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ARTICLE *in* JOURNAL OF COMPLEMENTARY AND INTEGRATIVE MEDICINE · JANUARY 2012

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Recommended Citation:
Verma, Nandini; Chakrabarti, Rina; Das, Rakha H.; and Gautam, Hemant K. (2012) "Anti-Inflammatory Effects of Shea Butter through Inhibition of Inos, Cox-2, and Cytokines via the Nf-Kb Pathway in Lps-Activated J774 Macrophage Cells," *Journal of Complementary and Integrative Medicine*: Vol. 9: Iss. 1, Article 4.

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Anti-Inflammatory Effects of Shea Butter through Inhibition of Inos, Cox-2, and Cytokines via the Nf-Kb Pathway in Lps-Activated J774 Macrophage Cells

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Abstract

Shea butter is traditionally used in Africa for its anti-inflammatory and analgesic effects. In this study we explored the anti-inflammatory activities of the methanolic extract of shea butter (SBE) using lipopolysaccharide (LPS)-induced murine macrophage cell line J774. It was observed that SBE significantly reduced the levels of LPS-induced nitric oxide, Tumor necrosis factor-α (TNF-α), interleukins, 1β (IL-1β), and -12 (IL-12) in the culture supernatants in a dose dependent manner. Expression of pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were also inhibited by SBE. These anti-inflammatory effects were due to an inhibitory action of SBE on LPS-induced iNOS, COX-2, TNF-α, IL-1β, and IL-12 mRNA expressions. Moreover, SBE efficiently suppressed IκBα phosphorylation and NF-κB nuclear translocation induced by LPS. These findings explain the molecular bases of shea butter's bioactivity against various inflammatory conditions and substantiate it as a latent source of novel therapeutic agents.

KEYWORDS: inducible nitric oxide synthase, cyclooxygenase-2, pro-inflammatory cytokines, nuclear factor-kappa B

INTRODUCTION

Shea butter is an ivory-colored natural fat extracted from fruits of the shea tree (*Vitellaria paradoxa)* which is predominantly found in sub-Saharan Africa and a region of East Africa (Hall et al. 1996). Shea butter is traditionally used to treat various skin problems including dry skin, psoriasis, burns, eczema, wrinkles and rheumatism relief which suggest its potential anti-inflammatory properties (Thioune, Ahodikpe, Dieng, Diop & Ngom, 2000; Akihisa T., et al., 2010).

Shea butter is extracted from the seed of the shea tree by crushing and boiling. It contains substantial amounts of fatty acids out of which stearic and oleic acids together account for 85-90% (Badifu, 1989). The unsaponifiables constituents of shea butter are biologically active compounds which include phyto-sterols together with triterpenes (Badifu, 1989) and triterpenes having uv-absorbing properties (Wiesman et al., 2003). However, no striking regional difference in the composition of the triterpenes fractions was observed (Akihisa, T., et al., 2011). Studies revealed that shea kernel is rich in phenolic compounds with antioxidant and free radical scavenging properties (Steven et al., 2003). Unrefined shea butter has been studied as an anti-inflammatory tropical cream, being helpful in cases of arthritis (Kerharo, 1942). Even through shea butter is traditionally used for centuries in African countries to relieve congestion, sprains, and arthritis, its anti-inflammatory activity and mode of action are not evaluated. This study addresses the effect of shea butter extract (SBE) on LPS-induced inflammatory responses in the J774 murine macrophages and to gain insight into the molecular mechanisms underlying potential anti-inflammatory properties.

