Bioorganic & Medicinal Chemistry Letters 21 (2011) 2415–2418

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Anti-inflammatory effect of soyasaponins through suppressing nitric oxide production in LPS-stimulated RAW 264.7 cells by attenuation of NF-κB-mediated nitric oxide synthase expression

Long-ying Zha [a,*], Li-mei Mao [a], Xiao-cui Lu [b], Hong Deng [a], Ju-feng Ye [b], Xin-wei Chu [a], Su-xia Sun [a], Hai-ji Luo [a]

[a] Department of Nutrition and Food Hygiene, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, PR China
[b] Experiment Teaching Demonstration Center for Preventive Medicine, Southern Medical University, Guangzhou 510515, PR China

ARTICLE INFO

Article history:
Received 17 November 2010
Revised 28 January 2011
Accepted 16 February 2011
Available online 19 February 2011

Keywords:
Anti-inflammatory
Soyasaponins
Nitric oxide
Inducible nitric oxide synthase
Nuclear factor kappa B

ABSTRACT

The anti-inflammatory properties of soyasaponins (especially soyasaponins with different chemical structures) have scarcely been investigated. We investigated the inhibitory effects of five structural types of soyasaponins (soyasaponin A^1, A^2, I and soyasapogenol A, B) on the induction of nitric oxide (NO) and inducible NO synthase (iNOS) in murine RAW 264.7 cells activated with lipopolysaccharide (LPS). Soyasaponin A^1, A^2 and I (25–200 μg/mL) dose-dependently inhibited the production of NO and tumor necrosis factor α (TNF-α) in LPS-activated macrophages, whereas soyasapogenol A and B did not. Furthermore, soyasaponin A^1, A^2 and I suppressed the iNOS enzyme activity and down-regulated the iNOS mRNA expression both in a dose-dependent manner. The reporter gene assay revealed that soyasaponin A^1, A^2 and I decreased LPS-induced nuclear factor kappa B (NF-κB) activity. Soyasaponin A^1, A^2 and I exhibit anti-inflammatory properties by suppressing NO production in LPS-stimulated RAW 264.7 cells through attenuation of NF-κB-mediated iNOS expression. It is proposed that the sugar chains present in the structures of soyasaponins are important for their anti-inflammatory activities. These results have important implication for using selected soyasaponins towards the development of effective chemopreventive and anti-inflammatory agents.

© 2011 Elsevier Ltd. All rights reserved.

Soyasaponins (SS) are major phytochemicals found in soybeans and soy products with an average content ranging from 0.5 to 114 μmol/g.[1] Based on the oleanane-type triterpenoid aglycone structure, group A, B, E and DDMP (2,3-dihydro-2,5dihydroxy-6-methyl-4*H*-pyran-4-one) soyasaponins have already been identified and classified.[2] The group A and B soyasaponins are the most abundant type of saponins found in soybean and related products. The group A soyasaponins including soyasaponin A^1 (SS-A^1) and soyasaponin A^2 (SS-A^2) have soyasapogenol A (SG-A) as the aglycone. The group B soyasaponins having soyasapogenol B (SG-B) as the aglycone contain soyasaponin I (SS-I), soyasaponin II (SS-II) and soyasaponin III (SS-III).[3] In recent years, soyasaponins have attracted great interest because of their potential health-promoting functions such as anticarcinogenic,[4,5] plasma cholesterol lowering,[6,7] anti-viral,[8,9] hepatoprotective,[10,11] antioxidant,[12] and antimutagenic bioactivities.[13,14]

