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Antioxidative effect of sesame coat on LDL oxidation and oxidative stress in macrophages

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Abstract

The effects of ethanolic extract of sesame (*Sesamum indicum* L.) coat (EESC) on oxidation of low-density lipoprotein (LDL) and production of nitric oxide in macrophages were investigated. The results show that EESC in the range of 0.01–0.8 mg/ml markedly inhibited copper-induced LDL oxidation and H_2O_2-induced cell damage. In addition, EESC at 0.1 mg/ml not only enhanced glutathione (GSH) levels, but also increased activity of GSH peroxidase, GSH reductase, GSH transferase and catalase by 3.32-, 3.39-, 1.60- and 2.26-fold compared to the control, respectively. Moreover, EESC showed dose-dependent inhibitory effects on nitric oxide production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages and in sodium nitroprusside (SNP), a NO donor. The generation of prostaglandin E2 (PGE2) in stimulated macrophages was also reduced by EESC. The induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) proteins in activated macrophages was inhibited by EESC. In addition, the levels of iNOS and COX-2 mRNA in activated macrophages were decreased by EESC when determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis. Furthermore, LPS-induced degradation of IκB-α was down regulated by EESC, indicating that EESC prevented LPS-induced NF-κB activation. These results implied that EESC could exhibit a protective action on biomolecules and generation of inflammatory mediators in vitro.

Keywords: Sesame (*Sesamum indicum* L.); LDL; Glutathione; Inducible nitric oxide synthase (iNOS); Cyclooxygenase 2 (COX-2); IκB-α

1. Introduction

The threat of excessive oxidative stress (e.g., reactive oxygen species and reactive nitrogen species) under pathological events can promote disease progression by oxidizing biological molecules, including lipids, proteins and DNA (Burke & Fitzgerald, 2003; Nakao, Iwai, Kalil, & Augusto, 2003). These reactive species have been repeatedly claimed as a major founder in cancer (Oikawa, Murakami, & Kawanishi, 2003), diabetes (Niedowicz & Daleke, 2005), hypertension (Wilcox, 2005) and atherosclerosis (Heinecke, 1998). For example, the oxidation of low-density lipoprotein (LDL) by free radicals existing in arterial wall has been known to be a crucial event resulting in atherosclerosis (Heinecke, 1997). Under physiological conditions, non-enzymatic antioxidants (e.g., vitamin E) and a group of antioxidant enzymes such as glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), and catalase (CAT) clear these reactive species in tissues and protect cells from oxidative destruction (Anderson, 1996). And, a deficient cellular antioxidant system only make damages worse (Van Remmen et al., 2004). Unquestionably, for example, to decrease oxidation of LDL would be an initial step in preventing the damage caused by cardiovascular disorders. Unluckily, LDL is subject to oxidative modification due to its high content of unsaturated fatty acyl group (Kalyanaraman, Joseph, & Parthasarathy, 1993). Therefore, to depress the excess generation of radicals in vivo by using natural antioxidants or

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phytochemicals has been suggested to be beneficial for prevention of LDL oxidation and cardiovascular damage (Boyer & Liu, 2004).

On the other hand, a central factor in oxidative stress generation in vivo is the neutrophils and macrophages producing mass superoxide and nitric oxide (NO) in response to extracellular stimulants (Juliet et al., 2004). This excess production of NO would react with superoxide, yielding the more toxic peroxynitrite and hydroxyl radicals which destroy neighboring molecules, resulting in oxidation related diseases (Beckman, Beckman, Chen, Marshall, & Freeman, 1990; Behr-Roussel et al., 2000). Thus, to inhibit mass NO production is a critical goal in preventing the pathogenesis of coronary artery diseases (Aliev et al., 2001). Under physiological situation, NO is formed by nitric oxide synthase (NOS) presented in various mammalian cells (Knowles & Moncada, 1994). In contrast to the constituted neuronal NOS (nNOS) and endothelial NOS (eNOS) only generating a trace of NO, the mass NO production in activated macrophages is formed by inducible nitric oxide synthase (iNOS) and causes circulatory shock (Md, Moochhala, & Siew-Yang, 2003), Alzheimer's-like disease (Nathan et al., 2005) and colon carcinogenesis (Payne, Bernstein, Bernstein, Gerner, & Garewal, 1999). And, iNOS has been revealed to be an instigator during the initiation of atherosclerosis, particularly by macrophages (Aliev et al., 2001). Again, if there were no iNOS expression in tissues, the mass reactive nitrogen species generation would disappear. Thus, the inhibition of iNOS overexpression represents a critical consideration in protection against cell injury and cardiovascular disease.

