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# Anti-Inflammatory and Immunomodulatory Activities of Stevioside and Its Metabolite Steviol on THP-1 Cells

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Stevioside, a natural noncaloric sweetener isolated from *Stevia rebaudiana* Bertoni, possesses anti-inflammatory and antitumor promoting properties; however, no information is available to explain its activity. The aim of this study was to elucidate the anti-inflammatory and immunomodulatory activities of stevioside and its metabolite, steviol. Stevioside at 1 mM significantly suppressed lipopolysaccharide (LPS)-induced release of TNF-α and IL-1$\beta$ and slightly suppressed nitric oxide release in THP-1 cells without exerting any direct toxic effect, whereas steviol at 100 $\mu$M did not. Activation of IKK$\beta$ and transcription factor NF-$\kappa$B were suppressed by stevioside, as demonstrated by Western blotting. Furthermore, only stevioside induced TNF-α, IL-1$\beta$, and nitric oxide release in unstimulated THP-1 cells. Release of TNF-α could be partially neutralized by anti-TLR4 antibody. This study suggested that stevioside attenuates synthesis of inflammatory mediators in LPS-stimulated THP-1 cells by interfering with the IKK$\beta$ and NF-$\kappa$B signaling pathway, and stevioside-induced TNF-α secretion is partially mediated through TLR4.

**KEYWORDS: IKK$\beta$; IL-1$\beta$; NF-$\kappa$B; nitric oxide; stevioside; TNF-α**

## INTRODUCTION

Stevioside (**Figure 1A**) is a natural noncaloric sweetener isolated from *Stevia rebaudiana* Bertoni (Compositae), a wild herb occurring in northern Paraguay and parts of Brazil where its leaves have traditionally been used as a sweetener. Pure stevioside is approximately 300 times sweeter than sucrose (*1*). Stevioside is a diterpenic carboxylic alcohol with three glucose molecules and has a molecular weight of 804.9. Steviol (**Figure 1B**) is a major metabolite of stevioside obtained by enzymatic hydrolysis, with a molecular weight of 318.44 (*2*). Extracts of *Stevia rebaudiana* Bertoni leaves and its processed substances, including stevioside, have been used as a sugar substitute to sweeten a variety of foods, including beverages, confectionery, pickled vegetables and seafoods in Japan and other parts of the world. It has particular advantages for those suffering from obesity, diabetes mellitus, heart disease, and dental caries (*3*). Stevioside has been suggested to exert beneficial effects on human health. It has been found to be antihypertensive (*4*, *5*), antihyperglycemic (*6*, *7*), antioxidant (*8*), anti-human rotavirus (*9*), and anti-inflammation and antitumor promoting (*10*). Stevioside has also been reported to influence glucose metabolism (*11*, *12*) and renal function (*13*).

Inflammation is an early host immune reaction mediated via immune cells and their cytokines. Various in vivo and in vitro experimental models have been established to assess the inhibitory effects of various natural products on the synthesis and releasing of inflammatory cytokines. The gram-negative