Inflammation, usually caused by microbial infections, chemicals and immunological reactions, can provide protection to human body by enclosing injury, destroying invaded microorganisms and restoring the tissue or organs for recovery.[15] However, excessive inflammation mediated by pro-inflammatory cytokines has been linked with tumor promotion and regression.[16] Pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6) and interferon γ (IFN-γ) are prominently involved in the stimulation of inducible nitric oxide synthase (iNOS), one of the three vital enzymes generating nitric oxide (NO) from the amino acid L-arginine within mammalian immune, cardiovascular and neural systems.[17,18] Uncontrolled overproduction of NO by iNOS is considered as an important mediator of carcinogenesis. Over-expression of iNOS has been observed in a variety of human malignant tumors.[18] The NO/iNOS pathway plays important role in tumorigenesis and its inhibitors can be used as chemoprevention agents. Saponins from plant resources such as *Gynostemma pentaphyllum*,[19] *Platycodon grandiflorum*[20] have been shown to exhibit anti-inflammatory properties by targeting NO/iNOS pathway. A recent study by Kang et al.[21] indicated that a crude extract of soybean saponins show anti-inflammatory properties by suppressing the transcription of inflammatory cytokine genes through the nuclear factor kappa B (NF-κB) signaling pathway in BALB/c mice peritoneal macrophages. However, neither the anti-inflammatory properties of different chemical structures

* Corresponding author. Tel.: +86 20 62789127; fax: +86 20 61648309.
E-mail address: lyzhasmu@126.com (L. Zha).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.02.071

of soyasaponins nor their mechanisms are fully understood. Therefore, in the present study, we investigated the effect of five different structural types of soyasaponins (SS-A[1], SS-A[2], SS-I, SG-A and SG-B, see Fig. S1 in Supplementary data for chemical structures) on NO production and iNOS expression in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells.

Soyasaponins A[1], A[2] and I (25–200 μg/mL) markedly suppressed NO production in LPS-stimulated RAW 264.7 macrophage cells in a dose-dependent manner (P <0.01) (Fig. 1). However, the same concentration of soyasapogenol A and B produced no effect on the production of NO (P >0.05). In order to determine whether this inhibition was due to the cytotoxicity of soyasaponins, the effects of all five soyasaponins on RAW 264.7 cell viability were further assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay under the same experimental treatment and conditions. None of the five soyasaponins had any effect on cell viability at the various concentrations used (Fig. S2).

We investigated iNOS enzyme activity and mRNA expressions in order to elucidate the mechanism of suppressive effect of soyasaponins on NO production. The basal enzyme activity of iNOS in RAW 264.7 macrophage cells was extremely low (Table 1). Stimulation of 1 μg/mL LPS markedly increased the enzyme activity (approximately 12-fold higher compared to the basal). The treatment of 25–200 μg/mL of soyasaponins A[1], A[2] and I dose-dependently decreased the iNOS enzyme activity in LPS (1 μg/mL) stimulated RAW 264.7 macrophages (P <0.01). However, treatment of soyasapogenol A and B produced no effect on iNOS enzyme activity in LPS-stimulated macrophages (P >0.05). Aminoguanidine (10 μg/mL), a known inhibitor of NOS enzymes, significantly suppressed the iNOS enzyme activity in LPS-stimulated RAW 264.7 macrophages. Real-time fluorescent quantitative PCR was performed to examine whether the inhibition of NO production by soyasaponins was involved with iNOS mRNA expression (as shown in Table 1). LPS-stimulated RAW 264.7 macrophage cells induced expression of iNOS mRNA, but not that of housekeeper gene β-actin mRNA. Similarly, the presence of soyasaponins A[1], A[2] and I, but not soyasapogenol A and B in LPS-stimulated RAW 264.7 macrophage cells suppressed expression of iNOS mRNA in a dose-dependent manner. However, soyasaponins treatment did not affect the expression of β-actin mRNA.

Macrophages activated with LPS have been known to secrete many cytokines. Therefore, the pro-inflammatory cytokines of TNF-α, IL-1 and IL-6 were assessed by ELISA in cultures in order

Table 1
Effects of soyasaponins (SS-A[1], SS-A[2], SG-A, SG-B and SS-I) on enzyme activities and mRNA levels of iNOS in LPS-stimulated RAW 264.7 macrophages

