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GyeongshinhaeGihwan T1 has Controlling Effects on the Factors Associated with Obesity

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Abstract – GyeongshinhaeGihwan T1 (GGT1) is a newly developed oriental medicine to help weight control. We investigated nitric oxide production and cytokine secretion in mouse peritoneal macrophages. According to recent reports, macrophages are participated in fat accumulation and closely related with obesity. In this study, using mouse peritoneal macrophages, we have examined whether GGT1 affects the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin (IL)-12 by the stimulation of interferon-γ and lipopolysaccharide (LPS). GGT1 inhibits LPS-induced NO production in a dose-dependent manner. The decrease in NO synthesis was reflected as a decreased amount of inducible NO synthase protein. We also found that GGT1 inhibits pro-inflammatory cytokines, TNF-α and IL-12 production. In mouse embryo preadipocyte 3T3-L1, GGT1 reduced the viability in a dose-dependent manner. These findings suggest that GGT1 may have potential effects in preventing and controlling adipogenesis and obesity.

Keywords □ GyeongshinhaeGihwan T1, peritoneal macrophages, preadipocyte, obesity, nitric oxide, cytokine

Obesity is one of public health dilemma, especially in developed countries, and has steadily increased at an alarming rate in recent years. Morbid obesity increases the risk of hypertension, coronary artery disease, diabetes mellitus, cancer, sleep apnea, and osteoarthritis (Balsiger *et al.*, 2000).

GyeongshinhaeGihwan T1 (GGT1) is an oriental medicine consisted of a mixture of different herbs. It is invented for weight loss and will be used for weight loss. However, it has not been cleared GGT1 is effective in an experimental model.

In 2000, Kapur *et al.* reported that nitric oxide (NO) is a new player in the modulation of energy metabolism (Kapur *et al.*, 2000). NO is a highly reactive molecule produced from a guanidine nitrogen of NO synthase (NOS). Three isoforms of NOS have been identified and are classified into two major categories, namely constitutive and inducible NOS. Neuronal and endothelial NOSs, which are constitutively expressed, are activated by calcium and calmodulin and are called constitutive NOSs (Nathan, 1992). Of the three NO synthases, inducible NOS (iNOS), the high-output isoform, is the most widely expressed in various cell types after its transcriptional activation (Xie *et al.*, 1992). Most importantly, iNOS is highly expressed in lipopolysaccharide (LPS)-activated macrophages (Petros *et al.*, 1991).

The effect of experimental hyperlipidemia on functional activity of macrophages was studied in CBA and C57Bl/6 mice resistant. Two-month atherogenic diet increased the content of cholesterol in the serum and cells of peritoneal exudate in mice of both strains. In parallel, production of nitrites and 5'-nucleotidase activity in peritoneal macrophages increased (Kiseleva *et al.*, 2002). Furthermore, expression analysis of macrophage and nonmacrophage cell populations isolated from adipose tissue demonstrates that adipose tissue macrophages are responsible for almost all adipose tissue tumor necrosis factor (TNF)-α expression and significant amounts of iNOS and interleukin (IL)-6 expression. Adipose tissue macrophage numbers increase in obesity and adipose tissue macrophages participate in inflammatory pathways that are activated in adipose tissues of obese

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individuals (Weisberg *et al.*, 2003).

In relation with this concept, we studied the inhibitory effects of LPS-induced NO, TNF-α and IL-12 by GGT1 in mouse peritoneal macrophages. In addition, using preadipocyte, we showed that GGT1 inhibits the preadipocyte proliferations.

MATERIALS AND METHODS

Materials

Murine recombinant interferon (rIFN)-γ (1×10^7 U/ml) was purchased from R&D Systems (Minneapolis, MI, USA). *N*- (1-naphtyl)-ethylenediamine dihydrochloride, LPS, and sodium nitrite were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal antisera to iNOS were obtained from Transduction Laboratories (Lexington, KY, USA). Recombinant (r) TNF-α and IL-12, biotinylated anti-murine TNF-α and IL-12, anti-murine TNF-α and IL-12 were purchased from R & D System Inc, USA. Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI, USA). Dulbecco's Modified Eagle's Medium (DMEM) containing L-arginine (84 mg/l), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA).

