphenolics were determined as gallic acid equivalents (Parejo et al., 2003). The different concentrations of WOE were transferred to a 10 ml volumetric flask, to which 2 ml sodium carbonate (20% (w/v)) were added. After 5 min, 0.1 ml Folin–Ciocalteu reagent (50% (v/v)) was added and the volume were made up to 10 ml with H_2O. After a 1 h incubation at 30 °C, the absorbance were measured at 750 nm and compared to a gallic acid calibration curve.

2.7. Measurement of nitric oxide/nitrite

Nitrite levels in the cultured media, which reflect intracellular nitric oxide synthase activity, were determined by Griess reaction (Kim et al., 1995). Briefly, cells were dispensed into 96-well plates and 100 μl of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined by using an Anthos 2010 microplate reader. Nitrite concentration was determined, using sodium nitrite as a standard.

2.8. MTT assays

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide) assay was performed as previously described. MTT is a tetrazolium salt and is converted to an insoluble formazan by mitochondrial dehydrogenase of living cells. Briefly, cells were dispensed into 96-well plates and WOE was added with or without LPS present for 24 h. Then, 20 μl MTT (5 mg/ml stock solution) were added to each well. After 1 h, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of dimethyl sulfoxide. The optical density of each well was measured with an Anthos 2010 microplate reader at 570 nm.

2.9. Western blot

The method described previously, was followed (Lin, Tsai, Lin-Shiau, Ho, & Lin, 1999). After stimulation, cells were washed with ice-cold phosphate buffer saline, and then cells were treated with lysis buffer. Cellular lysates were centrifuged at 10,000*g* at 4 °C for 20 min. The supernatants were collected and the protein contents were determined by using the BCA protein assay kit (Piece, USA). Each sample, which contained 50 μg protein, was separated on 8% SDS–polyacrylamide gels. After electrophoresis, gels were transferred to nitrocellulose paper. After washing with distilled water, the membrane was incubated with 5% albumin in phosphate buffer saline (containing 0.1% Tween-20) and then immunoblotted as described with mouse monoclonal anti-iNOS antibody (Santa Cruz, USA), rabbit polyclonal anti-COX-2 antibody (Upstate, USA), rabbit polyclonal anti-IκB-α antibody (Santa Cruz, USA) or mouse monoclonal anti-Tubulin antibody (Sigma, USA). Blots were then incubated with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Santa Cruz, USA) and visualized using an enhanced chemiluminescence (ECL) kit (Amersham, USA).

2.10. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells by using Trizol reagent (Life Technologies, California, USA) according to the manufacturer's instructions. Then, RNA concentrations were determined spectrophotometrically and 5 μg RNA was converted to cDNA with 1 μM oligo(dT)15, 500 μM of dNTP, 0.05 M Tris–HCl, pH 8.3, 0.075 M KCl, 0.003 M $MgCl_2$, RNase inhibitor (1 unit/μl) and moloney-murine leukemia virus reverse transcriptase (10 unit/μl) at 42 °C for 1 h. The same amount of the resulting cDNA was then used for amplification by specific primers for iNOS and GAPDH. The amplification of cDNA was performed by incubating in 0.1 M Tris–HCl buffer, pH 8.3, containing 0.5 M KCl, 0.015 M $MgCl_2$,

0.1% gelatin, 200 μM dNTPs and 50 units/ml of Super Taq DNA polymerase with the following mouse iNOS primers: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′. The cDNA sequence of GAPDH was also amplified as control in a similar way using as primers 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. A thermal cycle of 45 s at 95 °C, 45 s at 65 °C and 2 min at 72 °C was used for 30 cycles. PCR products were analyzed on 1.8% agarose gels. Amplified cDNA bands were detected by ethidium bromide staining (Lin & Lin, 1997).

2.11. Statistical analysis

All data were recorded as means ± SD and statistical significance between the groups was assessed by using one-way analysis of variance (ANOVA) followed by pairwise comparison with a post hoc test with Bonferroni correction. The level of significance was chosen as $P<0.05$.