Macrophages play a central role in inflammatory diseases through the release of inflammatory mediators such as prostaglandin (PGE2), nitric oxide (NO) and cytokines involved in the immune response (Lowenstein, Hill, Walker, Allen & Landavere, 1996; Harris, Padilla, Koumas, Ray & Phipps, 2002). LPS is an endotoxin, an integral outer membrane component of Gram-negative bacteria, and triggers inflammatory responses. Binding of LPS with Toll-like receptors causes the activation of the transcription factor NF-κB to induce the transcription of genes responsible for inflammation (Verstak et al., 2007). Generation of these mediators has been established in many inflammatory tissues, due to transcriptional activation of the genes following the exposure to various immune stimulants including bacterial lipopolysaccharide (LPS). Nuclear factor-κB (NF-κB) is a transcription factor that plays a primary role in the regulation of inflammatory mediators (Chen, Demers & Shi, 2002). In resting cells, NF-κB remains in the cytoplasm as heterodimeric complexes bound to one of the inhibitor IκB proteins. Stimulus-induced activation of the NF-κB-inducing kinase leads to the phosphorylation of IκB kinase complexes, followed by ubiquitination

and proteasome-mediated degradation of IκB that releases NF-κB to translocate in the nucleus. NF-κB interacts with its target motifs in the nucleus and regulates the synthesis of various inflammatory mediators (Ghosh & Karim, 2002). Overproduction of the NF-κB regulated inflammatory mediators is involved in many diseases, such as rheumatoid arthritis, chronic hepatitis, pulmonary fibrosis and cancer (Coker & Laurent, 1998; Lawrence, gilory, Colville & Willoughby, 2002). In the present study, we have investigated the effects of shea butter extract (SBE) using LPS-induced inflammatory responses in the J774 murine macrophages.

MATERIALS AND METHODS

Chemicals

Cell culture media (RPMI 1640 containing 2 mM L-glutamine) and antibiotic-antimycotic solution were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), trypan blue, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), rabbit anti-mouse iNOS, COX-2, NF-κB p65 subunit and phospo-IκBα (p-IκBα) antibodies and *Escherichia coli* lipopolysaccharide strain 055:B8 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Griess assay kit was obtained from Promega (Madison, WI, USA). Horseradish peroxidase conjugated anti-rabbit polyclonal antibodies were purchased from Santa Cruz biotechnology (Delaware Avenue Santa Cruz, CA, USA). Other chemicals and solvents were of reagent grade.

Preparation of methanolic extract of shea butter

Shea butter was acquired from Dr. Ajit Kumar from Durban University, Durban. One hundred grams of shea butter was vigorously shaken with 150 ml of methanol (MeOH) **at** room temperature and extracted after for 48 hours by centrifugation. The solvent fraction was isolated and dried under reduced pressure using rotary evaporator at 40° C. The dried crude methanolic extract (2.38 g) was dissolved in DMSO to make a stock solution of 50 mg/ml and the extract was designated as SBE. Filter sterilized SBE was used in in-vitro assays.

Cell culture and treatments

J774 murine macrophage cells (obtained from National Centre for Cell Science, Pune, India) were cultured at 37°C in a humidified incubator with 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma). LPS was prepared as a 1 mg/ml stock solution in sterile PBS [137.0 mM NaCl, 2.7 mM KCl, 4.3 mM

$Na_2HPO_4.7H_20$, 1.4 mM KH_2PO_4, pH 7.4] and stored at -20 °C. SBE was added to the culture medium 2 h before LPS treatment.

MTT assay

The MTT assays for cell viability were performed in a 96 well plate using 5×10^4 cells/well. Briefly, the cells were treated with different concentrations (0-1000 µg/ml) of SBE for 24 h. At the end of each experiment, the cells were washed and incubated with MTT (20 µl of 5 mg/ml per well) at 37°C for 4 h. The formazan product was solubilized by addition of 100 µl of DMSO and 100 µl of 10% SDS in 0.01 M HCl. Viable cells were estimated by recording the absorbance at 595 nm in an ELISA plate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA).

NO production assay

The production of NO, reflecting the cellular NO synthase activity was estimated from the accumulation of nitrite (NO_2^-), a stable oxidation product of NO in the medium. Nitrite was measured using the Griess reagent as described earlier (Chaudhury, Raghav, Gautam, Das & Das 2006). Briefly, after pre-incubation of the cells (1×10^5 cells/ml) with different concentrations of SBE for 2 h, cells were washed and incubated for 24 h either with or without LPS (1µg/ml), at 37˚C in a 5% CO_2 incubator. Nitric oxide was measured as NO_2^- in culture supernatant by reaction with Griess reagent. Absorbance of the reaction product was determined at 532 nm using a microplate reader (Molecular devices, Orleans Drive Sunnyvale, CA, USA). Sodium nitrite was used as a standard to calculate nitrite concentrations.