Animals

Male C57Bl/6 mice were purchased from Orient Co., LTD (Sungnam, Gyeonggi-do, Republic of Korea). They were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1°C and relative humidity of 55 ± 10% throughout the study.

Cell cultures

TG-elicited macrophages were harvested 3 - 4 days after i.p. injection of 2.5 ml TG to the mice and isolated, according to a procedure reported elsewhere (Chung *et al.*, 2004). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. The cells were then distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO_2. They were washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment. 3T3-L1 cells, obtained from the Korean Cell Line Bank (Seoul, Republic of Korea), were maintained in DMEM medium supplemented with 10% FBS.

Preparation of GGT1

Extract of GGT1 was prepared by decocting the dried prescription of herbs with boiling distilled water. The extraction decocted for approximately 3 h has been filtered, lyophilized and stored at 4°C. The ingredients 25.2 g of GGT1 include as follows: *Ophiopogonis Radix* 7.5 g, *Plantycodi Radix* 7.5 g, *Coicis Semen* 7.5 g, *Scutellariae Radix* 3.75 g, *Raphani Semen* 3.75 g, *Ephedrae Herba* 2.62 g etc. These plant materials were obtained from Professor S. S. Shin, College of Oriental Medicine, Dongeui University.

MTT assay

Cell aliquots (2.5×10^5 cells/well) were seeded in microplate wells and incubated with 20 μl of a MTT solution (5 mg/ml) for 4 h at 37°C under 5% CO_2 and 95% air. Consecutively, 250 μl of dimethylsulfoxide was added to extract the MTT formazan and the absorbance of each well at 510 nm was read by an automatic microplate reader.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations of GGT1. The cells were then stimulated with rIFN-γ (20 U/ml). After 6 hours, the cells were finally treated with LPS (10 μg/ml). NO synthesis in cell cultures was measured by a microplate assay method after 24 h and 48 h, as previously described (Jeong *et al.*, 2004). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N* - (1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were pretreated with GGT1 (1 mg/ml). The cells were then incubated for 6 h with rIFN-γ (20 U/ml). They were finally stimulated with LPS (10 μg/ml) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then

blocked with 5% skim milk in phosphate-buffered saline (PBS)-tween-20 (Sigma, St. Louis, MO, USA) for 1 h at room temperature and then incubated with anti-iNOS antibodies. After washing in PBS containing 0.05% tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized by an enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, USA).

Assay of cytokine production

Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (20 U/ml), GGT1, rIFN-γ plus LPS (10 μg/ml), and rIFN-γ plus at various concentrations of GGT1 for 24 h. The amount of TNF-α and IL-12 secreted by the cells were then measured by a modified enzyme-linked immunosorbent assay (ELISA), as described previously (Kim *et al.*, 2004). ELISA TNF-α and IL-12 were carried out in duplicate in 96-well ELISA plates. The plates were then coated with each of 100 μl aliquots of anti-mouse TNF-α and IL-12 monoclonal antibodies at 10 μg/ml in PBS at pH 7.4, and subsequently incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, sample or TNF-α and IL-12 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-mouse TNF-α and IL-12 were added and the plates were incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 20 min at 37 °C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α and IL-12 in serial dilutions.

Statistical analysis

Results were expressed as the mean ± SE of independent experiments, and statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey, and Duncan post hoc test to express the difference among the groups. All statistical analyses were performed using SPSS v10.0 statistical analysis software. A value of $P<0.05$ was considered to indicate statistical significance.

RESULTS

Effects of GGT1 on macrophage viability

To determine the effects of GGT1 on viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with GGT1 (0.01 - 2 mg/ml) during 48 h, the viability was not affected by GGT1 in mouse peritoneal macrophages(Fig. 1).

Effects of GGT1 on NO Production

To determine the effect of GGT1 on the production of NO by mouse peritoneal macrophages, the cells were pretreated with various concentrations of GGT1 (0.01, 0.1 and 1 mg/ml), and then stimulated them with rIFN-γ (20 U/ml) and LPS (10 μg/ml). The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 24 h and 48 h treatment. As shown in Fig. 3, when mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with LPS, NO production was increased about 5-folds after 24 h and 10-folds after 48 h. GGT1 had no effect on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When GGT1 was pretreated in a primed cell, GGT1 inhibited NO production dose-dependently after both of 24 h and 48 h (Fig. 2).