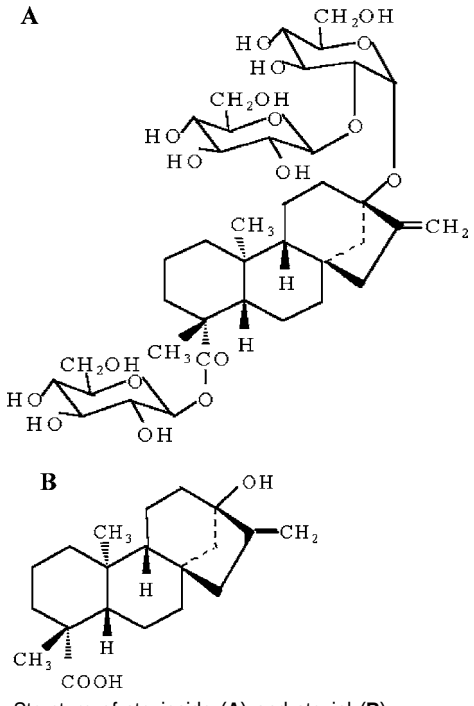


**Figure 1.** Structure of stevioside (**A**) and steviol (**B**).

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bacterial cell wall, in particular lipopolysaccharide (LPS), can stimulate monocytes and macrophages immune cells to release inflammatory cytokines. Among them, the proinflammatory cytokines TNF-α, interleukin (IL)-1β, and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS) are the important inflammatory mediators reported to be involved in the development of a number of inflammatory diseases (*14*). The release of these inflammatory cytokines is essential for host survival from infection and also is required for repair of tissue injury (*15*). Toll-like receptor 4 (TLR4) is the principal receptor for LPS and plays a key role in intracellular signal transduction (*16*). Stimulation of monocyte by LPS leads to the phosphorylation of the inhibitor of NF-κB, IκBs, by IκB kinase (IKKs), resulting in the rapid translocation of NF-κB to the nucleus (*17*). NF-κB activation is involved in expression of cytokine genes, such as TNF-α and IL-1β. Stimulation by LPS is required for NF-κB-dependent expression of TNF-α in human monocytes and THP-1 cells (*18*).

The aim of this study was to investigate the effects of stevioside and its metabolite, steviol, on immunomodulatory activity using an in vitro model LPS-stimulated human monocytic THP-1 cells and monitoring of the production of inflammatory cytokines, TNF-α, IL-1β, and NO. The intracellular signaling pathway was studied by analysis of IKKβ and NF-κB activation, and the direct effect of stevioside on TNF-α secretion mediated by TLR4 was also investigated.

## MATERIALS AND METHODS

**Preparation of Stevioside and Steviol.** Stevioside (approximately 98% purity) was extracted and purified from dried *S. rebaudiana* leaves as described by Adduci et al. (*19*). Steviol (approximately 90% purity) was obtained by oxidation of stevioside as described by Ogawa et al. (*20*). Stevioside and steviol purities were determined by high-performance liquid chromatography (Waters model 510 liquid chromatograph, Millipore Corp., Milford, MA).

**Cell Line and Tissue Culture Media.** THP-1 (TIB-202) cell line was purchased from ATCC (Manassas, VA). Cells were grown in RPMI 1640 medium containing 25 mM HEPES, 2 mM l-glutamine (Sigma, St. Louis, MO), 10% heat inactivated fetal calf serum (Gibco BRL, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% $CO_2$ at 37 °C.

**MTT Assay.** To detect viability of cells, the MTT colorimetric assay was performed. In brief, THP-1 cells ($1 \times 10^6$ cells/mL) were incubated for 6 h in a humidified atmosphere of 5% $CO_2$ at 37 °C with varying concentrations stevioside or steviol in the absence or presence of LPS (1 μg/mL)(Sigma). After addition of MTT solution (5 mg/mL), cells were further incubated for 4 h in a humidified atmosphere of 5% $CO_2$ at 37 °C. A 200-μL aliquot of dimethyl sulfoxide was added, and the absorbance of each well was measured at 540 nm in a Wallac Victor 1420 automatic microplate reader (Perkin-Elmer).

**Determination of TNF-α, IL-1 β, and NO Production.** THP-1 cells ($1 \times 10^6$ cells/mL) were incubated with different concentrations of stevioside or steviol in the absence or presence of LPS (1 μg/mL) for 6 h in a humidified atmosphere of 5% $CO_2$ at 37 °C. Supernatant fluids were collected and stored at −80 °C until measurements of TNF-α, IL-1β, and NO level were performed.

TNF-α and IL-1β levels in supernatant were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN) according to manufacturer's instructions.

NO production was determined by measuring the stable reaction product of NO with molecular oxygen, using a commercial assay kit (Cayman, Ann Arbor, MI) following manufacturer's instructions.