Measurement of TNF-α, IL-1β and IL-12

Macrophage cells (1×10^5 cells/ml) were incubated with different concentrations of SBE for 2 h, washed with fresh medium and then stimulated with 1 µg/ml of LPS. The culture supernatants were collected after 24 h of LPS stimulation. The levels of TNF-α, IL-1β and IL-12 in the culture media were measured by ELISA kits (BD Biosciences, San Jose, California, USA) according to the manufacturer's instructions. Quantitation of the ELISA results was performed using a microplate spectrophotometer (Molecular devices, USA) set to a wavelength of 450 nm and corrected for absorbance at 540 nm, according to the manufacturer's instruction.

Western blot analysis

For cytoplasmic protein extract preparation, $1x10^6$ adherent cells were washed three times with ice-cold PBS and then scraped on ice into a solution containing

10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.4 mM phenylmethyl sulfonyl fluoride (PMSF), leupeptin (10 mg/ml), aprotinin (10 mg/ml), 0.1 mM Na_3VO_4 and 1 mM DTT, and kept on ice for 15 min.. After the addition of NP-40 to a final concentration of 0.15%, the lysate was vigorously mixed for 15 seconds and then centrifuged at 16 000 r.p.m. for 1 min at 4˚C. The resulting supernatant was stored at -80˚C as cytoplasmic extract, and the nuclear pellet was resuspended in a solution containing 50 mM HEPES—KOH (pH 7.9), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.2 mM NaF, leupeptin (10 mg/ml), aprotinin (10 mg/ml), 0.4 mM PMSF, 0.1 mM Na_3VO_4, 1 mM DTT and 10% glycerol. The resulting suspension was incubated for 30 min on ice with occasional vortexing and then centrifuged at 16 000 r.p.m. for 30 min at 4˚C and collected the supernatant as nuclear fraction. Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). The supernatants containing 50 µg of protein were solubilized by boiling in SDS-PAGE sample buffer for 5 min and were separated by 8% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The cellular levels of iNOS, p-IκBα, and NF-κB were determined by immunoblotting using anti-mouse iNOS, COX-2, p-IκBa and NF-κB antibodies (Sigma-Aldrich Co.). The bands were visualised by incubating with anti-rabbit HRP conjugated secondary antibody for 1 h followed by developing with HRP substrate (Sigma, USA).

RNA isolation and RT-PCR

Macrophages (1×10^5 cells/ml) were incubated with different concentrations of SBE for 2 h, washed with fresh medium and were then stimulated with 1 µg/ml of LPS. Cells were harvested after 4 h of LPS treatment and then used to isolate the total RNA (using RNA isolation kit, Qiagen, Germantown, Maryland, USA) following the supplier's protocol. RNA was quantified spectrophotometrically and 2 µg of total RNA from each sample was used for cDNA synthesis (using cDNA synthesis kit, Clontech, Mountain View, CA, USA) following the manufacturer's protocol. Gene specific PCR for the inflammatory mediators and glyceraldehyde-3-phosphatedehyrogenase (GAPDH) as housekeeping gene were carried out separately. Primer sequences were designed from cDNA sequences of the specific gene (www.ncbi.nlm.nih.gov/locuslink) using DNASTAR software. The primer sequences, annealing temperatures (T_m) and the amplicon lengths used to amplify the genes were as follows: TNF-α (accession no. NM_013693.2) sense: 5′-CAGGGGCCACCACGCTCTTC-3′, antisense: 5′-CTTGGGGCAGGGGCTCTTGAC-3′, 60 °C, 419 bp; IL-1β (accession no. NM_008361.3) sense: 5′-CAGGCTCCGAGATGAACAACAAAA-3′, antisense: 5′- TGGGGAACTCTGCAGACTCAAACT-3′, 60 °C, 332 bp; IL-12 (accession no. NM_008352.2) sense: 5′- GTGACACGCCTGAAGAAGATGACA-3′, antisense: 5′-CGGCAGTTGGGCAGGTGAC-3′, 60° C, 433 bp; iNOS (accession