Effects of GGT1 on iNOS expression

Data in Figure 3 show the effects of GGT1 treatments on the expression of iNOS protein in mouse peritoneal macrophages. GGT1 alone did not induce iNOS protein in the peritoneal macrophages. Upon IFN-γ (20 U/ml) and LPS (10 μg/ml) treatment for 12 h, iNOS protein drastically increased in these cells, and pretreatment of cells with GGT1 (1 mg/ml) for 12 h significantly inhibited iNOS induction in mouse peritoneal macrophages(Fig. 3).

Effects of GGT1 on TNF-α and IL-12 production

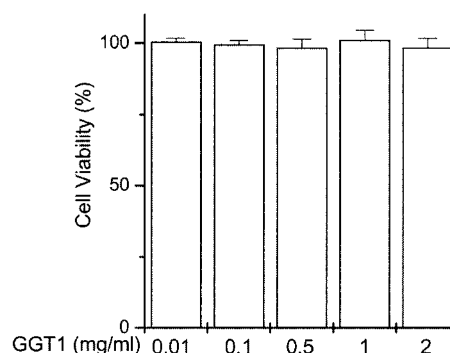

Fig. 1. Effects of GGT1 on the macrophage cell viability. Cell viability was evaluated by MTT colorimetric assay 48 h after GGT1 treatment in peritoneal macrophages. Values are the mean ± SE of three independent experiments duplicate in each run.

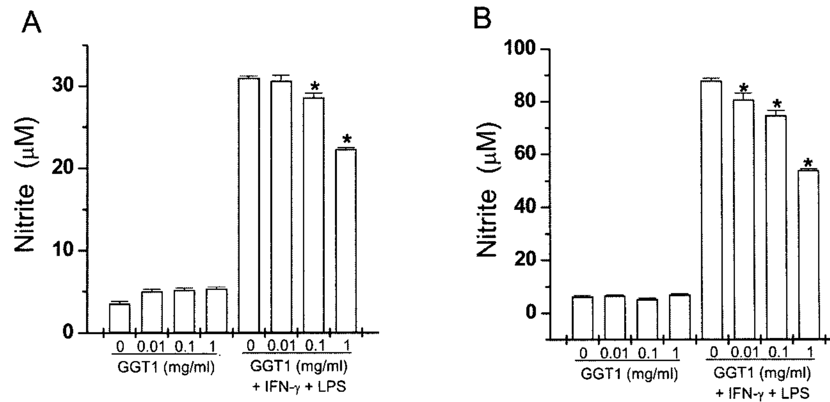

Fig. 2. Dose-dependent effects of GGT1 on NO inhibition in rIFN-γ and LPS-treated peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations GGT1. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 24 h (**A**) and 48 h (**B**) of culture, NO production was measured by the Griess method (nitrite). NO (nitrite) produced into the medium is presented as the mean ± SE of three independent experiments duplicate in each run. *P<0.05 compared to rIFN-γ plus LPS.

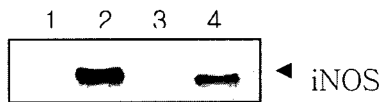

Fig. 3. Effects of GGT1 on the expression of iNOS. Peritoneal macrophages (5×10^6 cells/well) were incubated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with GGT1 (1 mg/ml) or LPS (10 μg/ml) for 12 h. The protein extracts were prepared, and then samples analyzed for iNOS expression by western blotting as described in the method. Lane 1, control; lane 2, rIFN-γ + LPS; lane 3, GGT1 alone and lane 4, GGT1 + rIFN-γ + LPS.