3. Results

3.1. Inhibition of LDL oxidation in vitro and scavenging $ABTS^{\cdot+}$ radicals

Fig. 1 shows the antioxidant capacity of WOE on LDL oxidation induced by Cu^{2+}. In the range of 0.1–1.0 mg/ml of WOE used, the protective effect against LDL oxidation increased with increasing concentration of WOE. The antioxidant capacity of trolox was more effective than WOE on LDL oxidation. WOE at 1.0 mg/ml inhibited the copper-induced LDL oxidation by 100% compared with the control, indicating that WOE had a remarkable inhibitory effect on LDL oxidation.

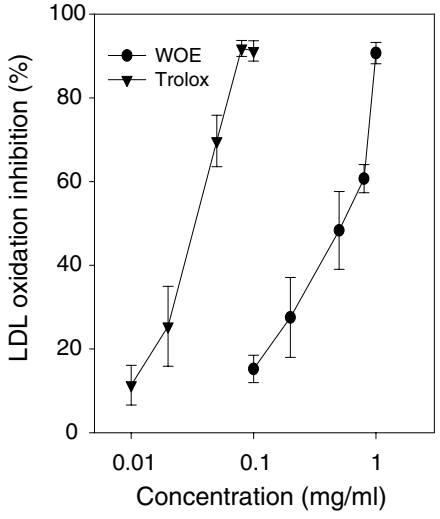

Fig. 1. Antioxidant capacity of aqueous extracts of WOE and trolox on cupric-catalyzed LDL-oxidation in vitro. LDL-oxidation was measured by the protocol described in Section 2. Each point represents means ± SD of six experiments. Results were analyzed by ANOVA ($P<0.05$).

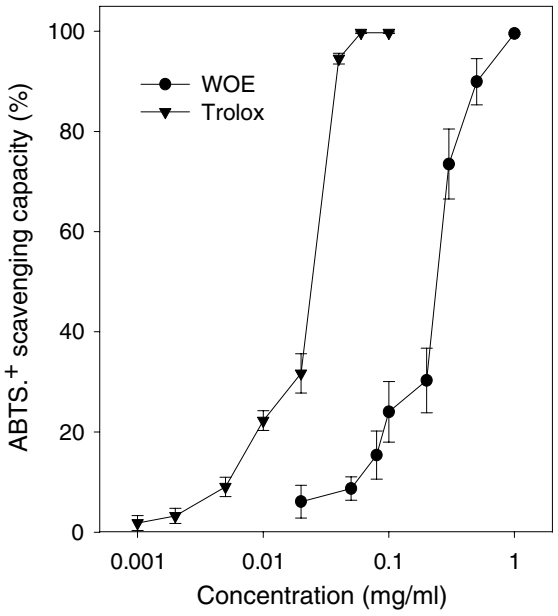

Fig. 2. Total antioxidant activity of aqueous extracts of WOE and trolox on scavenging $ABTS^{\cdot+}$ radical assay. Extent of scavenging $ABTS^{\cdot+}$, measured as described above. Each point represents means ± SD of six experiments. Results were analyzed by ANOVA ($P<0.05$).

The total antioxidant activity of WOE was evaluated by TEAC assay, and the results are shown in Fig. 2. The total antioxidant activity was increased by WOE in a concentration-dependent manner. This result also shows that WOE scavenged $ABTS^{\cdot+}$.

Fig. 3 shows the polyphenolic contents in WOE and their total antioxidant activity. As can be seen, the polyphenolic contents and total antioxidant activity were increased in a concentration-dependent manner. The equation of polyphenolic contents (X) and the total antioxidant activity (Y) used is $Y=5.070X-3.675$ ($r=0.968$, $P<0.05$), indicating that the total antioxidant activity correlated well with polyphenolic contents. This implied that a high positive correlation existed between antioxidant activity of WOE and polyphenolic contents.