no. NM_010927.2) sense: 5′-TCACTGGGACAGCACAGAAT-3′, antisense: 5′-TGTGTCTGCAGATGTGCTGA-3′, 60 °C, 510 bp; COX-2 (accession no. NM_011198.3) sense: 5′- TCCAGATCATATTTGATTGACAGT-3′, antisense: 5′-AGACCAGGCACCAGACCAAAGA-3′, 59 °C, 539 bp. The PCR mixture consisted of 25 mM 10X Taq buffer containing 15 mM Mg^{2+}, 5 mM dNTPs, 10 pM each of the sense and the antisense primers, 2 units Taq DNA polymerase enzyme and 2 µl of 1:5 diluted cDNA in a 25 µl reaction volume. PCR conditions to amplify the genes included an initial denaturation at 94 °C for 4 min and 34 cycles at 94 °C for 30 seconds, 60 °C (or the specific primer set T_m) for 30 seconds, 72 °C for 1 min and then a final extension for 7 min at 72 °C. The amplified PCR products were separated on 1.2% agarose gel and the densitometric analysis of the gene specific PCR products with respect to GAPDH gene was carried out using Digidoc 1201 software.

Nuclear translocation assays of p65 protein

Inhibition of the LPS induced NF-*κ*B nuclear translocation in the macrophages in response to SBE treatment was visualized by immunocytochemistry. Cells were seeded into 4-well chamber slides at the concentration of 5×10^3 cells/well and allowed to adhere for 24 h in RPMI containing 10% FBS. The cells were then washed 3 times with 1 ml of RPMI and incubated with different concentrations of SBE for 2 h. The cells were then washed and treated with LPS (1µg/ml) for 1 h followed by 3 times washing with 1 ml of cold PBS, fixed with 3.7% formalin in PBS for 10 min, and permeabilized with 1% Triton X-100 in PBS for 30 min. The immunostaining for NF-*κ*B p65 (Santa Cruz, USA) was carried out following the manufacturer's instructions supplied with the antibody. The cells were subsequently incubated with FITC conjugated secondary anti-rabbit IgG (diluted 1:200 in PBS containing 1% BSA) for 1 h. After washing the cells thrice with PBS the slides were observed under inverted fluorescence microscope (Nikon, Japan) at 40 X magnifications and photographed using digital camera DXM 20 1200F and ACT-1 software system.

Statistical analyses

The values of three separate sets of experiments are expressed as mean ± S.D in each figure. Data were analyzed for the significance of differences from the respective control using Student's t-test for each paired experiments. $p\leq0.05$ was considered as significant.

RESULT AND DISCUSSION

SBE treatment prior to LPS stimulation showed dose dependent suppression of LPS induced NO production in the culture supernatants of J774 macrophages without any significant changes in cell viability (Fig. 1A). Treatment of macrophages with SBE (50-150 µg/ml) significantly inhibited protein (Fig. 1B) and mRNA (Fig. 1D) expressions of iNOS and COX-2 in a concentration dependent manner as evident from the densitometric analysis (Fig. 1C, E).