In fact, natural phytochemicals present in our diet have been shown to protect LDL oxidation and atherosclerosis progression (Campbell, Efendy, Smith, & Campbell, 2001). For example, sesame (*Sesamum indicum* L.), an important edible oil source in Asia, has been suggested to decrease blood pressure (Sankar, Sambandam, Ramakrishna Rao, & Pugalendi, 2005), increase the lipolytic activity in the liver (Sirato-Yasumoto, Katsuta, Okuyama, Takahashi, & Ide, 2001) and lower the cholesterol level in blood (Sankar et al., 2005). Numerous studies have indicated that vitamin E present in sesame contributes to these healthy benefits (Yamashita, Nohara, Katayama, & Namiki, 1992). Besides vitamin E, other natural lipid-soluble lignans occurring in sesame have been reported to possess anti-oxidation activity and bioactive efficacy (Nakano et al., 2003; Sirato-Yasumoto et al., 2001). However, the sesame oil processed from intact seeds is more resistant to oxidation than those prepared from dehulled sesame seeds (Budowski, 1964). In general, the conventional process for preparing sesame oil commonly discards the sesame coat after dehulling. Therefore, more rational uses of sesame coat as a source of natural antioxidants to provide a means for recycling have attracted much attention. In our previous report, the ethanolic extracts of sesame coat (EESC) have shown remarkable antioxidant activities, including scavenging oxygen radicals, chelating metal ions, and protecting liposome from peroxidation reaction (Chang, Yen, Huang, & Duh, 2002). Although the antioxidant function of EESC has been demonstrated, whether EESC could be considered as an inhibitor of LDL oxidation and NO production in stimulated macrophages is still unclear. Thus, the purpose of this study was to further investigate the protective properties of EESC on LDL oxidation and nitric oxide production in macrophages.

2. Materials and Methods

2.1. Materials

The sample of white sesame (*S. indicum* L.) in this study was donated by Tainan District Agriculture Improvement Station, Taiwan, Republic of China. After harvesting, the coat was removed from sesame seeds, sealed in a plastic bottle, and stored at 4 °C until used. Sesame coat (10 g) was extracted overnight with 100 ml of ethanol, in a shaking incubator at room temperature. The extracts were filtered, and the residue was re-extracted under the same conditions. The combined filtrates were evaporated under vacuum below 40 °C using a rotary evaporator to a final volume of 5 ml. The final sample was named as ethanolic extracts of sesame coat (EESC). Tocopherol (Toc) and Trolox were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT), sodium nitroprusside (SNP), sulfanilamide, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Antioxidant action of EESC on low-density lipoprotein (LDL) oxidation

Human LDL ($d = 1.02$–1.06 g/ml) was prepared from fasting plasma, routinely pooled from five healthy normolipemia individuals. Lipoproteins were isolated by sequential preparative ultra-centrifugation and dialyzed overnight as previously described (Viana et al., 1996). Freshly prepared native LDL (0.1 mg/ml) was treated with $CuSO_4$ (10 μM) in the presence of EESC (0–800 μg/ml) or not for 24 h at 37 °C and oxidation was stopped by addition of BHT. LDL oxidation was analyzed by TBA-reactive substance assay as previously described, by measuring absorbance at 532 nm. The results were expressed as percentage inhibition of LDL oxidation.

2.3. Effect of EESC on 3T3 cells viability

3T3 fibroblasts (ATCC number: CRL-1658) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate and maintained in humidified 5% CO_2/95% air at 37 °C. After cells were cultured with 200 μM H_2O_2 in the presence of EESC (0–0.2 mg/ml) or

not for 24 h, cell viability was determined by colorimetric measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Briefly, the original medium was removed, then, MTT (final 0.5 mg/ml) 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 (Wang, Chen, Liang, & Duh, 2005).

2.4. Effect of EESC on glutathione and antioxidant enzyme activity in 3T3 cells

After 3T3 cells were cultured in the presence of EESC (0–0.1 mg/ml) for 24 h, cells (1×10^7 cells) were collected and centrifuged at 1000*g* for 10 min. The pellets were then resuspended in 10 mM phosphate buffer, pH 7.4, containing 1.5% (w/v) KCl and 1 mM EDTA, and lysed by three cycles of freezing, thawing and sonication for 10 s at 60 W. The cellular homogenates were centrifuged at 10,000*g* for 30 min and the supernatants for the subsequent determination of antioxidant enzyme activities were stored at −80 °C. Intracellular activities of GPX (Flohe & Gunzler, 1984) and GR (Cohen & Duvel, 1988) were measured by following the decrease in the absorbance due to oxidation of NADPH. Briefly, in a reaction mixture containing 1 mM GSH, 1 unit/ml GR, 1 mM NaN_3, 1 mM EDTA, 0.2 mM NADPH and 100 μl of cellular extract added 100 μl of 2.5 mM H_2O_2 for GPX activity determination. In another reaction containing mixture 1 mM $MgCl_2$, 1 mM GSSG, 0.2 mM NADPH added 100 μl of cellular extract for GR activity determination. The decreased absorbance at 340 nm was measured for 3 min. Cellular GST activity was assayed by measuring the increment of absorbance at 340 nm due to the formation of 2,4-dinitrophenyl-S-glutathione from 1-chloro 2,4-dinitrobenzene (CDNB) and GSH (Habig, Pabst, & Jakoby, 1974). Briefly, a reaction mixture containing 1 mM GSH, 1 mM CDNB added 100 μl of cellular extract. The activity of CAT was measured as described previously (Armstrong & Browne, 1994). Cellular extracts were reacted with 20 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The change in absorbance at 240 nm at 30 s and 120 s was monitored. The cellular GSH levels were measured as trichloroacetic acid soluble sulfhydryl group by measurement at 412 nm using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.2 M Tris–HCl buffer, pH 8.9 (Anderson, 1985).

