

Six-shogaol Inhibits Production of Tumour Necrosis Factor Alpha, Interleukin-1 Beta and Nitric Oxide from Lipopolysaccharide-stimulated RAW 264.7 Macrophages

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ABSTRACT

Objective: We previously reported that 6-shogaol, a phenolic compound from ginger, has anti-inflammatory properties in a Complete Freund's Adjuvant (CFA) model of mono-arthritic rats. In the present study, we investigated the effects of 6-shogaol on the production of inflammatory mediators from lipopolysaccharide (LPS) activated RAW 264.7 macrophages. These mediators (TNF- α , IL-1- β and NO) and their output from macrophages are involved in various pathophysiological events of chronic inflammation and arthritis.

Methods: Effects of 6-shogaol were investigated on the production of the mediators TNF- α , IL-1- β and NO (measured as nitrate) from macrophages. Lipopolysaccharide activated RAW 264.7 macrophages were cultured in the presence and absence of 6-shogaol (2 μ M, 10 μ M and 20 μ M) and ELISA was used to quantify the output of the mediators.

Results: 6-shogaol (2 μ M, 10 μ M and 20 μ M) significantly inhibited the production of nitric oxide (NO), IL-1 β and TNF- α from the LPS activated RAW264.7 macrophages.

Conclusion: The results suggest that macrophages are targets for the anti-inflammatory effects of 6-shogaol. Also, the inhibitory effects against TNF- α , IL-1 β and NO production from LPS activated macrophages are cellular mechanisms by which 6-shogaol produced its anti-inflammatory effects. These mechanisms provide an explanation of the protection by 6-shogaol against development of joint inflammation and cartilage degradation in CFA induced mono-arthritis that we previously demonstrated (1). Based on these results with 6-shogaol, there is evidence that it exhibits exploitable anti-inflammatory properties.

El 6-shogaol Inhibe la Producción del Factor de Necrosis Tumoral Alfa, la Interleuquina-1 Beta, y el Óxido Nítrico de los Macrófagos 264.7 RAW Estimulados por Lipopolisacáridos

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RESUMEN

Objetivo: Con anterioridad reportamos que el 6-shogaol – un compuesto fenólico del jengibre – posee propiedades anti-inflamatorias en un modelo CFA de ratas monoartríticas. En el presente estudio, investigamos los efectos del 6-shogaol sobre la producción de mediadores inflamatorios de macrófagos 264.7 RAW estimulados por lipopolisacáridos. Estos mediadores (TNF- α , IL-1- β y NO) y su producción de macrófagos están involucrados en varios eventos patofisiológicos de inflamación crónica y artritis.

Métodos: Se investigaron los efectos del 6-shogaol sobre la producción de los mediadores TNF- α , IL-1- β y NO (medidos como nitratos) de los macrófagos. Macrófagos 264.7 RAW estimulados por

lipopolisacáridos fueron cultivados en presencia y ausencia de 6-shogaol (2 μ M, 10 μ M y 20 μ M) y se usó la técnica de ensayo por inmunoabsorción ligado a enzimas (ELISA) a fin de cuantificar la producción de mediadores.

Resultados: *El 6-shogaol (2 μ M, 10 μ M y 20 μ M) inhibieron significativamente la producción de óxido nítrico (NO), IL-1 β y TNF- α de los macrófagos RAW 264.7 activados mediante LPS.*

Conclusión: *Los resultados sugieren que los macrófagos son blancos de los efectos anti-inflamatorios del 6-shogaol. Por otro lado, los efectos inhibitorios contra la producción de TNF- α , IL-1 β y NO a partir de macrófagos activados por LPS, son mecanismos celulares mediante los cuales 6-shogaol produjo sus efectos anti-inflamatorios. Estos mecanismos ofrecen una explicación de la protección mediante 6-shogaol contra el desarrollo de inflamación en las articulaciones y la degradación de los cartílagos en la monoartritis inducida mediante CFA que demostramos con anterioridad (1). Sobre la base de estos resultados con 6-shogaol, hay evidencia de que posee propiedades anti-inflamatorias explotables.*

West Indian Med J 2009; 58 (4): 296

INTRODUCTION

We previously reported that 6-shogaol (1-4-hydroxy-3-methoxyphenyl-4-decen-3-one), a phenolic compound from ginger has significant anti-inflammatory properties in a Complete Freund's adjuvant (CFA) model of mono-arthritic rats (1). Reduction of macrophage infiltration in the knee joint was a significant finding in that study: macrophages are therefore considered to be targets for the anti-inflammatory effects of 6-shogaol. Consequently, in the present study, RAW 264.7 macrophages were used to investigate the effects of 6-shogaol on the production of pro-inflammatory mediators that are known to contribute to the pathological features of chronic inflammation.