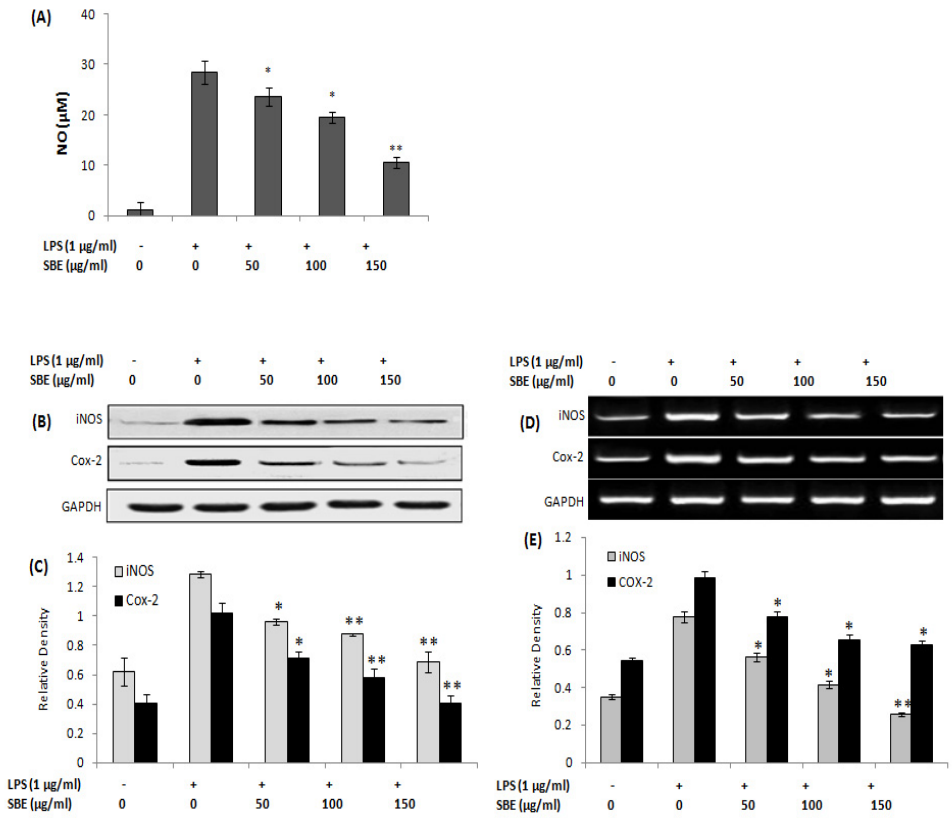

Figure 1. (A) Effect of SBE on LPS-induced NO production in J774 murine macrophages. NO was measured by the Griess reaction. Effect of SBE on LPS-induced expression of iNOS and COX-2 proteins in J774 macrophages. (B) The protein and (D) mRNA expression levels of iNOS and COX-2 as determined Western blotting and RT-PCR respectively, one of three representative set of experimental image is shown. (C) Relative densities of iNOS and Cox-2 proteins and (E) nRNAs to the housekeeping protein GAPDH. Data represent the mean±S.D.; *p < 0.05, **p < 0.01 compared with LPS alone.

SBE at its highest concentration (150 µg/ml) reduced the protein expression levels of iNOS and COX-2 respectively by 1.868 and 2.48 folds

(Fig.1C) whereas their gene transcripts were reduced by 3.002 (p<0.01) and 1.57 folds (p<0.05) respectively (Fig.1E). LPS induced production of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-12 (IL-12) in cells were found to be suppressed by SBE. As shown in the figure 2A, treatment of macrophages with SBE (50 µg/ml) led to a significant decrease in TNF-α (1.5 folds) and IL-1β (1.32 folds) levels. However, no significant change was seen in IL-12 level with SBE upto100 µg/ml. Further increase of SBE concentration (150 µg/ml) resulted in 2.64 and 2.129 folds (p<0.01) suppression of TNF-α and IL-1β respectively, while the IL-12 secretion was also found to be inhibited (1.44 folds) significantly (p<0.05). The transcriptional reduction of these pro-inflammatory genes was also evident from the densitometric scans of their RT-PCR products obtained (Fig. 2B and C) by their respective gene specific primer sets. These changes in the pro-inflammatory cytokines are in agreement to the inhibitory effect of SBE on their mRNAs and appeared in a dose-dependent manner.