2.5. Scavenging action of EESC on nitric oxide

Sodium nitroprusside (SNP) at physiological pH, spontaneously produces NO, which interacts with oxygen to generate nitrite. 0.005 M SNP and various concentrations of EESC (0–1000 μg/ml) in PBS (pH 7.4) were incubated at 25 °C for 150 min. After incubation, nitrite produced from SNP was measured by the Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water]. Each supernatant (0.1 ml) was mixed with the same volume of Griess reagent and absorbance of the mixture at 550 nm was determined (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994).

2.6. Inhibitory action of EESC on nitric oxide and prostaglandin E2 production in RAW 264.7 cells

RAW 264.7 cells (ATCC number: TIB-71) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate and maintained in humidified 5% CO_2/95% air at 37 °C. Nitrite levels in the cultured media, which reflect intracellular nitric oxide synthase activity, were determined by Griess reaction. Briefly, cells were cultured with EESC (0–1000 μg/ml) with or without LPS (0.2 μg/ml) for 24 h. Then, the growth medium was mixed with the same volume of Griess reagent; absorbance of the mixture at 550 nm was determined by using an Anthos 2010 microplate reader. Prostaglandin E2 levels in the cultured media were determined by using the prostaglandin E2-monoclonal enzyme immonoassay (EIA) kit (Cayman Chemical, Michigan, USA) according to the manufacturer's instructions.

2.7. Western blot

After RAW 264.7 cells were cultured with EESC (0–1000 μg/ml) in the presence of LPS (0.2 μg/ml) for 24 h, cells were washed with ice-cold PBS, and then 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, contained 50 μg protein, was separated on 8% SDS–polyacrylamide gels. After electrophoresis, the gels were transferred to nitrocellulose paper. After washing with distilled water, the membrane was incubated with 5% albumin in PBS (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). The 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).

2.8. Reverse transcription-polymerase chain reaction

Total RNA was isolated from RAW 264.7 cells by using Trizol reagent (Life Technologies, CA, 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, COX-2 and GAPDH. The amplification of cDNA was performed by incubation 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, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ and mouse COX-2, forward primer 5′-GGAGAGACT-ATCAAGATAGTGATC-3′, reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′. The cDNA sequence of GAPDH was also amplified as control in a similar way using as primers 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCAC-CAC-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 (Wang et al., 2005).

2.9. Statistical analysis

All data were recorded as means ± SD (standard deviation). Statistical analysis involved use of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $P < 0.05$.

3. Results

3.1. Inhibitory effects of EESC on LDL oxidation

Fig. 1 shows the inhibitory effects of EESC on copper induced LDL oxidation. In the range of 0–0.8 mg/ml, EESC showed protective effects against LDL oxidation. EESC at 0.5 and 0.8 mg/ml showed 74% and 77% inhibitory effects, respectively, on LDL oxidation. The protective capacity of EESC on LDL oxidation was inferior to Trolox. Upon the whole, EESC had a remarkable inhibitory effect on LDL oxidation.

3.2. Protective effects of EESC on H_2O_2-induced cytotoxicity in 3T3 cells

Fig. 2 showed the protective effects of EESC on cell damage induced with hydrogen peroxide. The cell viability of 3T3 cells in the presence of $H_2O_2$was decreased (data not shown). In the range of 0–0.2 mg/ml, EESC increased the 3T3 cell viability in the presence of 200 μM H_2O_2 in a dose-dependent manner. At 0.1 and 0.2 mg/ml, EESC showed 60% and 65% protective effects, respectively, on

