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Inhibitory Effects of Microwave Radiation on LPS-Induced NFκB Expression in THP-1 Monocytes

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Abstract

Microwave radiations can be encountered regularly in daily lives. When WHO announced that microwave radiations were a kind of environmental energy which interfere with the physiological functions of the human body, great concerns have been raised over the damages microwave frequencies can do to human physiology. The immunological performance and the activities of the cellular inflammatory factor NFκB have been closely related in monocyte. Due to the effect of phorbol 12-myristate 13-acetate (PMA) on THP-1 monocytes, THP-1 monocytes would differentiate into macrophages and would then react with lipopolysaccharides (LPS), and the amount of NFκB increased in the THP-1 monocytes. Expression of cytokine is affected when cells are exposed to a frequency of 2450 MHz and at 900 W. Thus, in our experiments, an observation was made when THP-1 monocytes were stimulated with PMA and LPS to differentiate into macrophage, the amount of NFκB in cells increased exponentially, and the levels of NFκB expression were decreased by the exposure of microwave radiation. In conclusion, microwave radiations were found to inhibit the activity functions of THP-1 monocytes stimulated with PMA and LPS.

Key Words: microwave radiation, monocyte, NFκB, PMA, LPS

Introduction

In the past 30 years, microwave radiations are a major application aspect of necessities, and are used in a variety of ways, including in the industry, science, cooking and medical therapies. Microwave radiations belong to the range of the electromagnetic spectrum with wavelengths from 1 mm to 1 m with corresponding frequencies between 300 MHz and 300 GHz (27). In general, the major frequencies of the applied microwave radiations for industrial and scientific purposes are 915 ± 25, 2450 ±13, 5800 ± 75 and 24125 ± 125 MHz (14). Recently, the major frequency and wavelength of the applied microwave radiation in medical therapy is 2450 MHz and 12 cm, respectively. It has been reported that the percutaneous microwave radiation coagulation is widely used in treatment of hepatocellular carcinoma (5, 17, 18, 24). Today, microwave radiations and daily lifestyles have a close relationship, but there are only a handful of reports concerning the impact of microwave radiation on human health.

Many households use microwave radiations to cook, and the most commonly used frequency of

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microwave ovens is 2450 MHz. There are a few reports that discuss the effects of microwave radiation on physiological activation of cells (20, 26, 29). Microwave radiation is the most common type of energy people come into contact with as it is used in households but not in industry or medical environment. Concerning microwave sterilization, US Federal Communication Commission (FCC) has allocated 2450-MHz bands for domestic microwave heating applications (27). The electromagnetic theory of microwave radiation for heating is well-known. Microwave radiations can be utilized for selective heating of materials. The molecular structure affects the ability of the microwave radiations to interact with materials and transfer energy (27).

Moisture content in the blood can be affected when coming into contact with microwave radiation sources. However, it is uncertain if blood cells are also affected. A few research papers have pointed out how microwave radiation affects immunity. Wang *et al*. (29) indicated that with contacts of microwave radiations (5 mW/cm^2 for 8 h) at 2450 MHz in cultured epithelial cells of rabbit lens might cause inhibition of DNA synthesis and DNA repair suppression. They stated that the subchronic microwave radiation illumination (2450 MHz, 15 mW/cm^2 respectively 1 h everyday for 30 days) in mice could induce apoptosis in the thymocytes causing thymus pathological changes and affecting the mitotic cycle (26). Immunologic functions are possibly suppressed (20). In addition, pregnant mice when exposed to microwave radiation (2450 MHz, 2 mW/cm^2 for 90 min) reduced the activities of natural killer cells (20). The reason for this difference was due to the fact that studies of Wang *et al*. (29) and Nakamura *et al*. (20) used animal models instead of human THP 1 monocytes which were used in our experiment. After exposure to microwave radiation, blood samples were collected from the model animals and changes in the expression of the immune cells were measured. In our experiments, human THP-1 monocytes were exposed to microwave radiation directly, thus, the exposure time would vary as well.