We examined the effects of GGT1 on LPS induced TNF-α and IL-12 production. Mouse peritoneal macrophages secreted low levels of TNF-α after 24 h incubation with medium alone. The basal level of TNF-α was little increased when incubated GGT1 only. Upon IFN-γ (20 U/ml) plus LPS (10 μg/ml) treatment for 24 h, TNF-α drastically increased in these cells, and pretreatment of cells with various concentrations of GGT1 (0.01, 0.1, and 1 mg/ml) for 24 h had inhibitory effects on TNF-α and IL-12 induction. As shown in Fig. 4, the inhibitory effects was dose-dependently and significant at 0.1 and 1 mg/ml.

Effects of GGT1 on preadipocyte proliferation

To test inhibitory effect of GGT1 on adipogenesis, we performed MTT assay in preadipocyte 3T3-L1 cells. 3T3-L1 cells were pre-treated with GGT1 (0.01 - 2 mg/ml) for 24 h or 48 h.

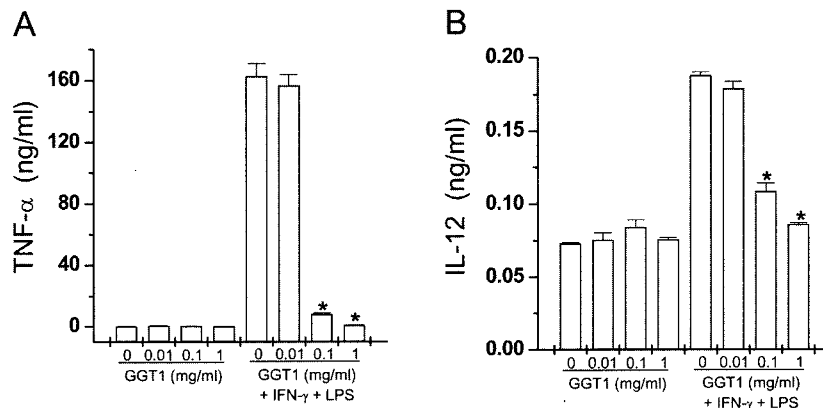

Fig. 4. Effects of GGT1 on the rIFN-γ and LPS-induced TNF-α (**A**) and IL-12 (**B**) production in peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells /well) were stimulated with various concentrations of GGT1 or rIFN-γ (20 U/ml) and LPS (10 μg/ml) plus GGT1. The amount of TNF-α and IL-12 secreted by peritoneal macrophages was measured by ELISA method after 24 h incubation. Values are mean ± SE of three independent experiments duplicate in each run. *P<0.05 compared to rIFN-γ plus LPS.

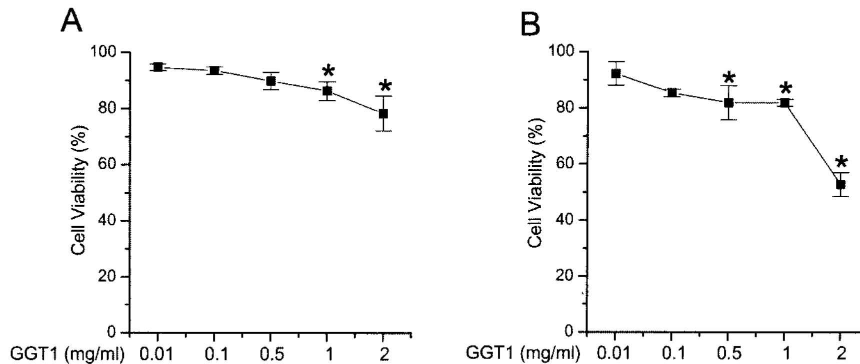

Fig. 5. Effects of GGT1 on the 3T3-L1 cell viability. Cell viability was evaluated by MTT colorimetric assay 24 h (**A**) and 48 h (**B**) after G-41 treatment in peritoneal macrophages. Values are the mean ± SE of three independent experiments duplicate in each run. $^*P$ <0.05 compared to non-treated cell.

After incubating for 24 h or 48 h, cell viability was measured by the MTT assay. The preadipocyte viability was decreased dose and time dependent (Fig. 5).

DISCUSSION

One of the mechanisms of the influence of activated immunocompetent cells on cholesterol metabolism in macrophages was, supposedly, the stimulation of sphigmomyelinase activity by a complex of anti-inflammatory cytokines produced by these cells on their activation (Linton and Fazio, 2003). Thus, macrophages activity is in concert with lipid, fat accumulation and obesity.