**Western Blotting.** THP-1 cell ($1 \times 10^6$ cells/mL) were stimulated for 45 min with LPS (1 μg/mL), or stevioside (1 mM), or both. Cell lysate was extracted with RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/mL Aprotinin, 5 μg/mL Leupeptin, 1% Tritron X-100, 1% sodium deoxycholate, and 0.1% SDS). The protein concentrations of the lysates were determined using BCA protein assay reagent (Sigma). An equal amount of protein (30 μg) from each lysate was subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Membrane was incubated overnight with 5% non fat milk in TBS containing 1% Tween-20 (blocking TBST) overnight. After washing with TBST, the membrane was treated with rabbit anti-IKKβ antibody (Serotec, Oxford, UK) (1:500 dilution in TBST containing 5% non fat milk) or with rabbit anti-NF-κB p50 antibody (Serotec) (1:2000 dilution in blocking TBST solution) for 2 h and washed three times with TBST. The membrane was then incubated with horseradish peroxidase conjugated secondary antibody (Santacruz) (1:5000 dilution in blocking TBST solution) for 1 h, and the antigen−antibody complex was visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, UK) according to the manufacturer's recommendation.

**Blocking of TLR4.** THP-1 cells ($1 \times 10^6$ cells/mL) were incubated with 20 μg/mL of antihuman TLR4 (Serotec, Oxford, UK) or IgG2A (Serotec) isotype specific antibodies for 1 h at 37 °C. This preparation was subsequently added with stevioside (1 mM), and the solution was incubated for 6 h in a humidified atmosphere of 5% $CO_2$ at 37 °C. The supernatant was assayed for TNF-α.

**Statistical Analysis.** Data from at least three individual experiments were analyzed and presented as mean ± standard error measurement (SEM). Statistical significance was determined by using one-way ANOVA and student Newman-Keuls, with a value of $p < 0.05$ as being statistically significant.

## RESULTS

**Effect of Stevioside on TNF-α and IL-1β release.** Human monocytic THP-1 cell line has been widely used to characterize the actions of various immunomodulatory components (*21*). To study the anti-inflammatory and immunomodulatory effects of stevioside, proinflammatory cytokines, TNF-α and IL-1β, were measured in unstimulated and LPS-stimulated THP-1 cells. **Figure 2** demonstrates that unstimulated THP-1 cells cultured for 6 h produced 20.6 ± 15.4 pg/mL of TNF-α and negligible quantity of IL-1β. However, treatment of cells with stevioside for 6 h induced TNF-α and IL-1β release; only 1 mM stevioside significantly induced release of 1135 ± 193 pg/mL of TNF-α (**Figure 2A**) and 140 ± 21 pg/mL of IL-1β (**Figure 2B**). Stimulation with LPS (1 μg/mL) for 6 h caused a substantial increase in the release of proinflammatory cytokines (2986 ± 165 and 312 ± 35 pg/mL for TNF-α and IL-1β, respectively). When THP-1 cells were treated with LPS (1 μg/mL) in the presence of stevioside, significant inhibition of cytokine release was observed at 1 mM stevioside, 25.9 and 34.9% for TNF-α (**Figure 2A**) and IL-1β (**Figure 2B**), respectively.

To evaluate whether the inhibitory effects on cytokine release were due to direct toxicity on THP-1 cells, viability of THP-1 cells were evaluated by MTT assay. Stevioside was not cytotoxic either with or without LPS (1 μg/mL) (data not shown).

**Effect of Steviol on TNF-α and IL-1β release.** When THP-1 cells were stimulated with LPS (1 μg/mL) together with steviol (0.1−100 μM) for 6 h, TNF-α (**Figure 3A**) and IL-1β (**Figure 3B**) levels in the supernatant fluid remained unchanged compared to control.

**Effect of Stevioside and Steviol on NO Production. Figure 4A** shows that unstimulated THP-1 cells cultured for 6 h produced 2.1 ± 0.1 μM of nitrite, and stimulation with LPS (1 μg/mL) for 6 h caused a significant increase in the level of nitrite to 6 ± 0.4 μM. When THP-1 cells were stimulated with LPS (1 μg/mL) together with stevioside (1 mM) for 6 h, the level of nitrite was decreased to 4.7 ± 0.5 μM, but this was not significantly different when compared with LPS-stimulated cells alone. In addition, stevioside alone significantly induced NO production (4.2 ± 0.1 μM at 1 mM stevioside) when compared