Monocytes of the innate immunity system are activated when the infecting gram-negative bacteria release lipopolysaccharide (LPS) of endotoxin (7). The activated monocytes express many kinds of inflammatory factors to limit the infection area (11). Using the monocytes or the macrophages *in vitro*, the nuclear factor kappa B (NFκB) is identified with many functions to induce different kinds of inflammation cytokines (2). The transcriptional factor NFκB is made up of homodimeric and/or heterodimeric proteins Rel /NFκB family, including p50, p52, p65 (RelA), Rel B and c-Rel (2, 16). NFκB-p65 is a subunit of the NFκB transcription complex that plays a crucial role in inflammatory and innate immune responses (30). The inhibitory effect of IκB on NFκB in the cytoplasm is exerted primarily through interactions with p65 (9). In our studies, the expression level of NFκB p65 acted as a major element of monocyte activity for the inflammatory effect. The goal of this study is to focus on the effects of household microwave radiations on monocyte immunity and molecular mechanism.

Our hypothesis is that microwave radiation decreases NFκB expression of THP1 monocytes treated with phorbol 12-myristate 13-acetate (PMA) and LPS. The amount of NFκB p65 expression was investigated as the ability of immunologic function to assess the feasibility of human health dangers developed from microwave radiation.

Materials and Methods

Cell Culture Conditions

The THP-1 monocytes (BCRC number 60430), known as human monocyte leukemia cells, were obtained from the Bioresource Collection and Research Center of Taiwan (HsinChu, Taiwan). The cells were prepared and resuspended in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), 0.05 mM 2-mercaptoethanol (Gibco, Burlington, ON, USA), 1% Penicillin (100 IU/ml, Gibco) and 1 mg/ml Streptomycin (Gibco). The cells were maintained in a humidified CO_2 incubator (ThermoFisher Scientific, Waltham, MA, USA) at 37°C in presence of 5% CO_2.

PMA Treatment

One hundred nM PMA (P1585, Sigma, St. Louis, MO, USA) was added to the RPMI medium to induce differentiation of the THP-1 monocytes and they were incubated at 37°C for 24 h throughout the study. NFκB assays were used as criteria to study the differentiation and adhesion, morphological changes, and changes in expression of cell surface makers such as integrin, FcγRI, and MHC class II antigen of the THP-1 monocytes. The PMA solution was prepared by dissolving PMA in sterile dimethylsulfoxide (DMSO) (Sigma). The stock solution was stored at -20°C. Immediately prior to use, PMA stock solution was diluted in RPMI medium to 100 nM as the final concentration.

Macrophage Treatment with Microwave Radiation and Stimulation with LPS

THP-1 monocytes were seeded onto a 60 mm^2 culture dish (1×10^6 cells) for the experiments. The

cells were pre-treated with 100 nM of PMA (Sigma P1585) for 24 h and subsequently exposed to microwave radiation at 2450 MHz using a household microwave oven (Whirlpool brand AKM2680 type) for 0, 1, 2, 4, 6 or 8 sec. The cells were subsequently stimulated with 1 µg/ml of *E. coli* LPS (Sigma L4391) for 2 h in a CO_2 incubator at 37°C in the presence of 5% CO_2.

Cell Viability

Cell viability was measured in two ways: after stimulation with microwave radiation and after stimulation with PMA and LPS drugs as described above. The effects of different treatments on THP-1 monocytes were evaluated by 0.4% (final concentration) trypan blue dye (Gibco) exclusion analysis as described earlier (4). The numbers of cells were determined by counting the viable cells in a neubauer-improved haemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany) under an inverted microscope. The percentage of viable cells from each well after incubation with treated extracts was obtained by applying the following equation: % viable cells = (VC/TC) × 100%, where VC = viable cells counted (unstained cells) and TC = total cells counted (stained plus unstained cells).