In this study, exposure of IFN-γ-primed macrophages to LPS for several hours was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This LPS-induced NO production was inhibited by GGT1. Activated macrophages produce a large amount of NO as a result of the increase of iNOS activity. A previous report showed that activation of macrophages with LPS produced time-dependent expression of iNOS mRNA and protein. Protein expression peaked between 12 and 24 h and had decreased by 75% of the maximum within 48 h. The production of NO in LPS induced macrophages rapidly increased until 24 h and steadily increased till 96 h (Conelly *et al.*, 2001). In accordance with previous reports (Conelly *et al.*, 2001, Jeong *et al.*, 2004), our results showed that NO production in LPS induced macrophages increased about 3 times higher at 48 h later than at 24 h later, and the inhibitory effect of GGT1 became apparent at 48 h later. iNOS protein expression peaked between 12 and 24 h in IFN-g and LPS-induced peritoneal macrophages. However, pretreatment of GGT1 resulted in reduction of IFN-γ and LPS-induced iNOS protein expression. Thus the reduction of NO production by GGT1 can be attributed to inhibition of iNOS protein expression. As is mentioned in the introduction section, NO is known as the player in the modulation of energy metabolism (Kapur *et al.*, 2000). These urge us to investigate the effect of GGT1 on obesity controlling in further study.

In relation with cytokines, we evaluated TNF-α and IL-12 production. TNF-α and IL-12 cytokines are correlated with activations of macrophages. TNF-α regulates systemic responses to microbial infection or tissue injury (Tracey and Cerami, 1994). The role of TNF-a in obesity in human is still unclear. But studies in women have shown that subcutaneous adipose tissue TNF-α mRNA levels in obese subjects are higher than those found in lean subjects, but are normalized after weight loss (Hostamisligil *et al.*, 1995). These findings are paralleled in plasma, where TNF-α levels are increased in obese women compared with lean controls (Bastard *et al.*, 2000). The *db/db*, *ob/ob*, *tub/tub* and *fa/fa* genetic mouse models of obesity all exhibit a similar obesity-related increase in expression of TNF-α mRNA in adipose tissue, compared with lean controls (Hostamisligil *et al.*, 1993). IL-12 is secreted by macrophages and other antigen-presenting cells and plays a critical role in determining the nature of immune response to exogenous or endogenous antigens (Barnes, 2003). In this study, GGT1 inhibited LPS-induced TNF-α, and IL-12 production in a dose-dependent manner. These means that GGT1 reduced the activity of macrophages, and GGT1 is able to reduce macrophage related obesity and fat accumulation.

Preadipocytes 3T3-L1 have the property to differentiatein to adipocytes. Therefore, inhibition of 3T3-L1 proliferations relates to prevention of obesity (Furuyashiki *et al*, 2004). The viability of 3T3-L1 was decreased dose and time dependent by

GGT1 (0.01 - 2 mg/ml). Therefore, GGT1 can be used as a preventive medicine of adipogenesis.

In this study, we tried to estimate the effects of GGT1 in mouse peritoneal macrophages associated with anti-obesity factors. In adipose tissue, the main cellular components are mature, lipid-filled adipocytes, lipid-free preadipocytes and endothelial cells. Nerve fibers and monocytes/macrophage have been identified as additional components (Hauner, 2004), and increased macrophages are connected directly with obesity (Weisberg *et al.*, 2003). Moreover, there are a lot of reports that obesity is an inflammatory condition (Das, 2001, Detrich and Jialal, 2005, Lamas *et al.*, 2003).

We have demonstrated that GGT1 acts as a NO synthesis inhibitor of peritoneal macrophages activation by LPS and that it is through inhibition of iNOS expression. Furthermore, GGT1 inhibited the production of TNF-α, IL-12, and preadipocytes proliferation. These findings suggest that GGT1 might have potential activity of preventing fat accumulation, adipogenesis, or obesity, especially related with macrophages.

In connection with this study, using mouse, the weight reductive effects of GGT1 *in vivo* is currently under investigation. Additionally further investigation is required to clarify unknown constituents, which may be more active than GGT1 itself. The studies on the isolation and characterization of the active chemical constituents are in progress.