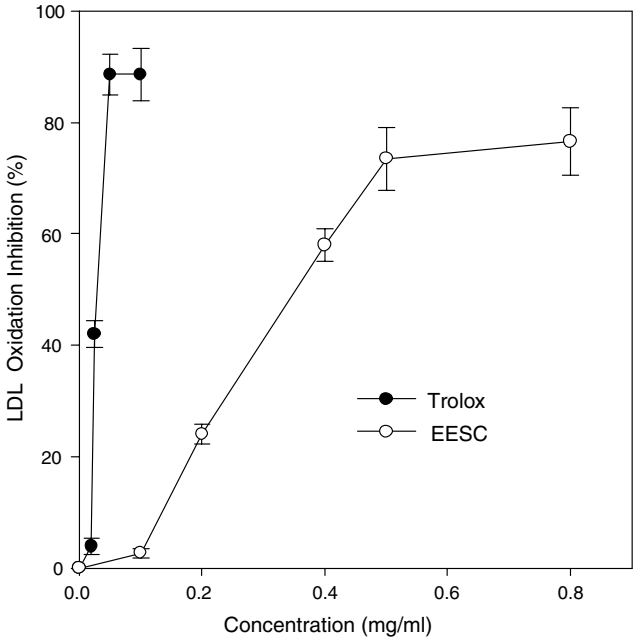

Fig. 1. The inhibitory effect of ethanolic extract of sesame coat (EESC) on oxidation of LDL. The data were displayed with mean ± SD ($n = 4$). Results were analyzed by ANOVA ($p < 0.05$).

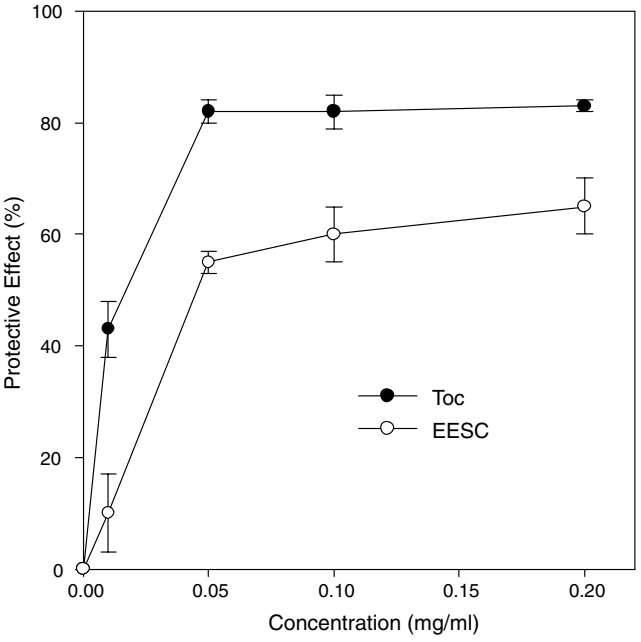

Fig. 2. Protective effect of ethanolic extract of sesame coat (EESC) on hydrogen peroxide induced cytotoxicity in 3T3 cells. Toc, tocopherol. The data were displayed with mean ± SD ($n = 3$). Results were analyzed by ANOVA ($p < 0.05$).

H_2O_2 induced cytotoxicity. In this study, the protective effects of tocopherol (Toc) were superior to EESC. Since cell viability in the presence of hydrogen peroxide was increased by EESC, this investigation further explored EESC's effect on intracellular antioxidant enzymes to clar-

ify the metabolism mechanism. Table 1 shows the effects of EESC on intracellular GSH level and antioxidant enzyme activity in 3T3 cells. With EESC in the range of 0–0.1 mg/ml, the GSH levels increased with increasing concentrations of EESC. The addition of 0.1 mg/ml EESC, the GSH level was 6.3-fold compared to the control. In addition, EESC in the range of 0–0.1 mg/ml could increase the activities of GPX, GR, GST, and CAT in 3T3 cells in concentration-dependent manners. With EESC at 0.1 mg/ml, the activities of GPX, GR, GST, and CAT were 3.3-, 3.4-, 1.6- and 2.3-fold, respectively, compared to the control. This data indicates that EESC might be a potential positive regulator of intracellular GSH content and antioxidant enzyme activity in 3T3 cells.

3.3. EESC decreased NO production in cell and cell-free system

Many studies have suggested that except for reactive oxygen species, mass reactive nitrogen species (e.g., nitric oxide) are produced in artery wall during the progression of atherosclerosis. The ability of EESC to affect nitric oxide production in stimulated RAW 264.7 macrophages was further investigated. Fig. 3 shows the inhibitory effects of EESC on cellular nitric oxide production in the presence of LPS. EESC in the range of 0–0.08 mg/ml inhibited nitric oxide production in stimulated macrophages. EESC at 0.04 and 0.08 mg/ml showed 51% and 89% inhibitory effects, respectively, on nitric oxide production in activated cells. Furthermore, the inhibitory activity of EESC on the production of prostaglandin E2 (PGE2), another important inflammatory mediator in activated macrophages, was also determined. As shown in Fig. 3, EESC at 0.04 and 0.08 mg/ml showed 14% and 33% inhibitory effects, respectively, on PGE2 production in stimulated macrophages. Since EESC decreased the nitric oxide levels present in the culture medium of macrophages, whether EESC could scavenge nitric oxide directly was examined. Fig. 4 shows the scavenging effect of EESC on nitric oxide in the SNP system. SNP was able to produce cyanide and NO by one-electron reduction reaction under physiological condition. In the range of 0–1.0 mg/ml, EESC decreased the production of nitric oxide in a dose-dependent manner. EESC at 1.0 mg/ml reduced nitric oxide by 39%, which was inferior to Trolox (89%).