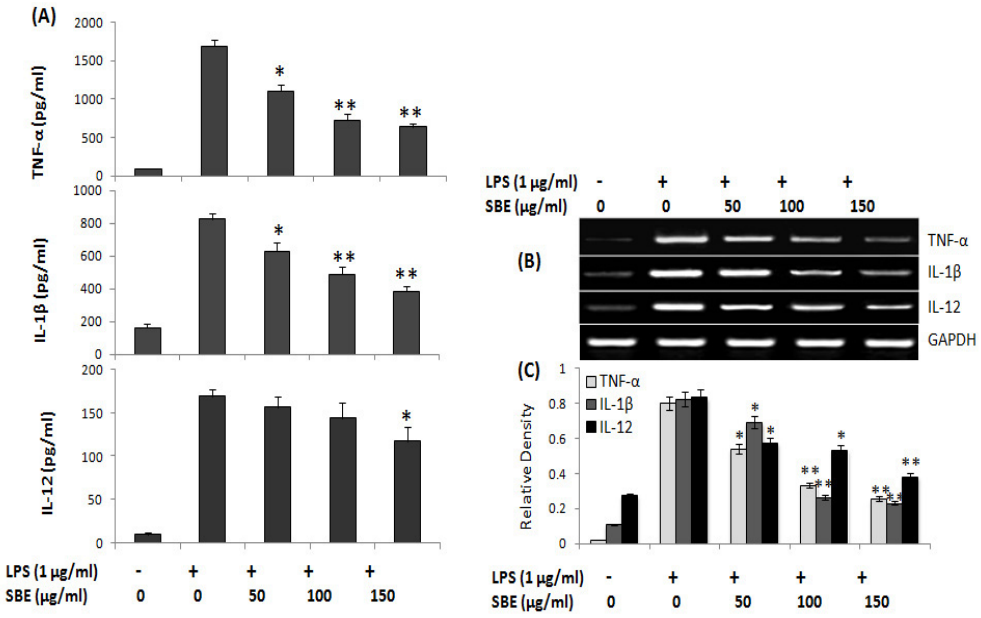

Figure 2. (A) Effects of SBE on LPS stimulated inflammatory cytokines production in J774 cells. The concentration of cytokines in the culture medium was determined by ELISA. (B) Effect of SBE on LPS-induced mRNA expression of pro-inflammatory cytokines in J774 murine macrophages as observed in RT-PCR amplified products. (D) Relative density (to the housekeeping gene GAPDH) of the PCR products observed in agarose gels from three independent sets of experiments, data represent the mean±S.D.; *p < 0.05, **p < 0.01 compared with LPS alone.

LPS stimulation is known to induce the activation and nuclear translocation of NF-κB which involves the production of pro-inflammatory mediators. It was observed that SBE inhibited NF-κB activation, and the inhibitory effect increased with the increase of its concentration (Fig. 3).