Treatment items		iNOS enzyme activity (pmol/min/mg protein)	iNOS mRNA relative expression
Control		2.4 ± 0.4*	1.0 ± 0.0*
LPS (1 μg/mL)		27.9 ± 1.7	15.3 ± 1.2
LPS (1 μg/mL) + SS-A[1] (μg/mL)	25	22.3 ± 2.0*	11.6 ± 2.4#
	50	18.3 ± 1.0*	11.3 ± 2.7*
	100	11.4 ± 1.0*	7.5 ± 1.0*
	200	6.2 ± 0.5*	5.1 ± 1.1*
LPS (1 μg/mL) + SS-A[2] (μg/mL)	25	23.6 ± 2.1*	11.4 ± 1.7*
	50	19.0 ± 1.6*	10.7 ± 1.6*
	100	14.0 ± 1.7*	5.7 ± 1.1*
	200	9.3 ± 0.3*	4.4 ± 1.0*
LPS (1 μg/mL) + SS-I (μg/mL)	25	24.0 ± 1.6*	11.1 ± 2.7*
	50	16.6 ± 2.5*	8.8 ± 1.5*
	100	11.7 ± 1.3*	6.8 ± 1.6*
	200	9.2 ± 0.8*	5.6 ± 1.0*
LPS (1 μg/mL) + SG-A (μg/mL)	25	27.7 ± 2.3	15.1 ± 1.8
	50	27.0 ± 1.4	15.0 ± 2.1
	100	26.9 ± 2.2	15.1 ± 2.4
	200	27.0 ± 1.6	15.3 ± 1.8
LPS (1 μg/mL) + SG-B (μg/mL)	25	27.4 ± 2.3	14.7 ± 1.6
	50	26.5 ± 1.7	14.8 ± 2.2
	100	26.6 ± 2.9	14.7 ± 1.6
	200	27.1 ± 2.2	15.4 ± 1.9
LPS (1 μg/mL) + AG (10 μg/mL)		2.7 ± 0.4*	

RAW 264.7 cells were treated for 24 h with either vehicle (L, LPS stimulation alone with no addition of soyasaponin) or increasing concentrations of soyasaponins (25–200 μg/mL) in the presence of LPS (1 μg/mL). Unstimulated cells were used as a negative control. Aminoguanidine (AG, 10 μg/mL) acted as a positive control. The iNOS enzyme activities in cell homogenates were determined by conversion of L-[^{14}H] arginine to L-[^{14}H] citrulline at 37 °C. The absolute copies of iNOS mRNA expression were detected by real-time fluorescent quantitative PCR assay and normalized by the copies of housekeeper gene β-actin in the same sample. Results were expressed as iNOS mRNA relative expression (fold difference compared to the negative control which denoted as 1). All data were obtained from three independent experiments and analyzed by using one-way ANOVA and LSD multiple comparison tests. Results are presented as means ± SD values. * and # indicate significant differences (P <0.01 and P <0.05, respectively) compared with the LPS alone stimulated group.

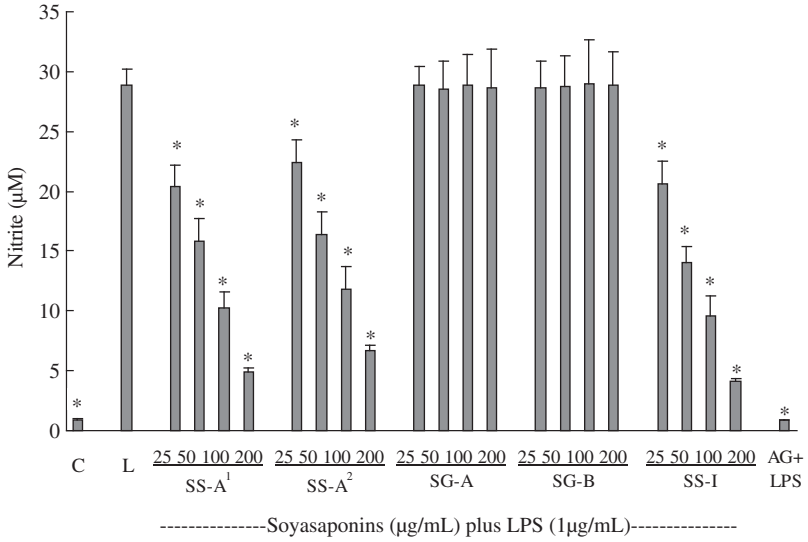

Figure 1. Effects of various soyasaponins (SS-A[1], SS-A[2], SG-A, SG-B and SS-I) on nitrite production in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were treated for 24 h with either vehicle (L, LPS stimulation alone with no addition of soyasaponin) or soyasaponins (25–200 μg/mL) in the presence of LPS (1 μg/mL). Unstimulated cells were used as a negative control (C). Aminoguanidine (AG, 10 μg/mL) acted as a positive control (AG + LPS). Nitrite production was measured by the Griess reaction. Data were obtained from three independent experiments and analyzed by using one-way ANOVA and LSD multiple comparison tests. Results are presented as means ± SD values. * Indicate significant differences (P <0.01) compared with the LPS alone stimulated group.