3.2. Inhibition of NO production in LPS-stimulated macrophages

The ability of WOE to influence NO production in RAW 264.7 macrophages was investigated. The efficacy of WOE for nitrite production in macrophage is

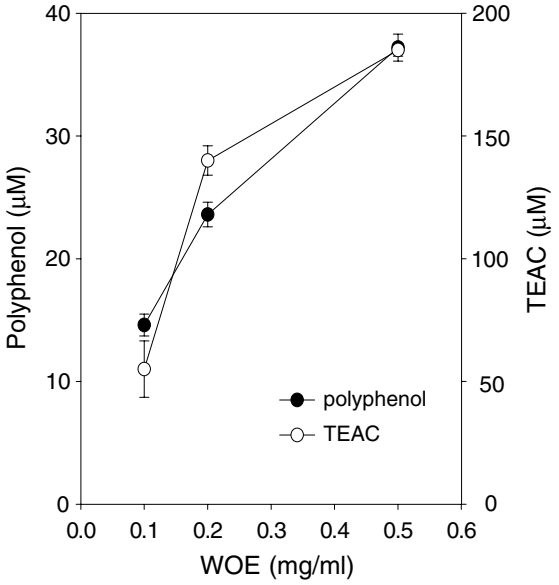

Fig. 3. The polyphenolic content and total antioxidant activity in aqueous extracts of WOE. The activity was measured by TEAC protocol and calculated as described above. Data were analyzed by ANOVA ($P<0.05$).

shown in Fig. 4A. RAW 264.7 cells were cultured in 96-well plates. When LPS (1 μg/ml) was added to RAW 264.7 cells, NO production, measured as nitrite, was increased dramatically up to 142 ± 11 nmole/10^6 cells for 24 h from the basal level of 11 ± 1 nmole/10^6 cells without LPS. NO production from LPS-activated RAW 264.7 cells decreased with increasing concentration of WOE in the range of 0.05–1.0 mg/ml. When RAW 264.7 cells were cultured with 1.0 mg/ml WOE, the NO production was inhibited by 100%. In addition, no effect on cell viability was detected, in the presence of WOE (0.05–0.5 mg/ml), as measured by the MTT assay (Fig. 4B). When WOE, at 1.0 mg/ml, was added to LPS-activated RAW 264.7 cells and incubated for 24 h, it reduced viability of RAW 264.7 cells by 17%.

3.3. Suppression of iNOS and COX-2 proteins production in LPS-stimulated macrophages

Fig. 5 illustrates the inhibitory effect of WOE on iNOS and COX-2 protein induction in LPS-activated macrophages. RAW 264.7 cells were cultured in 6-well plates. LPS, at concentrations of 0.02–0.2 μg/ml, significantly induced iNOS and COX-2 protein production in cells treated without WOE. As shown in Fig. 5, WOE at 0.5 mg/ml decreased LPS (0.02–0.2 μg/ml)-induced iNOS protein production by 98%, 68% and 50%, and decreased LPS- induced COX-2 protein by 98%, 50% and 36%, respectively, compared with the LPS alone (Fig. 5, lanes 5–7).