A large body of evidence suggests that tumour necrosis factor-alpha (TNF- α), interleukin-one beta (IL-1- β) and nitric oxide (NO) are involved in various pathophysiological events of chronic inflammation and arthritis (2). Therefore, effects on the release of these mediators from RAW 264.7 cells were investigated in this study. Specific functions of NO in arthritis include activation of metalloproteinases such as collagenases in chondrocytes (3) and inhibition of osteoblast proliferation (4). Nitric oxide also promotes the apoptotic death of synovial cells and chondrocytes (5). It enhances cytokine induced osteoclastic bone resorption (6) and indirectly promotes cartilage catabolism through free radical damage to the matrix (7, 8). Similar effects are produced by TNF α and IL-1 β leading to cartilage and bone destruction in rheumatoid arthritis (RA). The mechanisms include induction of collagenase release from chondrocytes (9), osteoclasts and synovial fibroblasts (10). The cytokines also inhibit proteoglycan synthesis and stimulate resorption of cartilage and bone (11). Additionally, IL-1 β serves as a trigger for chemotactic activity of neutrophils and lymphocytes (10).

Macrophages are major sources of nitric oxide (NO) and cytokines during the pathological manifestations of arthritis (10, 12, 13). Macrophages are also normal constituents of synovial tissue, and at this site their concentrations increase during inflammatory synovitis and CFA

arthritis (1, 14). The activities of these synovial macrophages can be reproduced by the RAW 264.7 cell line which has many similarities. For example, they respond to LPS stimulation by increasing either the expression or production of NO (15), IL-1 (16) and TNF- α (17, 18). These cultured macrophages were therefore used in this study as targets for investigation of the effects of 6-shogaol on the production of TNF α , IL-1 β and NO.

MATERIAL AND METHODS

Propagation of the RAW 264.7 cell line

RAW 264.7 macrophage cells (ECACC) were propagated in T25 flasks containing Dulbecco's modified eagle's medium (DMEM from Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS from Sigma Aldrich), 2mM L-glutamine (Sigma Aldrich) and 100 units mL⁻¹ penicillin/streptomycin/fungizone antibiotic mixture (Cambrex, USA). Cells were incubated at 37°C in humidified air containing 5% CO₂ (19). The cells were adherent and they reached confluence within 48 hours. Confluent flasks were scraped with a cell scraper and the contents of one flask split into two flasks. This propagation procedure was repeated until enough cells were obtained for the experiment.

Assessment of the viability of the cell line

The viability of the RAW 264.7 cell line after propagation was determined using the trypan blue exclusion technique (20). For this, cells were harvested from each of the culture flasks using a cell scraper and they were centrifuged (5RPM/5minutes) to a plug then resuspended in DMEM media to produce a concentration of 10⁶ cells/ml. This concentration was achieved by counting the cells on a Coulter counter. One hundred microlitres (100 μ l) of the cell suspension was then mixed with an equal volume of trypan blue (0.4%). The mixture was allowed to stand for 1–2 minutes to facilitate absorption into the cells. Five to ten microlitres (5–10 μ l) of the mixture were then used to fill one of the chambers of a haemocytometer, and the cells were viewed under a light microscope using x 20 magnification. The total

number of cells was counted and those that were stained dark blue (indicating dead cells) were identified for determination of viability.

The percentage viability was determined using the following formula:

$$\% \text{ viability} = \frac{\text{total cells counted}/1\text{mm}^2 - \text{stained cells}}{\text{total cells counted}} \times 100$$

Assessment of cytotoxic potential of 6-shogaol on the RAW 264.7 cell line

The colorimetric assay using methyl thiazolyl tetrazolium (MTT) was performed to titrate cell viability after treatment with 6-shogaol. This was done to assess the potential cytotoxicity of 6-shogaol on the RAW 264.7 cells. The assay was performed using a Cell quanti-MTT kit according to the manufacturer's instructions (BioAssay Systems).

RAW 264.7 cells at a density of 5.0×10^4 cells mL^{-1} in DMEM containing 10% FBS were dispensed into 96 well plates in volumes of 80 μl . Six-shogaol was diluted in DMEM containing 10% FBS to final concentrations of 2 μM , 10 μM , 20 μM , 40 μM , 60 μM and 80 μM . A volume of 20 μl of each concentration of 6-shogaol was added in duplicate to selected wells. Control wells were treated in duplicate with a known cytotoxic compound, saponin, (20 μl) at concentrations of 0.1, 0.01, 0.001 and 0.0001 weight % (where 1% = 10 mg/ml). A second set of control wells was used containing cells only in DMEM (20 μl) supplemented with 10% FBS. Fifteen microlitres (15 μl) of the MTT labelling reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were then added to each well and incubated in a humidified atmosphere at 37°C for four hours. Following incubation, 100 μl of the solubilizing reagent was added to each well and mixed gently for one hour at room temperature. The absorbance was then measured at 570 nm using a microplate reader (MF iEmS, Labsystems).