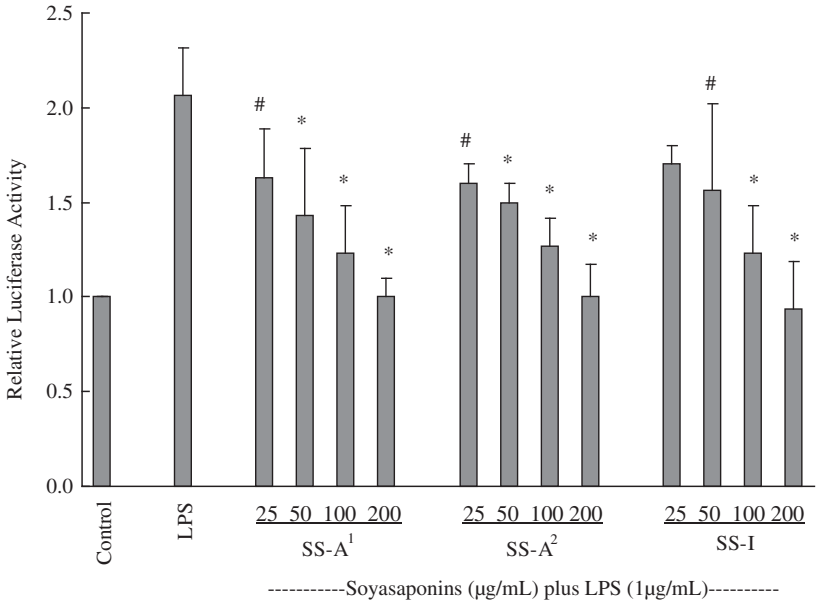

Figure 2. Effects of soyasaponins (SS-A^1, SS-A^2 and SS-I) on NF-κB activity in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were transiently transfected with pNF-κB-Luc reporter gene, and then treated for 24 h with either vehicle (LPS stimulation alone with no addition of soyasaponin) or increasing concentrations of soyasaponins (25–200 μg/mL) in the presence of 1 μg/mL LPS. Unstimulated cells were used as a negative control. Results were expressed as relative luciferase activity (fold difference compared to the negative control which denoted as 1). Data were obtained from three independent experiments and analyzed by using one-way ANOVA and LSD multiple comparison tests. Results are presented as means ± SD values. * and # indicate significant differences (P <0.01 and P <0.05, respectively) compared with the LPS alone stimulated group.

to investigate whether soyasaponins inhibits the production of cytokines by LPS-activated RAW 264.7 macrophages. As evident in Figure S3, soyasaponins A^1, A^2 and I, but not soyasapogenol A and B inhibited the release of TNF-α into the medium of LPS-activated macrophages in a dose-dependent way similar to that they inhibit NO production. However, the production of IL-1 and IL-6 were not affected by any of the five types of soyasaponins under the same treatment and conditions (data not shown).

The activation of transcription factor NF-κB is an essential step in the induction of iNOS gene expression in macrophages in response to stimulation by LPS. A reporter gene assay with a commercially available plasmid pNF-κB-Luc was performed to further assess whether or not soyasaponins A^1, A^2 and I block the NF-κB activation. As evident in Figure 2 and 25–200 μg/mL of soyasaponins A^1, A^2 and I significantly decreased LPS-induced NF-κB transcriptional activity in a dose-dependent manner.