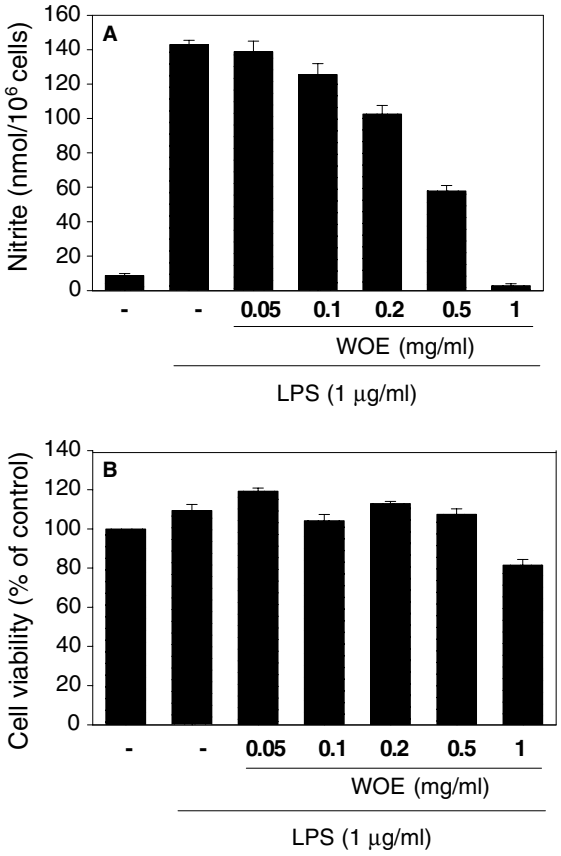

Fig. 4. Effects of aqueous extracts of WOE on nitrite production and cell viability in RAW cells stimulated with LPS. Cells were with or without WOE (0.05–1.0 mg/ml) prior to the addition of LPS (1 μg/ml). Nitrite production (a) and cell viability (b) were measured after 24 h of treatment by the protocol described in above. Each bar represents means ± SD of six experiments. Data were analyzed by ANOVA ($P<0.05$).

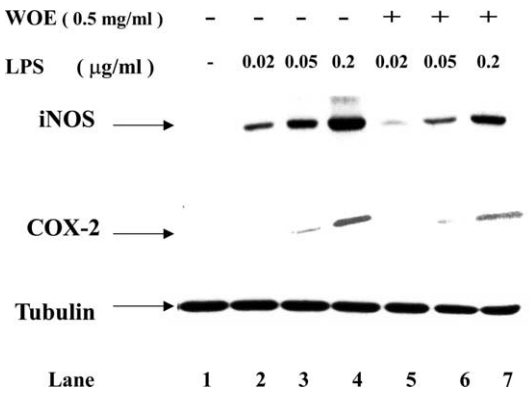

Fig. 5. Effects of aqueous extracts of WOE on iNOS and COX-2 protein expression in RAW cells stimulated with LPS. Cells were with or without WOE (0.5 mg/ml) prior to the addition of LPS (0.02–0.2 μg/ml). After 24 h of treatment, cell lysates were subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblot analysis. One representative blot of three independent experiments is shown.

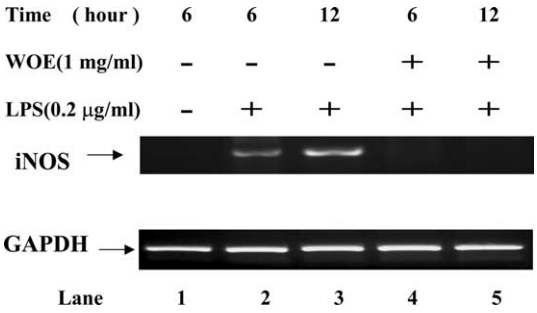

Fig. 6. Effect of WOE on iNOS mRNA production in RAW cells stimulated with LPS. Cells were with or without WOE (1.0 mg/ml) prior to the addition of LPS (0.2 μg/ml). After 3 or 6 h of treatment, respectively, mRNA of iNOS and GAPDH in cells were analyzed by the protocol described in above. A representative of three experiments is shown.

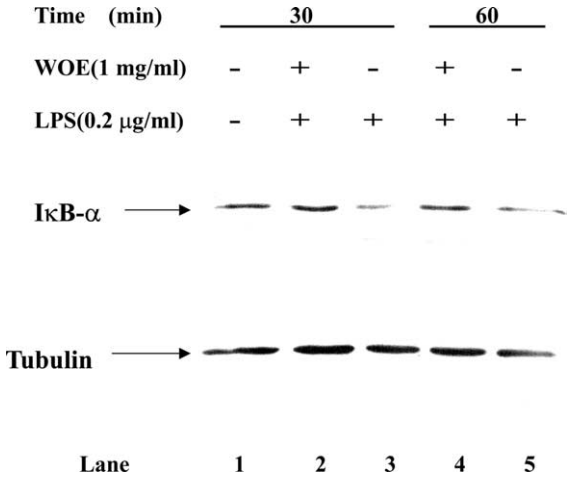

Fig. 7. Effect of WOE on IκB-α protein degradation in RAW cells stimulated with LPS. Cells were with or without WOE (1.0 mg/ml) prior to the addition of LPS (0.2 μg/ml). After 30 or 60 min treatment, respectively, cell lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis. One representative result of three experiments is shown.