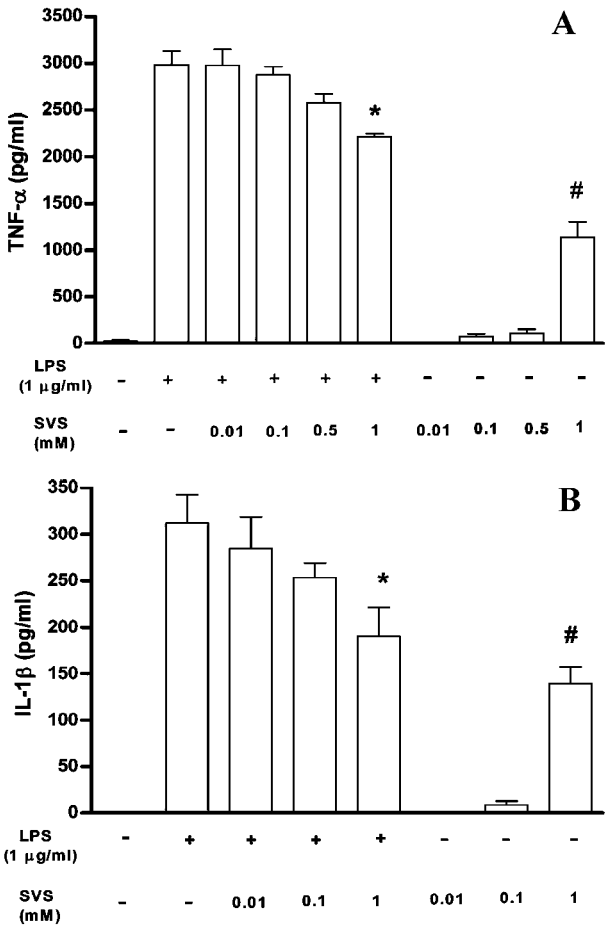


**Figure 2.** Effects of stevioside on the production of TNF-α (**A**) and IL-1β (**B**) in THP-1 cells. Data are expressed as the mean ± SEM of three independent experiments. Statistically significant difference in cytokine release ($p < 0.05$), as compare with the LPS-treated (*) and untreated (#) groups, respectively.

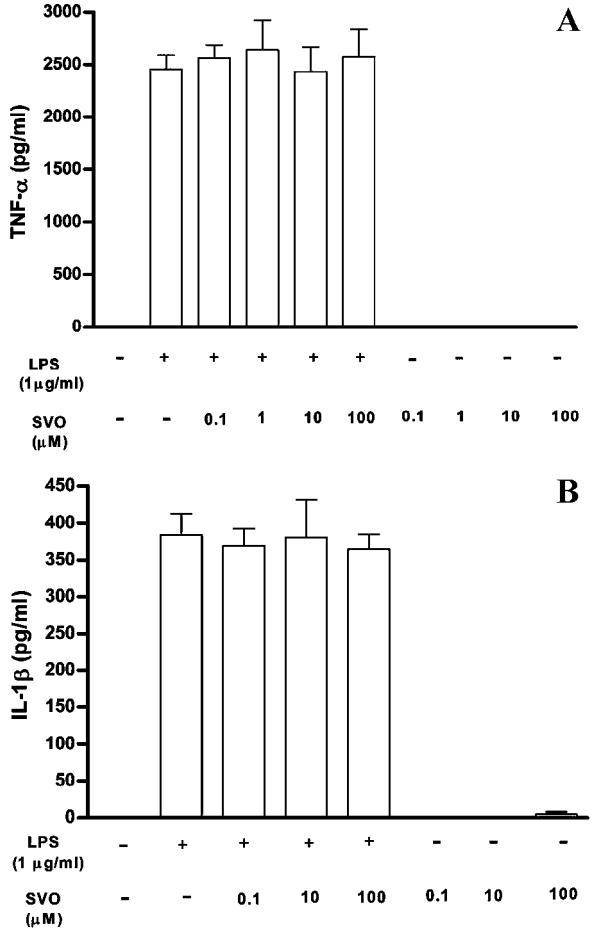


**Figure 3.** Effects of steviol on the production of TNF-α (**A**) and IL-1β (**B**) in THP-1 cells. Data are expressed as the mean ± SEM of three individual experiments.

with unstimulated THP-1 cells but with less magnitude than that of LPS alone. On the other hand, steviol had no effect on NO production in LPS (1 μg/mL)-stimulated THP-1 cells, although steviol alone at 100 μM slightly induced NO production (2.7 ± 0.4 μM) (**Figure 4B**).