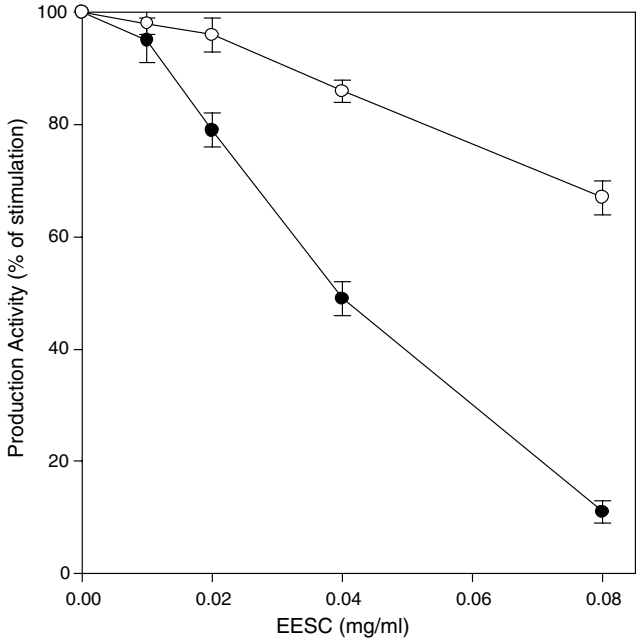

Fig. 3. Effect of ethanolic extract of sesame coat (EESC) on production of nitric oxide (NO, ●) and prostaglandin E2 (PGE2, ○) in RAW 264.7 cells stimulated with lipopolysaccharide (LPS). The data were displayed with mean ± SD ($n = 6$). Results were analyzed by ANOVA ($p < 0.05$).

3.4. Effects of EESC on LPS-induced iNOS and COX-2 protein expression

According to the data mentioned above, EESC did inhibit NO and PGE2 production in stimulated macrophages. The expression of iNOS and COX-2 protein in activated macrophages was further analyzed by means of immunoblot with a view to this inhibitory mechanism. As shown in Fig. 5, EESC in the range of 0.01–0.08 mg/ml decreased iNOS protein expression in the presence of LPS in a concentration-dependent manner. EESC at 0.04 and 0.08 mg/ml showed 53% and 94% inhibitory effects, respectively, on iNOS protein expression in stimulated macrophages. On the other hand, EESC at 0.04 and 0.08 mg/ml showed 7% and 53% inhibitory effects, respectively, on COX-2 protein expression. These data implied that the inhibitory action of EESC on iNOS and COX-2 protein could be responsible for inhibition of nitric oxide and PGE2 production in stimulated macrophages.

Table 1
The effects of ethanolic extracts of sesame coat (EESC) on glutathione (GSH) and enzyme activity in 3T3 cells

Concentration (mg/ml)	GSH (nmol/mg)	Activity (nmol/min/mg protein)			
		GSH peroxidase	GSH reductase	GSH transferase (10^{-3})	Catalase
0	40 ± 8^{a}	0.70 ± 0.02^{a}	1.45 ± 0.33^{a}	2.00 ± 0.20^{a}	138.6 ± 17.4^{a}
0.01	56 ± 9^{a}	0.95 ± 0.01^{a}	2.16 ± 0.42^{a}	2.90 ± 0.10^{b}	175.4 ± 34.2^{a}
0.05	128 ± 59^{a}	1.71 ± 0.16^{b}	3.01 ± 0.12^{b}	3.50 ± 0.40^{b}	186.7 ± 16.7^{a}
0.1	252 ± 65^{b}	2.33 ± 0.31^{b}	4.92 ± 0.28^{c}	3.20 ± 0.20^{b}	313.7 ± 27.3^{b}

The data were displayed with mean ± SD ($n = 3$).
Values with different superscripts in a column are significantly different ($p < 0.05$).