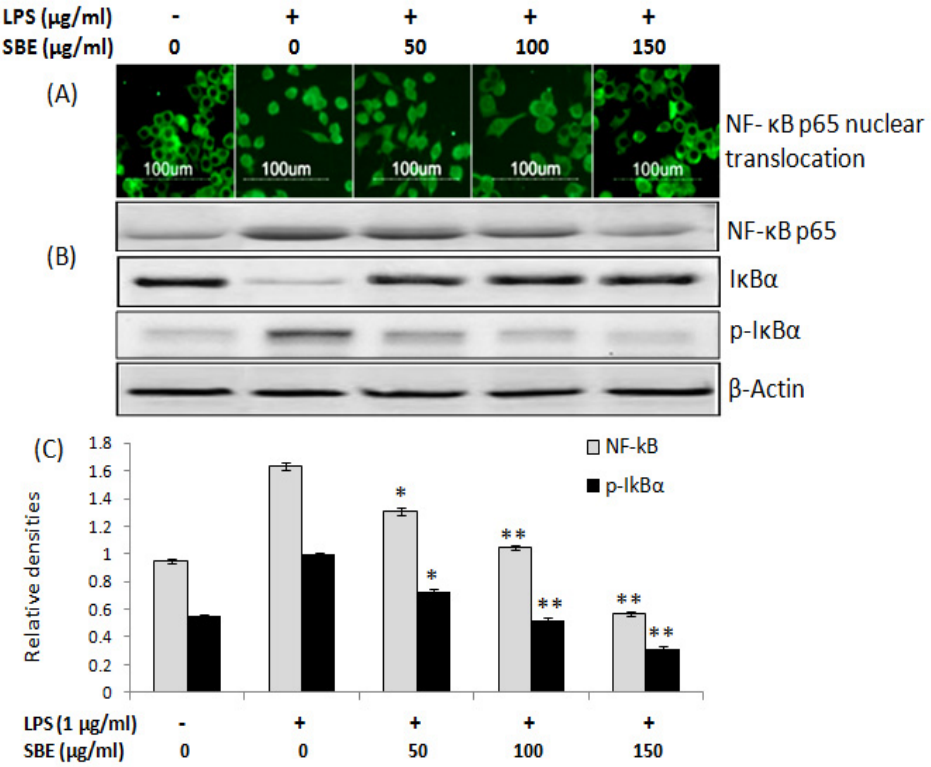

Figure 3. Effect of SBE on LPS-induced nuclear translocation of NF-κB p65 subunit in J774 cells. (A) Cells pre-incubated with different concentrations of SBE (μg/ml) followed by LPS (1 μg/ml) treatment for 30 min were analysed immunohistochemically (B) The levels of NF-κBp65 and phospo-I-κBα proteins in the nuclear and cytoplasmic fractions respectively, were analyzed by Western blotting. (C) Relative densities of specific proteins (to β-actin) observed in Western blots from three independent sets of experiments, data represent the mean±S.D.; *p < 0.05, **p < 0.01 compared with LPS alone.

Immunostaining of SBE treated cells with anti-NF-κB p65 IgG showed the increased inhibition of NF-κB p65 translocation in the nuclei with the increase of SBE dosage. Densitometric analysis of the p65 subunit immunoblot showed that the SBE inhibited translocation of NF-κB by 1.24, 1.56 and 2.89 folds (p<0.05) with 50, 100, and 150 μg/ml of SBE respectively (Fig. 3B and C). The activation and translocation of NF-κB was also found to be correlated with the proteolytic degradation of the inhibitory subunit of NF-κB (IκBα) in the cytoplasm after

phosphorylation (Fig. 3B and C). Comparison of cytoplasmic IκBα and p-IκBα in SBE treated and untreated cells showed no differences in IκBα levels but the levels of its phosphorylated form (p-IκBα) decreased significantly ($p<0.05$) with the increase of SBE concentration (Fig. 3B). Densitometric analysis of the immunoblot (Fig. 3C) indicated upto 3.13 folds decrease ($p<0.01$) in the cytoplasmic concentration of p-IκBα on SBE (150 μg/ml) treatment. This could only happen when SBE inhibits LPS induced phosphorylation of the IκBα subunit in the cytoplasm.

Inflammation is an essential physiological process which helps in pathogen clearance and facilitates wound healing. Many plant-derived compounds showing significant anti-inflammatory effects are now gaining more pharmacological attention (Harvey, 2008; Verma, Tripathi, Sahu, Das & Das, 2009). Identification of new compounds from unexplored natural sources to develop new drugs, especially for the treatment and/or control of chronic inflammatory states such as rheumatism, asthma, inflammatory bowel diseases, and atherosclerosis is challenging. To explore the anti-inflammatory activity of shea butter we studied the effects of its methanolic extract in suppressing the levels of inflammatory mediators elevated by LPS in murine macrophages. SBE effectively inhibits iNOS and COX-2 production and secretion of pro-inflammatory cytokines at concentrations much below of its cytotoxic effects on macrophages. Suppression of LPS induced inflammatory mediators by SBE is due to the downregulation of their respective genes at the transcriptional level. The molecular mechanism by which SBE suppresses the expressions of inflammatory mediators appears to involve the inhibition of LPS induced NF-κB activation by modulating the phosphorylation of IκBα regulatory subunit. All these results suggest that shea butter contains potential therapeutic agent(s) for treating inflammatory conditions by the regulation of NF-κB activation.

Conclusion

The results suggested that SBE exerts its anti-inflammatory activity by downregulating the mRNA and protein expressions of established pro-inflammatory cytokines and interleukins. SBE was found to have regulatory effects on LPS induced inflammatory responses by inhibiting the NF-κB activation by suppressing the phosphorylation of IκBα. Thus the suppression of NF-κB activation dependent inflammatory genes is responsible for the anti-inflammatory effects of SBE. It can be concluded that the herbal product shea butter has potential anti-inflammatory principle(s) which need to be identified by further studies.

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