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REFERENCES

Balsiger, B.M., Murr, M.M., Poggio, J.L. and Sarr, M.G. (2000) Bariatric surgery. Surgery for weight control in patients with morbid obesity. *Med Clin North Am*, **84**, 477-489.

Barnes, P.J. (2003) Cytokine-directed therapies for the treatment of chronic airway diseases. *Cytokine Growth Factor Rev*, **14**, 511-522.

Bastard, J.P., Jardel, C., Bruckert, E., Blondy, P., Capeau, J., Laville, M., Vidal, H. and Hainque, B. (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab*, **85**, 3338-3342.

Chung, H.S., An, H.J., Jeong, H.J., Won, J.H., Hong, S.H. and Kim, H.M. (2004) Water extract isolated from Chelidonium majus enhances nitric oxide and tumour necrosis factor-alpha production via nuclear factor-kappaB activation in mouse peritoneal macrophages. *J Pharm Pharmacol*, **56**, 129-134.

Connelly, L., Palacios-Callender, M., Ameixa, C., Moncada, S. and Hobbs, A.J. (2001) Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol*, **166**, 3873-3881.

Das UN. (2001) Is obesity an inflammatory condition? *Nutrition* **17**, 953-966.

Dietrich M, Jialal I. (2005) The effect of weight loss on a stable biomarker of inflammation, C-reactive protein. *Nutr Rev*, **63**, 22-28.

Furuyashiki, T., Nagayasu, H., Aoki, Y., Bessho, H., Hashimoto, T., Kanazawa, K. and Ashida, H. (2004) Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells. *Biosci Biotechnol Biochem*, **68**, 2353-2359.

Hauner H. (2004) The new concept of adipose tissue function. *Physiol Behav*, **83**, 653-658.

Hotamisligil, G.S., Arner, P., Caro, J.F., Atkinson, R.L. and Spiegelman, B.M. (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest*, **95**, 2409-2415.

Hotamisligil, G.S., Shargill, N.S. and Spiegelman, B.M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science*, **259**, 87-91.

Jeong, H.J., Chung, H.S., An, H.J., Seo, S.W., Kim, T.G., Won, J.H., Shin, J.Y., Ahn, K.S. and Kim, H.M. (2004) The immune-enhancing effect of the herbal combination Bouum-Myunyuk-Dan. *Biol Pharm Bull*, **27**, 29-33.

Kapur, S., Picard, F., Perreault, M., Deshaies, Y. and Marette, A. (2000) Nitric oxide: a new player in the modulation of energy metabolism. *Int J Obes Relat Metab Disord*, **24 Suppl 4**, S36-40.

Kim, S.J., Jeong, H.J., Lee, K.M., Ryu, H.H., Lee, K.S., Moon, B.S., Cho, K.H. and Kim, H.M. (2005) Zingansikpoongtang modulates beta-amyloid and IL-1beta-induced cytokine production and cyclooxygenase-2 expression in human astrocytoma cells U373MG. *J Ethnopharmacol*, **96**, 279-285.

Kiseleva, E.P., Puzyreva, V.P., Ogurtsov, R.P. and Kovaleva, I.G. (2002) Effect of hyperlipidemia on functional activity of peritoneal macrophages in CBA and C57Bl/6 mice. *Bull Exp Biol Med*, **134**, 288-290.

Lamas O, Moreno-Aliaga MJ, Martinez JA, Marti A. (2003) NF-kappa B-binding activity in an animal diet-induced overweightness model and the impact of subsequent energy restriction. *Biochem Biophys Res Commun*. **311**, 533-539.

Linton, M.F. and Fazio, S. (2003) Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord*, **27 Suppl 3**, S35-40.

Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *Faseb J*, **6**, 3051-3064.

Petros, A., Bennett, D. and Vallance, P. (1991) Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet*, **338**, 1557-1558.

Tracey, K.J. and Cerami, A. (1994) Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med*, **45**, 491-503.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. and Ferrante, A.W., Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*, **112**, 1796-1808.

Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. and Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256**, 225-228.