Preparation of Cell Lysates and Western Blot Analysis

THP-1 monocyte lysates were prepared as described earlier (25) with minor modifications. After treatment, cells were washed with cold PBS containing 137 mM NaCl, 2.7 mM KCl, 10.2 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 (Washing Buffer), and the cells were then lysed in 80 µl of lysis buffer [10 mM Tris-Base, pH 8.0; 50 mM NaCl; 2 mM EDTA; 1% (w/v) Triton X-100; 1 mM Na_3VO_3; 50 mM sodium fluorid; 20 mM $Na_4P_2O_7$ • D10 H_2O; 1% (w/v) protease inhibitor cocktail (Sigma P8340)] for 10 min at 4°C and the cells were then shaken on sonics (VCX 500/750). Insoluble materials in the detergent were pelleted by centrifugation (12,000 rpm, 20 min, 4°C). The protein concentration in the soluble fraction was determined by BCA (Pierce, Rockford, IL, USA) protein assay kit. The solubilized proteins were separated on 10% SDS-polyacrylamide gels (30 µg protein/lane) at 20 mA. The separated proteins were transferred to both a PVDF membrane for 2 h at 350 mA and immunoblots as described (1). Immunoblots with separated proteins were blocked for 1 h at room temperature in TTBS (0.1% Tween 20), 5% skim milk. Immunoblots were treated with the NFκB monoclonal anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblots were washed in TTBS (0.1% Tween-20) and incubated with goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). Visualization of the immunocomplex was conducted by enhanced chemiluminescence (ECL Plus) (PerkinElmer, Waltham, MA, USA). The cellular p65 level was used as a parameter of total cellular NFκB expression.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine production assay for detection of human interleukin-1β (IL-1β) sandwich ELISAs was performed according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). In brief, THP-1 at 4×10^5 cells/ml in a 60 mm^2 culture dish was pre-incubated with PMA at 100 nM for 24 h and subsequently exposed to microwave irradiation at 2450 MHz at various times and subsequent LPS treatment as described above. After the medium was collected from the wells, concentrations of human IL-1β in the supernatant were measured by a commercially available ELISA (R&D Systems). ELISAs were quantitated by absorbance at 450 nm on a ELISA reader (TECAN Sunrise, Männedorf, Switzerland).

Densitometry and Statistical Analysis

Densitometry analysis was performed using the Gel-Pro Analyzer version 4.0 (SynGene, Cambridge, England) for quantification of the western blot band. Results were expressed as the means ± standard error (SE), and the difference between the control and experimental groups was analyzed statistically by the one-way analysis of variance (ANOVA) method. A P-value < 0.05 was considered statistically significant.

Results

Stimulation of Microwaves Radiation, PMA and LPS on the Viability of THP-1 Monocytes

After treating THP-1 monocytes with LPS and PMA inflammatory mediators grown in RPM1 1640 medium, 98% of the original floating THP-1 monocytes were observed to attach to the culture flask and the rate of proliferation stopped. When cell division increased and the cell bodies grew larger, bubbles were formed inside the nucleus and cytoplasmic extensions were enlarged to become pseodopods. Cells were exposed to microwave radiation at different times under the circumstances that cell death was prevented, and it was observed that cell viability did not decrease significantly. It was important to prevent cell death as an experiment control since viable cells were required for measurement of NFκB expression. Cell viability was observed to be above 80% for cells that had been exposed to microwave radiation for 8 seconds (Fig. 1).