Activation and treatment of RAW 264.7 cell line for effects on mediator production

RAW 264.7 cells were plated in 10cm diameter dishes at a density of 5×10^6 cells/ml in DMEM containing 10% fetal bovine serum (19). The cells in each dish were incubated for 18 hours with different concentrations of 6-shogaol (2 μM , 10 μM and 20 μM) and LPS (1 $\mu\text{g}/\text{ml}$) in triplicates. Following these treatments, the culture medium was harvested and aliquots were stored at -20°C for use in different assays (21).

Colorimetric assay for NO production in RAW 264.7 cells incubated in the absence and presence of 6-shogaol

Nitric oxide (NO) levels were measured in the cell culture supernatants obtained after activation with LPS in the absence and presence of 6-shogaol. Nitric oxide levels were determined using a nitrate/nitrite colorimetric assay kit according to the manufacturer's instructions (Cayman Chemical).

Immunoassay for TNF- α and IL-1 β production in RAW 264.7 cells incubated in the absence and presence of 6-shogaol

TNF- α and IL-1 β levels were measured in the cell culture supernatants obtained after activation with LPS in the absence and presence of 6-shogaol. TNF- α and IL-1 β levels were determined by ELISA according to the manufacturer's instructions (Pierce Biotechnology).

Statistical procedures and format of presentation of experimental data

Data were obtained from experiments carried out in triplicate and are expressed as means \pm SEM. Test for statistical significance was executed by the unpaired *t* test. Values of $p < 0.05$ were considered to be statistically significant. Graphs were generated using Sigma Plot 2000.

RESULTS

Viability of cultured RAW 264.7 cells

RAW 264.7 cells used in this study were determined to be 90% viable by the trypan blue exclusion analysis.

MTT cytotoxicity assay in RAW 264.7 cells

The IC_{50} determined for 6-shogaol was $40.0 \pm 2.7 \mu\text{M}$. These findings indicated that the concentrations of 6-shogaol (2 μM , 10 μM , 20 μM) used in the study were non-cytotoxic and therefore suitable for studying the release of mediators from RAW 264.7 cells.

Effects of 6-shogaol on nitric oxide (NO) production in LPS activated RAW 264.7 cells

Treatment of RAW 264.7 cells with LPS (1 $\mu\text{g}/\text{ml}$) increased the production of NO, measured as nitrate, to $42.2 \pm 3.0 \mu\text{M}$ from a basal concentration of $19.3 \pm 1.2 \mu\text{M}$ in the absence of LPS treatment (Fig. 1). Six-shogaol (20 μM) significantly ($p = 0.008$) inhibited the production of NO from the LPS stimulated cells causing a reduction in the concentration of nitrate from $42.2 \pm 3.0 \mu\text{M}$ to $32.4 \pm 10.7 \mu\text{M}$ (Fig. 1). Lower

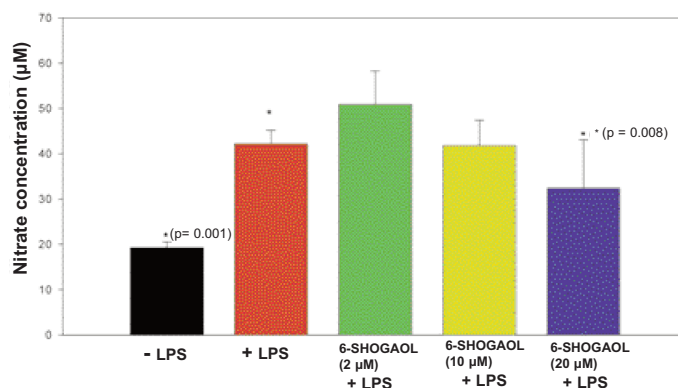


Fig. 1: Graph showing the effect of 6-shogaol (2 μM , 10 μM , 20 μM) on nitric oxide (NO) production in LPS activated RAW 264.7 cells. Each bar represents the mean SEM value from triplicate experiments. Asterisks indicate values significantly different from the control (+ LPS = LPS stimulated cells) value.

concentrations of 6-shogaol (2 μM and 10 μM) did not produce significant inhibition of NO release from the LPS stimulated cells (Fig. 1).