Inflammation is linked with tumor promotion and regression.[16,17] The chemopreventive activities of plant saponins are associated with their anti-inflammatory properties.[19,21,22] Soyasaponins has drawn interest because of their potential chemopreventive bioactivities.[3–5,23,24] However, the anti-inflammatory properties of soyasaponins with different chemical structures have scarcely been investigated. In this study, we examined the anti-inflammatory activities of five structural types of soyasaponins (i.e., soyasaponin A^1, A^2, I and soyasapogenol A, B) by targeting NO/iNOS pathway in a well-characterized murine macrophage cell line, RAW 264.7 since NO production and iNOS expression are considered to be related to inflammation and carcinogenesis. Soyasaponins A^1, A^2 and I inhibited NO production in LPS-induced RAW 264.7 macrophages by suppressing iNOS enzyme activity and/or attenuating iNOS gene expression. However, soyasapogenol A and B exhibited no effects on the NO/iNOS pathway. Activation of NF-κB is critical for LPS induction of iNOS gene expression in macrophages.[17] Our data showed soyasaponin A^1, A^2 and I significantly decreased LPS-induced NF-κB transcriptional activity in a dose-dependent manner. The present results suggested that soyasaponin A^1, A^2 and I inhibit the LPS-induced NO production and iNOS expression through, at least in part, blocking of NF-κB activation. A previous study by Kang et al.[21] indicated that crude soybean saponins extracts containing soyasaponin I and II as major saponins exhibited anti-inflammatory properties by suppressing the release of NO and transcription of iNOS genes through the NF-κB signaling pathway. Oda et al.[25] reported that soyasaponins bearing sugar chains showed adjuvanticity, while their corresponding aglycones soyasapogenol A and B did not. We postulated that the sugar residues present in the structures of soyasaponins may be important for their anti-inflammatory or pharmacological activities. This structure/function relationship has also been witnessed by studies using saponins from other plant sources such as *Panax ginseng*,[26] *Korean red ginseng*,[27] *Platycodon grandiflorum*.[20]

Pro-inflammatory cytokines play an important role in regulating inflammation and tumor progression.[21] The production of pro-inflammatory cytokines TNF-α, IL-1 and IL-6 by macrophages in response to inflammatory stimuli and microbial products is well established.[28] Our data revealed that soyasaponins A^1, A^2 and I suppressed the production of TNF-α in LPS-stimulated RAW 264.7 macrophages, whereas soysapogenol A and B, did not. Furthermore, none of the five structural types of soyasaponins exhibited effect on the production of IL-1 and IL-6 in LPS-stimulated RAW 264.7 macrophages. The decrease in the level of TNF-α observed in the present experiment is consistent with Kang et al.[21] and Zhang et al.[29] Because TNF-α released by inflammatory cells can influence neoplastic growth and metastasis,[16] the inhibitory activity of soyasaponins against TNF-α suggested that soyasaponins may be a potential candidate for attenuating inflammatory response as well as suppressing tumor progression.

In conclusion, soyasaponins A^1, A^2 and I exhibit anti-inflammatory properties through suppressing NO production in LPS-stimulated RAW 264.7 cells by attenuation of NF-κB-mediated iNOS expression. It is proposed that soyasaponins A^1, A^2 and I are potential chemopreventive agent and may be used in the future to treat inflammation-associated tumorigenesis. To our knowledge, this is the first report concerning the evaluation of the anti-inflammatory properties of soyasaponins with various chemical structures.

Acknowledgements

We gratefully thank Jingwen Zeng Ph.D. for her kind technical assistance in real-time fluorescent quantitative PCR and reporter gene assay.

Grants, sponsors and funding sources: This work was financially supported by Guangdong Province '211 Project' (Grant No. GW201020), Special Foundation of President of the School of Public Health and Tropical Medicine (Grant No. GW200823), and Startup Foundation for Advanced Ph.D. Talents in Southern Medical University (Grant No. B1000350).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.071.