**Effect of Stevioside on NF-κB Activation.** The common pathway involved in the induction of inflammatory gene expression, including that of TNF-α, IL-1β, and NO, is via NF-κB. To investigate whether the inhibitory action of stevioside on LPS-treated THP-1 cells was due to an effect on NF-κB activation, levels of NF-κB (p50) were examined in THP-1 cells lysates by Western blot analysis. The expression level of NF-κB was increased in LPS-stimulated THP-1 cells but was decreased in the presence of 1 mM stevioside (**Figure 5A**).

**Effect of Stevioside on IKKβ Protein Activation.** LPS-activation of IKKβ has been shown to be required for the phosphorylation of IκBs, resulting in the rapid translocation of NF-κB to the nucleus (*18*). To investigate whether the stimulatory effect of stevioside on cytokines release was due to an effect on IKKβ activation, the levels of IKKβ were examined in THP-1 cells lysates after LPS (1 μg/mL) stimulation for 45 min using Western blot anlysis. In LPS-stimulated cells, the expression level of IKKβ was increased, whereas the IKKβ level was decreased when 1 mM stevioside was cultured together with LPS for 45 min (**Figure 5B**).

**Effect of Blocking of TLR4 on Stevioside-Induced TNF-α Release.** Toll-like receptor 4 (TLR4), an important receptor for LPS, has been implicated in inflammatory cytokine production. We therefore asked whether stevioside-induced TNF-α release is mediated through TLR4. THP-1 cells were preincubated with anti-TLR4 antibody for 1 h before adding 1 mM stevioside for a further 6 h. The culture supernate was assayed for TNF-α level. THP-1 cell pretreated with anti-TLR4 antibody showed significant decrease of TNF-α release from 860 ± 25.5 pg/mL to 470 ± 35.3 pg/mL (**Figure 6**). Treatment with IgG2A antibody, used as a negative control, had no effect on TNF-α level.

## DISCUSSION

This study shows for the first time the effects of stevioside on the production of inflammatory cytokines, TNF-α, IL-1β, and NO. It is known that pathogenic bacteria and other infectious agents can activate monocytes or macrophages directly, initiating a cytokine cascade in the inflammatory process and the immunological response (*22*). Stimulated monocytes release a broad spectrum of cytokines. TNF-α and IL-1β are biologically active peptides produced by monocytes, induced by endotoxin and other stimuli (*23*). In addition, the reactive free radical NO also plays a role in inflammation (*24*). Thus, the interference in the production of TNF-α, IL-1β, and/or NO can be employed as criteria to evaluate anti-inflammatory effects of natural products.

Moderate levels of inflammatory mediators are important for host survival from infection, while overproduction has deleterious effects. Therefore, synthesis of inflammatory cytokines must