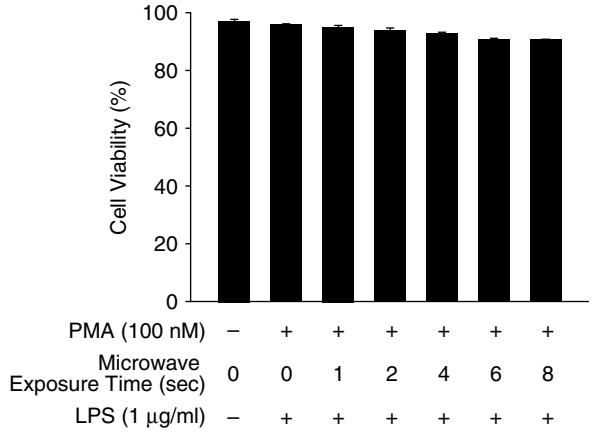

Fig. 1. Effects of microwave radiation on viability of THP-1 monocytes. THP-1 monocytes dividing into macrophages were grown in 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h, and then the THP-1 monocytes were exposed to microwave radiation at 2450 MHz for 0, 1, 2, 4, 6 or 8 sec. The treated THP-1 cells were then incubated with 1 μg/ml LPS for 2 h to collect cells for staining with trypan blue for determination of cell viability. Experimental results are shown as means ± SD with n = 3.

Effects of NFκB Expression on the Development of Monocytes in Comparison to Macrophage THP-1 Monocytes

Studies have shown that LPS is able to induce the expression of THP-1 monocytes NFκB after the differentiation of THP-1 monocytes induced by PMA (3, 11). Expression of NFκB in THP-1 cells was studied both with and without PMA and LPS treatments. Results showed that THP-1 monocytes without PMA treatment showed low levels of NFκB protein expression. After treatment with PMA, cells co-treated with LPS for 2 h expressed high levels of NFκB. As the concentration of LPS increased, the expression level of NFκB also increased (Fig. 2).

Relationship between the Induction Time of LPS in THP-1 Cells before Treatment with PMA and the Expression Level of NFκB

Based on Fig. 2, LPS at 1 μg/ml concentration was used for induction to increase the expression of NFκB in THP-1 monocytes. At the same concentration of LPS, various periods of time of induction in THP-1 monocytes triggered different levels of expression. Results showed that as LPS reaction time increased, expression level of NFκB also increased (Fig. 3).