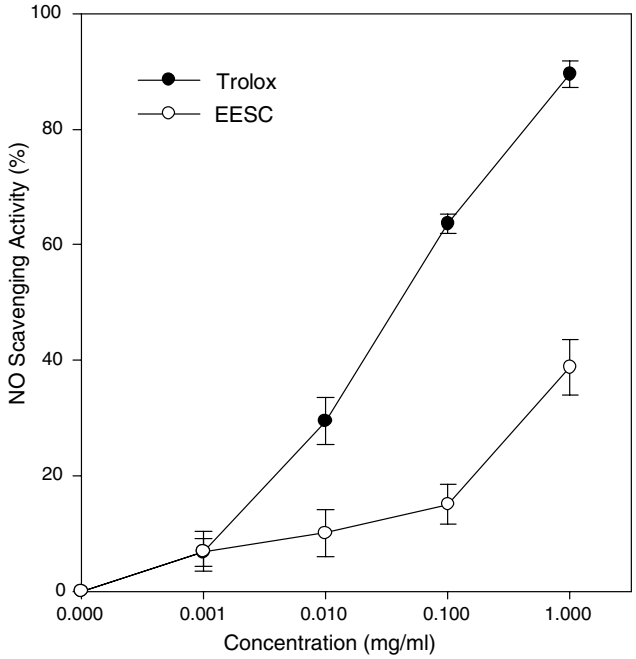

Fig. 4. Scavenging effect of ethanolic extract of sesame coat (EESC) on nitric oxide that generated with sodium nitroprusside (SNP) during incubation. The data were displayed with mean ± SD ($n = 5$). Results were analyzed by ANOVA ($p < 0.05$).

EESC (mg/ml)	0	0	0.01	0.02	0.04	0.08
LPS (200 ng/ml)	0	+	+	+	+	+

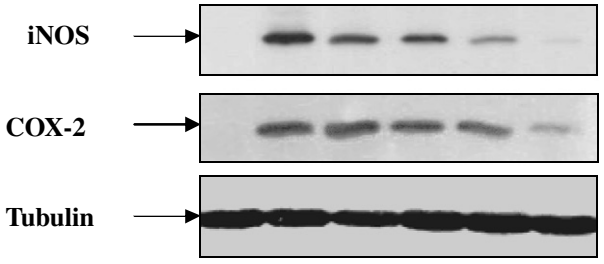

Fig. 5. Effects of ethanolic extract of sesame coat (EESC) on iNOS protein and COX-2 protein that incubated with lipopolysaccharide (LPS) in RAW 264.7 macrophages.

3.5. Effects of EESC on iNOS and COX-2 mRNA in stimulated RAW cells

To characterize the mechanism responsible for inhibition of iNOS and COX-2 protein production by EESC, we further determined whether EESC could affect the transcription of iNOS and COX-2 mRNA. Thus, the production of iNOS and COX-2 mRNA in activated cells was determined by means of RT-PCR analysis. As shown in Fig. 6, EESC at 0.08 mg/ml decreased iNOS and COX-2 mRNA production after 6 h treatment in the presence of LPS by 72% and 43%, respectively. It is apparent that

EESC (mg/ml)	0	0	0.04	0.08
LPS (200 ng/ml)	0	+	+	+

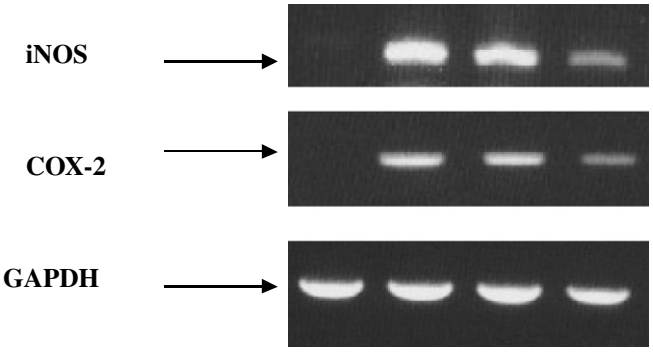

Fig. 6. Effects of ethanolic extract of sesame coat (EESC) on expression of iNOS mRNA and COX-2 mRNA which incubated with lipopolysaccharide (LPS) in RAW 264.7 macrophages.

EESC (mg/ml)	0	0	0.02	0.04	0.08
LPS (200 ng/ml)	0	+	+	+	+

Fig. 7. Effects of ethanolic extract of sesame coat (EESC) on IkB-α protein in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS).

EESC showed an inhibitory effect on the expression of iNOS and COX-2 mRNA in stimulated RAW cells.

3.6. EESC decreased IkB-α degradation in stimulated RAW cells

Fig. 7 shows the inhibitory effect of EESC on intracellular IκB-α protein degradation in LPS-activated cells. The IκB-α protein was detected in cells by means of immunoblot. After stimulating cells with LPS for 60 min, the band of IκB-α protein was obviously degraded in RAW 264.7 cells, in comparison with the control. On the other hand, as shown in Fig. 7, EESC at 0.04 and 0.08 mg/ml inhibited the degradation of intracellular IκB-α protein in LPS-stimulated cells, showing the protective effect of EESC on LPS-induced IκB-α protein degradation.