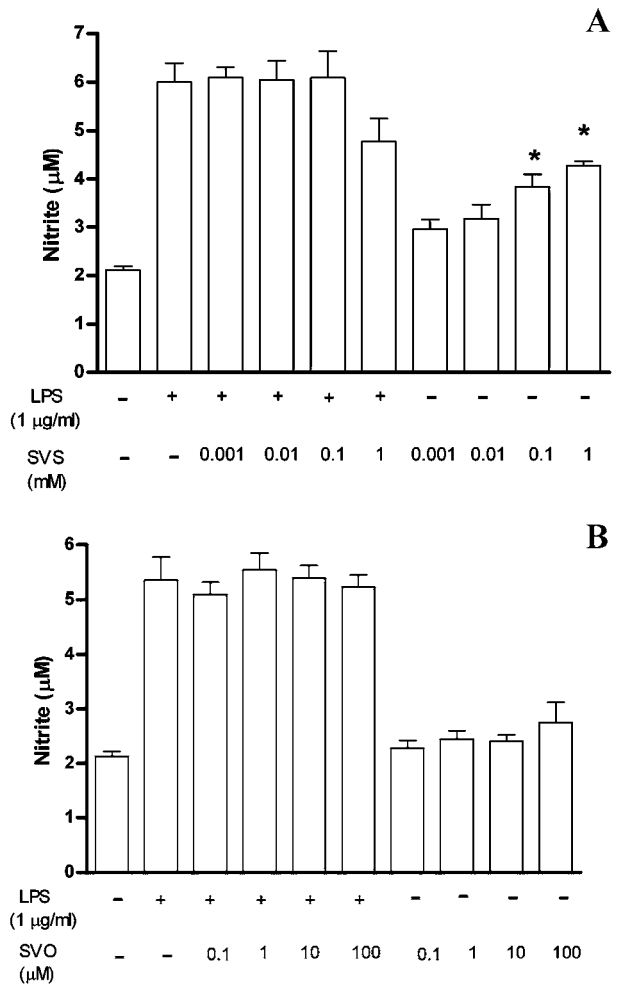


**Figure 4.** Effects of stevioside (**A**) and steviol (**B**) on the production of NO in THP-1 cells. Data are expressed as the mean ± SEM of four individual experiments. Statistically significant difference in cytokine release ($p < 0.05$), as compared with untreated (*) group.

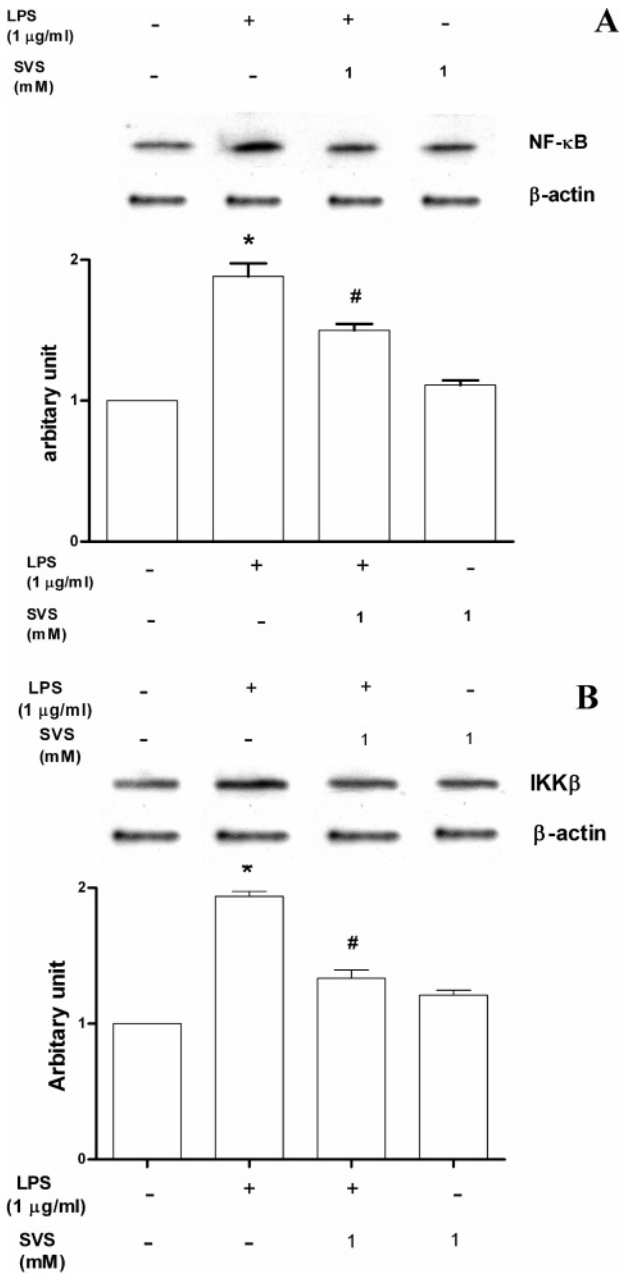


**Figure 5.** Effect of stevioside on activation of NF-$\kappa$B (**A**) and IKK$\beta$ (**B**) in THP-1 cells. Statistically significant difference in NF-$\kappa$B and IKK$\beta$ protein ($p < 0.05$), as compared with the untreated (*) and LPS-treated (#) groups, respectively.

be tightly governed (*15*). In the present study, we demonstrated that stevioside (1 mM) significantly decreased the production of TNF-α and IL-1$\beta$ and slightly decreased NO production, in LPS-stimulated THP-1 cells. These activities were not attributed to cell cytotoxicity. These results are consistent with earlier observation of stevioside being an anti-inflammation in mouse skin (*10*).