Effects of 6-shogaol on Interleukin-1 Beta (IL-1 β) production in LPS Activated RAW 264.7 Cells

Treatment of RAW 264.7 cells with LPS (1 $\mu\text{g}/\text{ml}$) stimulated the production of IL-1 β (7.7 \pm 0.5 pg/ml) in these cells which had no basal output in the absence of LPS stimulation. Six-shogaol (2 μM) significantly ($p = 0.0001$) inhibited the production of IL-1 β from the LPS stimulated cells causing a reduction in the concentration from 7.7 \pm 0.5 pg/ml to 0.4 \pm 0.3 pg/ml (Fig. 2). Higher concentrations of 6-shogaol (10

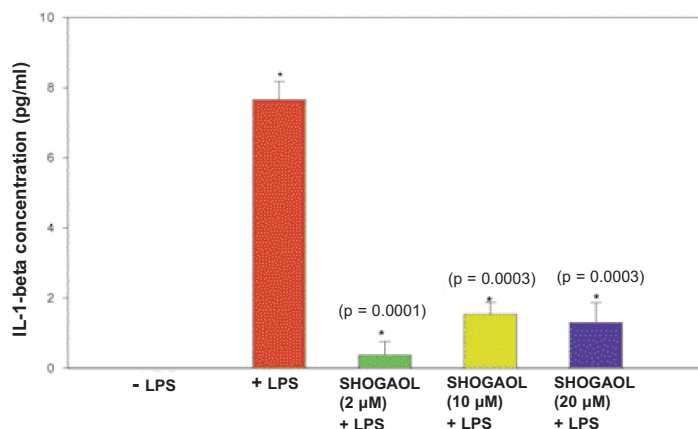


Fig. 2: Graph showing the effect of 6-shogaol (2 μM , 10 μM , 20 μM) on IL-1-beta production in LPS activated RAW 264.7 cells. Each bar represents the mean SEM value from triplicate experiments. Asterisks indicate values significantly different from the control (+ LPS = LPS stimulated cells) value.

μM and 20 μM) also caused significant inhibition of IL-1 β production from the LPS stimulated cells leading to reductions from 7.7 \pm 0.5 pg/ml to 1.5 \pm 0.3 pg/ml ($p = 0.0003$) and 1.3 \pm 0.6 pg/ml ($p = 0.0003$) respectively (Fig. 2).

Effects of 6-shogaol on tumour necrosis factor alpha (TNF- α) production in LPS activated RAW 264.7 cells

Treatment of RAW 264.7 cells with LPS (1 $\mu\text{g}/\text{ml}$) increased the production of TNF- α from a basal concentration of 29.3 \pm 6.3 μM to a significantly ($p = 0.02$) higher concentration of 49.3 \pm 0.9 μM (Fig. 3). Six-shogaol (2 μM) significantly ($p = 0.01$) inhibited this high production of TNF- α to 16.0 \pm 6.8 μM in the LPS stimulated cells (Fig. 3). Higher concentrations of 6-shogaol (10 μM and 20 μM) also caused significant inhibition of TNF- α production from the LPS stimulated cells leading to reductions from 49.3 \pm 0.9 μM to 14.3 \pm 2.2 μM ($p < 0.05$) and 11.0 \pm 5.2 μM ($p = 0.0004$) respectively (Fig. 3).

DISCUSSION

Increased production of the pro-inflammatory mediators NO, TNF- α and IL-1 β from macrophages is a major feature of the

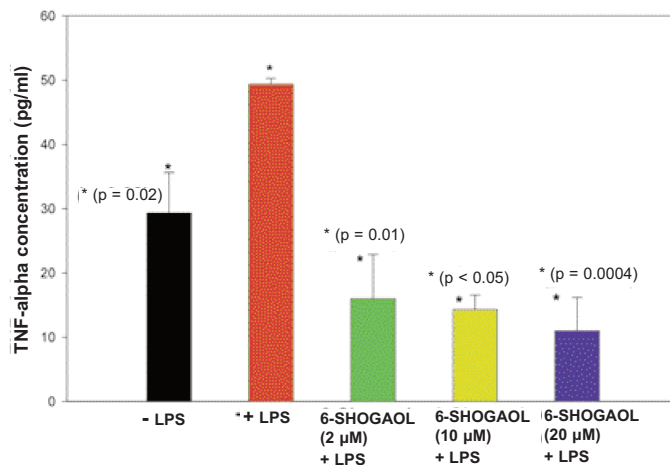


Fig. 3: Graph showing the effect of 6-shogaol (2 μM , 10 μM , 20 μM) on TNF-alpha production in LPS activated RAW 264.7 cells. Each bar represents the mean SEM value from triplicate experiments. Asterisks indicate values significantly different from the control (+ LPS = LPS stimulated cells) value.