References and notes

1. Hu, J.; Reddy, M. B.; Hendrich, S.; Murphy, P. A. *J. Nutr.* **2004**, *134*, 1867.
2. Güçlü-üstünda, Ö.; Mazza, G. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 231.
3. Gurfinkel, D. M.; Rao, A. V. *Nutr. Cancer* **2003**, *47*, 24.
4. Zhang, W.; Popovich, D. G. *J. Agric. Food. Chem.* **2008**, *56*, 2603.
5. Xiao, J. X.; Huang, G. Q.; Zhang, S. H. *Exp. Toxicol. Pathol.* **2007**, *59*, 35.
6. Lee, S.; Simons, A. L.; Murphy, P. A.; Hendrich, S. *Exp. Biol. Med.* **2005**, *230*, 472.
7. Oakenfull, D. G.; Sidhu, G. S. *Eur. J. Clin. Nutr.* **1990**, *44*, 79.
8. Hayashi, K.; Hayashi, H.; Hiraoka, N.; Ikeshiro, Y. *Planta Med.* **1997**, *63*, 102.
9. Nakashima, H.; Okubo, K.; Honda, Y.; Tamura, T.; Matsuda, S.; Yamamoto, N. *AIDS* **1989**, *3*, 655.
10. Kinjo, J.; Imagire, M.; Udayama, M.; Arao, T.; Nohara, T. *Planta Med.* **1998**, *64*, 233.
11. Kinjo, J.; Hirakawa, T.; Tsuchihashi, R.; Nagao, T.; Okawa, M.; Nohara, T.; Okabe, H. *Biol. Pharm. Bull.* **2003**, *26*, 1357.
12. Ishii, Y.; Tanizawa, H. *Biol. Pharm. Bull.* **2006**, *29*, 1759.
13. Berhow, M. A.; Wagner, E. D.; Vaughn, S. F. *Mutat. Res.* **2000**, *448*, 11.
14. Jun, H.; Kim, S.; Sung, M. *J. Med. Food* **2002**, *5*, 235.
15. Shin, E. M.; Zhou, H. Y.; Guo, L. Y.; Kim, J. A.; Lee, S. H.; Merfort, I.; Kang, S. S.; Kim, H. K.; Kim, S.; Kim, Y. S. *Int. Immunopharmacol.* **2008**, *8*, 1524.
16. Coussens, L. M.; Werb, Z. *Nature* **2002**, *420*, 860.
17. Aggarwal, B. B.; Shishodia, S.; Sandur, S. K.; Pandey, M. K.; Sethi, G. *Biochem. Pharmacol.* **2006**, *72*, 1605.
18. Jung, K. H.; Ha, E.; Kim, M. J.; Won, H. J.; Zheng, L. T.; Kim, H. K.; Hong, S. J.; Chung, J. H.; Yim, S. V. *Food Chem. Toxicol.* **2007**, *45*, 1545.
19. Aktan, F.; Henness, S.; Roufogalis, B. D.; Ammit, A. J. *Nitric Oxide* **2003**, *8*, 235.
20. Ahn, K. S.; Noh, E. J.; Zhao, H. L.; Jung, S. H.; Kang, S. S.; Kim, Y. S. *Life Sci.* **2005**, *76*, 2315.
21. Kang, J.; Sung, M.; Kawada, T.; Yoo, H.; Kim, Y.; Kim, J.; Yu, R. *Cancer Lett.* **2005**, *230*, 219.
22. Lee, J. Y.; Shin, J. W.; Chun, K. S.; Park, K. K.; Chung, W. Y.; Bang, Y. J.; Sung, J. H.; Surh, Y. J. *Carcinogenesis* **2005**, *26*, 359.
23. Ellington, A. A.; Berhow, M.; Singletary, K. W. *Carcinogenesis* **2005**, *26*, 159.
24. Chang, W. W.; Yu, C. Y.; Lin, T. W.; Wang, P. H.; Tsai, Y. C. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 614.
25. Oda, K.; Matsuda, H.; Murakami, T.; Katayama, S.; Ohgitani, T.; Yoshikawa, M. *Vaccine* **2003**, *21*, 2145.
26. Kiefer, D.; Pantuso, T. *Am. Fam. Physician* **2003**, *68*, 1539.
27. Jeon, B. H.; Kim, C. S.; Kim, H. S.; Park, J. B.; Nam, K. Y.; Chang, S. J. *Acta Pharmacol. Sin.* **2000**, *21*, 1095.
28. Kim, K. R.; Son, E. W.; Rhee, D. K.; Pyo, S. *Toxicol. In Vitro* **2002**, *16*, 517.
29. Zhang, W. J.; Wojta, J.; Binder, B. R. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 465.