Macrophage-derived mediators such as TNF-α and NO have been recognized for their cytostasis and/or cytotoxic properties against tumor cells and microorganisms (*25*). We demonstrated that stevioside alone could directly activate THP-1, especially at the dose of 1 mM, to release TNF-α and NO. The magnitude of induction of inflammatory mediator was consistently less than that of LPS (1 $\mu$g/mL) stimulation, suggesting a possible beneficial effect of stevioside on innate immunity. This may be one possible mechanism of stevioside and stevia mixture that has been reported to possess antitumor-promoting effect (*10*).

TLR4 is a critical receptor mediating effects of LPS in TNF-α secretion in THP-1 cells and blocking of TLR4 and LPS interaction by using anti-TLR4 antibody has been studied (*26*). We hypothesized that stevioside-mediated TNF-α secretion by extracellular interacting with the surface of THP-1 cells possibly TLR4. Our study demonstrated that TNF-α release from stevioside-treated THP-1 cells was partially neutralized by anti-TLR4 antibody. This effect was not observed with the stevioside metabolite, steviol. Thus, the induction of TNF-α secretion by stevioside may be partially mediated via TLR4 and the three glucose molecules that are present only in stevioside may play this role in its interaction.

It is well known that the expression of several genes involved in immune inflammatory responses is regulated at the transcriptional level by NF-$\kappa$B (*27*). Stimulation of monocytes by LPS leads to an activation of IKK$\beta$, which is required for NF-$\kappa$B-dependent transcription and TNF-α expression. (*18*). We found that stevioside decreased IKK$\beta$ and NF-$\kappa$B activation when cultured with LPS, concomitant with the reduction of TNF-α, IL-1$\beta$, and NO release from THP-1 cells. Therefore, stevioside can attenuate inflammatory mediator synthesis through the inhibition of IKK$\beta$ and NF-$\kappa$B activation.

In summary, this study provided evidence that stevioside induces TNF-α, IL-1$\beta$, and NO production in unstimulated

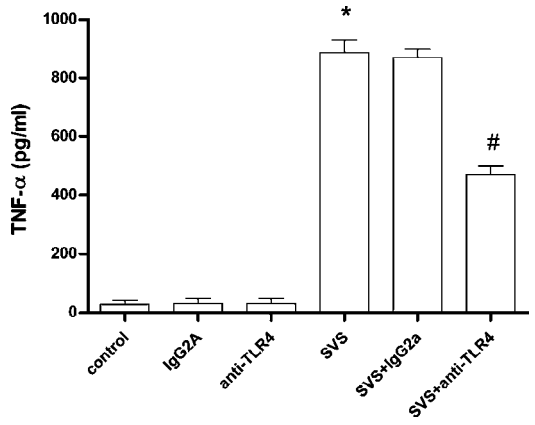


**Figure 6.** Effect of blocking of TLR4 on stevioside-induced TNF-α secretion in THP-1 cells. Data are expressed as the mean ± SEM of three individual experiments. Statistically significant difference in cytokine release ($p < 0.05$), as compared with the control (*) and stevioside-treated (#) groups, respectively.

human monocytic THP-1 cells. The induction of TNF-α, IL-1$\beta$, and NO may augment macrophage function and thus contribute to the enhancement of innate immunity. On the other hand, inhibition of TNF-α, IL-1$\beta$, and NO release in LPS-stimulated THP-1 cells could be of benefit in circumstances where there is a pathological effect resulting from the excessive of TNF-α, IL-1$\beta$, and NO production, which may indicate an anti-inflammatory effect of stevioside. Stevioside is widely used as sweetener and is contained in many foods and beverages, and therefore, consumption of stevioside may also be able to enhance innate immunity and protect against inflammatory diseases.

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