pathophysiological manifestations of the inflammatory processes involved in diseases such as rheumatoid arthritis (12, 13, 21, 22, 23). These features were demonstrated in the present study in which there was increased production of NO, TNF- α and IL-1 β from LPS stimulated RAW 264.7 macrophages. Significant infiltration of monocytes/macrophages in the synovial fluid of knee joints of Complete Freund's Adjuvant (CFA) mono-arthritic rats have been reported by us (1). Other studies have reported the release of similar pro-inflammatory mediators from synovial macrophages and RAW 264.7 cells in response to LPS stimulation (19, 20, 21). This similarity in response by RAW 264.7 cells and by synovial macrophages provided the basis for using LPS activated RAW 264.7 cells to study the effects of 6-shogaol, a phenolic compound, on the production of NO, TNF- α and IL-1 β .

According to results reported by Wright *et al* (24), cellular activation by LPS is initiated by LPS-binding protein (LBP), an acute-phase protein that forms complexes with the LPS molecules and transports them directly to the mCD14 receptor on macrophages. In the interaction with macrophages, LPS induces a variety of intracellular signalling cascades leading to the release of mediators such as NO, TNF- α , IL-1 β and PGE₂ (20, 25, 26).

In the present study, NO production from LPS stimulated RAW 264.7 macrophages was significantly decreased by 6-shogaol (20 μM). Other phenolic compounds such as eugenol and bibenzyls were also reported to have similar inhibitory effects on NO production (27, 28). The consequence of reduced NO production at inflammatory sites was reported to cause decreased production of peroxynitrite (29) and reduced susceptibility to its tissue damaging effects. In this regard, we have previously provided evidence that 6-shogaol protected the knee joint from damage during in-

duction of mono-arthritis with complete Freund's adjuvant in rats (1). This finding suggests that the inhibitory effect of 6-shogaol on NO production may have contributed to protection against damage to the knee joint during development of CFA mono-arthritis.

It was also shown in this study that 6-shogaol (2 μ M, 10 μ M and 20 μ M) inhibited the production of TNF- α and IL-1 β from LPS activated RAW 264.7 macrophages. These are among the major mediators involved in the production of the pathological features of chronic inflammatory joint diseases (30). For example, TNF- α and IL-1 β were reported to cause degradation of articular cartilage of arthritic joints by induction of collagenase release from chondrocytes and by inhibition of proteoglycan synthesis (9, 11). But we previously reported that 6-shogaol prevented such cartilage degradation induced by complete Freund's adjuvant in the knee joint of rats (1). This protective effect against damage to the articular cartilage of the knee joint may be explained in terms of the inhibitory action of 6-shogaol on the production of TNF- α and IL-1 β from LPS activated RAW 264.7 macrophages in the present study. Similar inhibitory effects on the production of these cytokines were reported for other phenolic compounds like oleuropein glycoside (31) and eugenol (32).

The anti-inflammatory activity of these phenolic compounds was suggested to be related to common molecular structural features such as the lipophilicity of the alkyl side chain, the substitution pattern of hydroxyl and carbonyl groups on the side chain and hydroxy and methoxy group substitution on the aromatic ring (33, 34). Six shogaol (1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one) has these molecular features (35) which may be the basis for its anti-inflammatory effects, especially the inhibitory effects on cytokine release from macrophages as demonstrated in this study.

The significance of these results with 6-shogaol is that a basis has been established for further investigation of this ginger compound as an anti-inflammatory drug with the potential to prevent progressive damage to inflammatory arthritic joints due to NO, TNF- α and IL-1 β .

CONCLUSION

It is concluded from these results that macrophages are targets for the anti-inflammatory effects of 6-shogaol. Also, the inhibitory effects against TNF- α , IL-1 β and NO production from LPS activated macrophages are cellular mechanisms by which 6-shogaol produced its anti-inflammatory effects. These mechanisms provide an explanation of the protection by 6-shogaol against development of joint inflammation and cartilage degradation in CFA induced mono-arthritis that we previously demonstrated (1). Based on these results with 6-shogaol, there is evidence that it has exploitable anti-inflammatory properties.

ACKNOWLEDGEMENT

This project was funded by a grant from the Graduate Studies and Research Committee at The University of the West Indies, Jamaica. Funding was also provided by a grant to Dr O Simon from the Organization of American States.

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