3.4. Inhibition of iNOS mRNA production in LPS-stimulated macrophages

To characterize the mechanism responsible for inhibition of NO production by WOE, we investigated whether WOE could directly affect iNOS mRNA production in macrophages. Fig. 6 shows the effect of WOE on iNOS mRNA production in LPS-activated macrophages. RAW 264.7 cells were cultured in 6-well plates. When RAW 264.7 cells were treated with 0.2 μg/ml LPS for 6 and 12 h, respectively, the iNOS mRNA expression was obvious, as expected. However, RT-PCR analysis of LPS-activated macrophages treated with 1.0 mg/ml WOE showed suppression of iNOS mRNA expression (Fig. 6, lanes 4–5).

3.5. Blocking the NF-κB activation pathway in LPS-stimulated macrophages

Fig. 7 shows the effect of WOE on the DNA binding activity of NF-κB in LPS-activated macrophages. RAW 264.7 cells were cultured in 6-well plates. An IκB-α band was detected in extracts obtained from RAW 264.7 cells without being treated with LPS (Fig. 7, lane 1). At 30 and 60 min after activating with 0.2 μg/ml LPS, the band of IκB-α was markedly decreased in macrophages. When the cells were cultured with 1.0 mg/ml WOE, along with LPS, the IκB-α band was increased, compared with cells treated with LPS, indicating that WOE reduced IκB-α degradation.

4. Discussion

The oxidation of LDL is now recognized to play the main role in the initiation and progression of atherosclerosis (Heinecke, 1997). It is well known that vegetables of the *Allium* species exhibit significant protection against LDL oxidation. For example, fresh garlic or garlic extracts have beneficial effects on blood lipid and LDL oxidation (Lau, 2001). In addition, S-allyl cystein and diallyl disulfide in garlic have been proved to scavenge reactive oxygen species (Rabinkov et al., 1998), inhibit LDL oxidation and suppress the formation of atherogenic lesions (Campbell et al., 2001; Kim et al., 2001). However, no reports on the protection against LDL oxidation of Welsh onion have been reported so far. As shown in Fig. 1, under the conditions of LDL copper-oxidation assay used, the addition of WOE had a marked protective effect against LDL oxidation. This result implies that WOE may have potential cardiovascular-protective effects since they reduce the level of LDL oxidation.

The TEAC assay, a radical-scavenging method, is increasingly used, not only to screen compounds, food products and extracts for their antioxidant capacity, but also to assess antioxidant status in biological fluids such as plasma (Re et al., 1999). In the present study, WOE showed antioxidant activity by means of scavenging $ABTS^{\cdot+}$ radicals. This result revealed that WOE contained unknown active compounds, which act as good radical scavengers and inhibit lipid peroxidation. Moreover, comparing the TEAC values with the total polyphenol contents (Fig. 3), it is found that the antioxidant activity of the WOE correlated significantly with the total polyphenol contents ($r = 0.968$). In addition, Miean and Mohamed (2001) have reported that abundant polyphenolic compounds are found in onion leaves. Furthermore, quercetin (130 μg/g) and kaempferol (90 μg/g) were identified in WOE (Chen & Tsai, 1999). Quercetin and kaempferol have been reported to possess greater antioxidant activities than traditional vitamins (Kim, Lee, Lee, & Lee,

2002; Noroozi, Angerson, & Lean, 1998). Therefore, quercetin and kaempferol have been proposed to decrease oxidative modification of LDL (Kostyuk, Kraemer, Sies, & Schewe, 2003) and increase intracellular antioxidant capacity (Myhrstad, Carlsen, Nordstrom, Blomhoff, & Moskaug, 2002). These findings implied that WOE might efficiently enhance the endogenous antioxidant levels due to the presence of quercetin and kaempferol in WOE. Rice-Evans, Miller, Bolwell, Bramley, and Pridham (1995) noted that polyphenolic compounds were mainly active due to their redox properties, allowing them to act as reducing agents, hydrogen donators, and reactive oxygen quenchers. Consequently, it is possible that the total polyphenolic compounds in the WOE were the mainly active compounds contributing to the antioxidant activity. Although previous reports noted that polysulfides, and some organosulfur compounds affecting biofunctional properties, existed in Welsh onion (Kuo, Chien, & Ho, 1990), whether the organosulfur compounds in Welsh onion exhibit antioxidant activity or not merits further study.