4. Discussion

In our previous study (Chang et al., 2002), EESC not only showed a significant scavenging action on free radicals, but also displayed an obviously inhibitory effect against the peroxidation of liposome in a $FeCl_3$–ascorbic acid system. And, sesamin and sesamolin, the lignan substances, have been identified in EESC by the means of

HPLC analysis (Chang et al., 2002). Some literatures have reported that sesamin and sesamolin did not exhibit obviously antioxidant function in DPPH radical system (Shyu & Sun-Hwang, 2002; Suja, Jayalekshmy, & Arumughan, 2004). On the other hand, Nakai et al. (2003) noted that sesamin is a pro-antioxidant, however, its metabolites containing the catechol moieties in their structures are responsible for the antioxidant effects of sesamin against oxidative damage in vivo. In addition, other antioxidant bioactive phenolic compounds and tetranortriterpenoids present in EESC have been identified and suggested to be responsible for the protective effects of EESC in acellular mode (Chang et al., 2002). Since EESC is still possessed of antioxidant activity, EESC may directly exhibit a protective effect on cellular molecules against oxidative damage. As shown in Fig. 1, EESC shows a protective effect under the condition of LDL copper-oxidation assay. The oxidation of LDL has been verified as an important factor in the initiation and progression of atherosclerosis (Heinecke, 1998). Therefore, if human intend to avoid the menace of coronary artery diseases, an urgent goal should be to decrease oxidation of LDL. In this study, EESC showed a clear inhibitory action on oxidation of LDL. This result implies that EESC might have potential effects in cardiovascular disease prevention. Although copper–LDL oxidation was seldom enforced in circular, as a high concentration of metal-chelating proteins was present in plasma, this should not prohibit LDL oxidation from occurring where spreading reactive oxygen species and reactive nitrogen species abuse (Berliner & Heinecke, 1996).

Hydrogen peroxide at low concentrations has positive effects in some physiological processes such as carbohydrate metabolism (Janero, Hreniuk, & Sharif, 1994), cell proliferation (Cai, 2005) and platelet activation (Redondo et al., 2005). However, a high concentration of hydrogen peroxide is regarded as a toxic stress. Studies of biochemical events in oxidant damage of cells have proven that H_2O_2 could directly inactivate cellular glyceradehyde-3-phosphate dehydrogenase (GAPDH; an enzyme of the glycolytic pathway) (Cochrane, 1991) and increase activity of poly(ADP-ribose) polymerase (Lautier et al., 1990). These data revealed that living cells damaged by H_2O_2 might be responsible for ATP depletion and DNA damage in target cells. Consequently, H_2O_2 molecules have been effectively used as in vitro oxidants for applications such as screening antioxidants and their mechanisms (Surh, 2002; Wijeratne, Cuppett, & Schlegel, 2005). As shown in Fig. 2, EESC exhibited a protective action against H_2O_2-induced cell damage. In our previous study (Chang et al., 2002), EESC showed obvious antioxidant activities. Besides scavenging hydroperoxide directly, it is possible that EESC could prevent H_2O_2 damage by activating intracellular antioxidant defense systems. According to the data in Table 1, EESC could induce an increase in cellular GSH level in a dose-dependent manner. GSH has been claimed to be a critical endogenous antioxidant source. GSH not only increases the reducing capacity in cells but also functions in the detoxification of every variety of xenobiotics (Hayes & McLellan, 1999). Prasamthi, Muralidhara, and Rajini (2005) noted the fact that sesame oil significantly attenuated oxidative damage in the liver, which was attributed to the increasing of GSH level and antioxidant enzymes. On the other hand, other intracellular antioxidant enzymes including GPX, GR, GST and CAT could depress the oxygen radical cascade and reduce radical-induced cellular damage (Anderson, 1996). As shown in Table 1, the activities of GPX, GR, GST and CAT were increased in cells in the presence of EESC. GPX could eliminate hydroperoxide by using GSH to produce glutathione disulfide that subsequently was reduced by GR. In other words, the regeneration of GSH from GSSG was catalyzed by GR. And, GST could catalyze the conjugation of GSH with other toxic xenobiotics. Furthermore, CAT mainly located in subcellular peroxisomes could reduce H_2O_2 to water and oxygen. Higher CAT activity would directly clean more hydroperoxide and could reduce the depletion of GSH in cells. Thus, the fact that EESC increased the activities of GPX, GR, GST and CAT might attenuate H_2O_2-induced oxidative damage in cells by increasing the activity of antioxidant enzymes and elevating GSH levels. This observation is in agreement with the report of Hemalatha, Raghunath, and Ghafoorunissa (2004), who found that activities of hepatic antioxidant enzymes were significantly increased in rats that were fed sesame lignans. These data imply that EESC containing sesamin and sesamolin might effectively prevent oxidative injury by promoting antioxidant enzymes.