It is well known that NO is synthesized from L-arginine by NOS in various animal cells and tissues. According to the data obtained from Fig. 4A, WOE inhibited NO production from LPS-activated RAW 264.7 cells. It is meaningful to consumers that WOE might inhibit NO overproduction, and consequently decrease NO-induced DNA damage as well as cytotoxicity (Inano & Onoda, 2003). In addition, Kumagai et al. (2000) reported that overproduction of COX-2 not only plays a central role in inflammation, but also discloses intracellular oxidative mediators mass production. Hernandez-Presa et al. (2002) noted that inhibition of COX-2 might improve endothelial function in a rabbit model of atherosclerosis. These observations revealed the importance of lower iNOS and COX-2 production in preventing cellular damage from oxidative stress. In order to obtain clear knowledge of the inhibitory mechanism of NO production, the effect of WOE on iNOS and COX-2 enzymes was evaluated. As seen in Fig. 5, WOE did reduce iNOS and COX-2 enzymes expression in a concentration-dependent manner, as revealed by western blotting. Hence, it is strongly suggested that inhibition of NO production by WOE may be attributed to their reduction of iNOS and COX-2 enzymes expression. Moreover, down-regulation of iNOS and COX-2 expression may offer protection against inflammatory reactions and cardiovascular diseases.

The gene expression of iNOS and COX-2 are regulated mainly at the transcriptional level. Both iNOS and COX-2 gene promoters contain a TATA box and numerous consensus sequences for the binding of various transcription factors (Lin, Chang, & McCormick, 1996; Tanabe and Tohnai, 2002). Of these potentially relevant transcription factors, NF-κB has been shown to be functionally important for both iNOS and COX-2 induction (Callejas, Casado, Bosca, & Martin-Sanz, 1999). Events leading to the activation of NF-κB depend on the phosphorylation of IκB-α, followed by its ubiquitination and proteolytic degradation into the proteasome.

According to the data presented above, WOE obviously reduced the iNOS and COX-2 enzyme expression; however, the cellular mechanism of WOE for down-regulating iNOS and COX-2 enzyme in RAW 264.7 cells is still unknown. As seen in Fig. 6, WOE displayed significant suppression of the iNOS mRNA expression. Kim et al. (1999) reported that the down regulation of iNOS enzyme may be due to the translational level of iNOS enzyme expression, or up-stream signal transduction, such as activation/translocation of transcription factors, including NF-κB, being affected. These results imply that, because the NF-κB transcription factor activation is the predominant response for induction of both iNOS and COX-2 expression by LPS in inflammatory cells, it is hypothesized that WOE might reduce iNOS and COX-2 expression by preventing NF-κB activation. As seen in Fig. 7, WOE might prevent NF-κB activation through down-regulated degradation of IκB-α. Liang et al. (1999) noted that kaempferol was a potential inhibitor of iNOS and COX-2 induction at the level of gene transcription. Cho et al. (2003) reported that quercetin suppressed iNOS expression through down-regulated degradation of IκB-α and NF-κB activation. These findings implied that WOE containing quercetin and kaempferol might effectively prevent organ or tissue injury during acute inflammation by suppressing NF-κB-mediated response.

Previous studies have noted that some antioxidants and flavonoids are able to inhibit the activation of NF-κB and production of inflammatory mediators (Cho et al., 2003; Lee et al., 2003). In addition, Cuzzocrea et al. (2002) noted that the inhibition of NF-κB, decrease in NO production, was associated with antioxidant activity. According to Fig. 7, the inhibitory effect of NF-κB by WOE was attributed to block of IκB-α degradation. In other words, WOE may act as a NF-κB inhibitor. Moreover, as mentioned above, WOEs act as antioxidants and their antioxidant action may in part be responsible for the inhibition of NO production.

In conclusion, WOE inhibited both iNOS and COX-2 expression under different LPS-stimulated and reduced LDL oxidation conditions. With these biological functions, WOE could influence anti-inflammation in vascular walls and prevent progress of atherosclerosis. Further investigation of the mechanisms underlying the anti-inflammatory effects of WOE may be helpful for the protection of patients with different cardiovascular lesions.

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