Besides hydroperoxide, the inhibitory action of EESC on NO was also examined. Excess NO in high enough concentrations could react with superoxide to yield peroxynitrite that is more toxic. Therefore, the scavenging and/or inhibitory activity of natural phytochemicals on disastrous NO production has attracted more attention. Sesamin and sesamolin have been indicated to be inhibitors of cytokine production in LPS-activated microglia by reducing p38 mitogen activated protein kinase (MAPK) activation pathway (Jeng, Hou, Wang, & Ping, 2005). Furthermore, EESC contains polyphenolic compounds which have been reported to exhibit greater antioxidant activities than vitamins C and E (Chang et al., 2002; Kim, Lee, Lee, & Lee, 2002). In the present study, EESC showed an inhibitory effect on NO production in cell mode systems. These findings suggest that EESC might be an efficient inhibitor of NO production in activated macrophages due to the presence of lignans and phenols in EESC. In addition, Salvemini et al. (1993) reported that enhanced release of PGE2 which detonate violent inflammatory reaction was almost driven by NO. As shown in Fig. 3, EESC could inhibit PGE2 production in LPS-stimulated macrophages. Thus, EESC's inhibitory effects on inflammatory progression might be partially attributed to decreased PGE2 production in stimulated macrophages. Inhibition of PGE2 production not only play a critical role in depressing excess oxidative stress production in cells (Kumagai et al.,

2000), but also improves endothelium function in an animal mode of atherosclerosis (Hernandez-Presa et al., 2002). Moreover, as shown in Fig. 4, EESC also exhibited direct scavenging activity on NO in SNP reduction process. These data reveal that EESC might decrease the crisis of reactive nitrogen species by inhibiting NO production in both direct and indirect pathway. According to the data in Figs. 2 and 4, EESC displayed a potent scavenging action toward reactive oxygen and nitrogen species. These data reveal that EESC might be helpful to protect tissues from nitration damage and inflammatory stress.

In order to investigate the mechanism of depression NO and PGE2 production in macrophages, the effect of EESC on iNOS and COX-2 enzymes expression was evaluated. As shown in Fig. 5, EESC did inhibit iNOS and COX-2 protein expression in LPS-stimulated macrophages in a dose-dependent manner. Hence, it is clear that inhibition of NO and PGE2 production by EESC was attributed to the reduction of iNOS and COX-2 enzymes production in cells. Furthermore, the expression of iNOS and COX-2 enzymes are regulated mainly at the transcriptional level. As shown in Fig. 6, EESC decreased the levels of iNOS and COX-2 mRNA in LPS-activated cells. This result suggests that EESC's inhibition of iNOS and COX-2 enzymes production in activated macrophages can be attributed to a decrease in the transcriptional expression of iNOS and COX-2. Since MAPK catalyzed phosphorylation reaction at specific serine and threonine residues of target protein substrates and regulated cellular activities including promoting iNOS and COX-2 gene expression (Huang, Rose, & Hoyt, 2004). In addition, Jeng et al. (2005) noted that LPS-activated p38 MAPK was reduced by sesamin and sesamolin. Consequently, it is possible that sesamin and sesamolin present in EESC were the main components contributing to the signal transduction inhibitory effect on MAPK activation and the gene expression of iNOS and COX-2.

On the other hand, various transcription factors have been claimed to play a critical role in promoting the gene expression of iNOS and COX-2. Of these potential relevant transcription factors, NF-κB was important for both iNOS and COX-2 induction (Xie, Kashiwabara, & Nathan, 1994). In most resting cells, NF-κB binding to one of the inhibitory IκB proteins retains inactive form in the cytoplasm. Extracellular signals, which make phosphorylation and degrade of IκB protein, would result in the activation of NF-κB and down-stream signal transduction in cells. As shown in Fig. 7, LPS could promote IκB protein degradation followed by increasing NF-κB activation in stimulated macrophages. EESC could inhibit the IκB protein degradation in LPS-stimulated macrophages. This data implies that EESC might be an inhibitor of NF-κB activation and iNOS gene transcription. Sesamin and sesamolin have been reported as NF-κB inhibitors (Jeng et al., 2005). Previous studies have noted that some polyphenolics could inhibit NF-κB activation and inflammatory mediator production (Cho et al., 2003). Cuzzocrea et al. (2002) also suggested that inhibitors of NF-κB were characterized by their antioxidant activity. As mentioned above, EESC acting as an antioxidant might be responsible for the inhibitory action on NF-κB and NO production.

According to the data presented above, EESC obviously reduces LDL oxidation and inhibits both iNOS and COX-2 expression under LPS-stimulated conditions. With these biological functions, EESC might retard inflammatory progression in vascular walls and reduce the risk of atherosclerosis.

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