Expression of NFκB under Induction of LPS after Exposure to Microwave Radiations

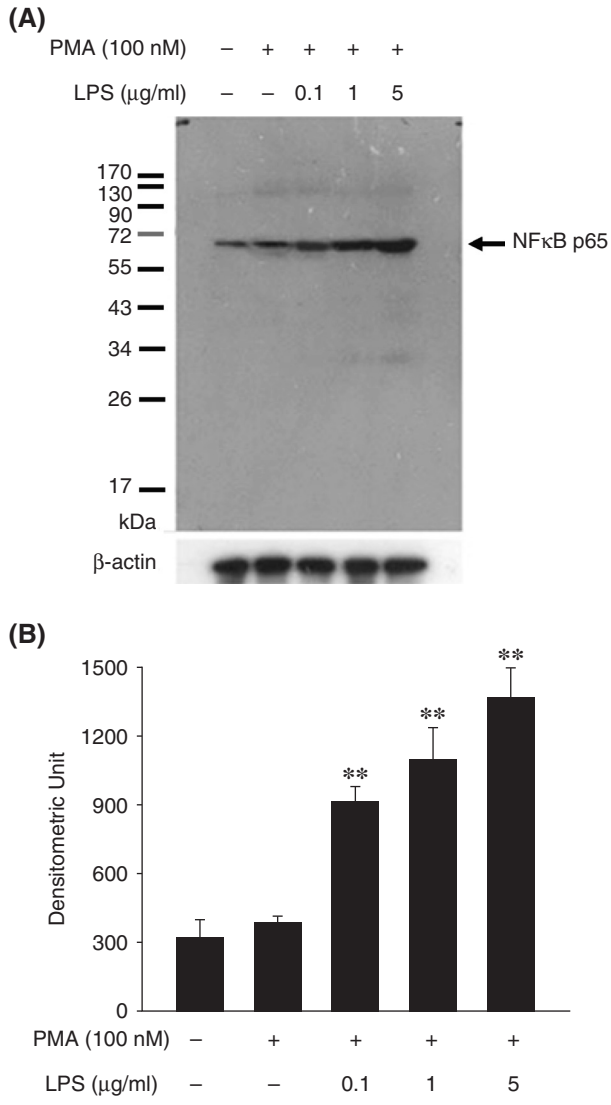

Fig. 2. Expression of NFκB in THP-1 monocytes at various LPS concentrations. Before the THP-1 monocytes differentiated into macrophage, they were grown in 100 nM PMA for 24 h, and were then treated with 0, 0.1, 1 or 5 μg/ml LPS for 2 h before analysis of expression of proteins and the amount of NFκB. (A) A typical Western blot of the p65 subunit of NFκB. The same experiment was performed three times. (B) Densitometric quantification of the NFκB p65 band in the Western blot from three independent experiments. Experimental results are shown as means ± SD with n = 3. $^{**}P < 0.01$ *vs.* 100 nM PMA only.

Based of the experimental results above, we further designed our experiments to stimulate cell division of THP-1 at 100 nM PMA to macrophages for 24 h. The cells were then exposed to microwave radiations for various periods, including 0, 1, 2, 4, 6 or 8 sec, respectively. With addition of 1 μg/ml LPS for 2 h, results showed that after THP-1 monocytes had been exposed to microwave radiations, inhibition

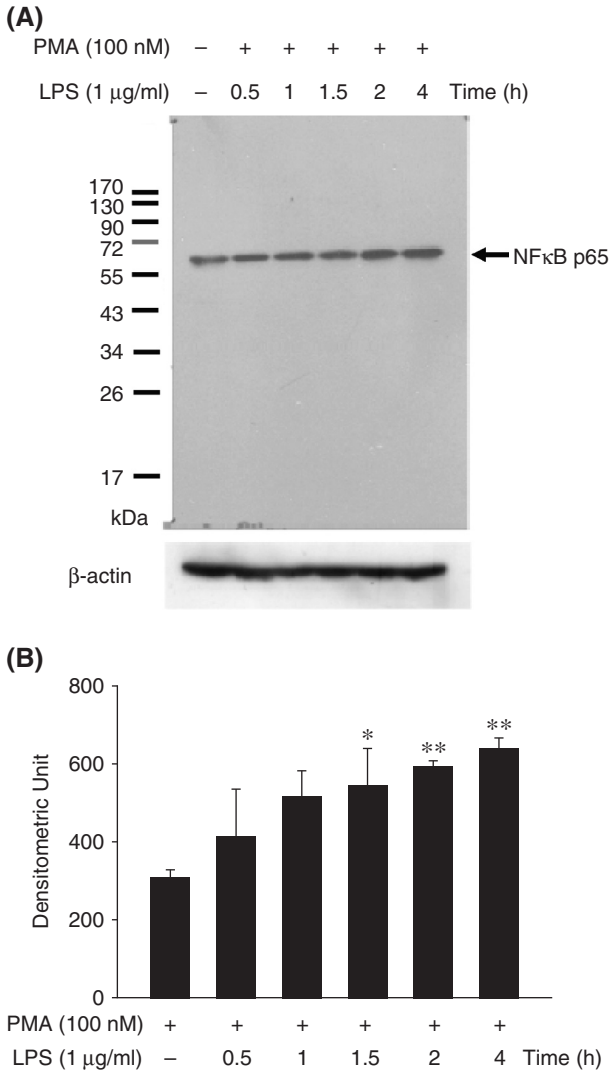

Fig. 3. Various time intervals of LPS in the expression of NFκB in THP-1 monocytes. THP-1 monocytes dividing into macrophages were grown in 100 nM PMA for 24 h, and the macrophages were treated with 1 μg/ml LPS for 0.5, 1, 1.5, 2 or 4 h before analysis of protein expression and NFκB. (A) Western blot and (B) densitometric quantification of the p65 subunit of NFκB as explained in the legend to Fig. 2. **P* < 0.05 and ***P* < 0.01 *vs*. 100 nM PMA only.

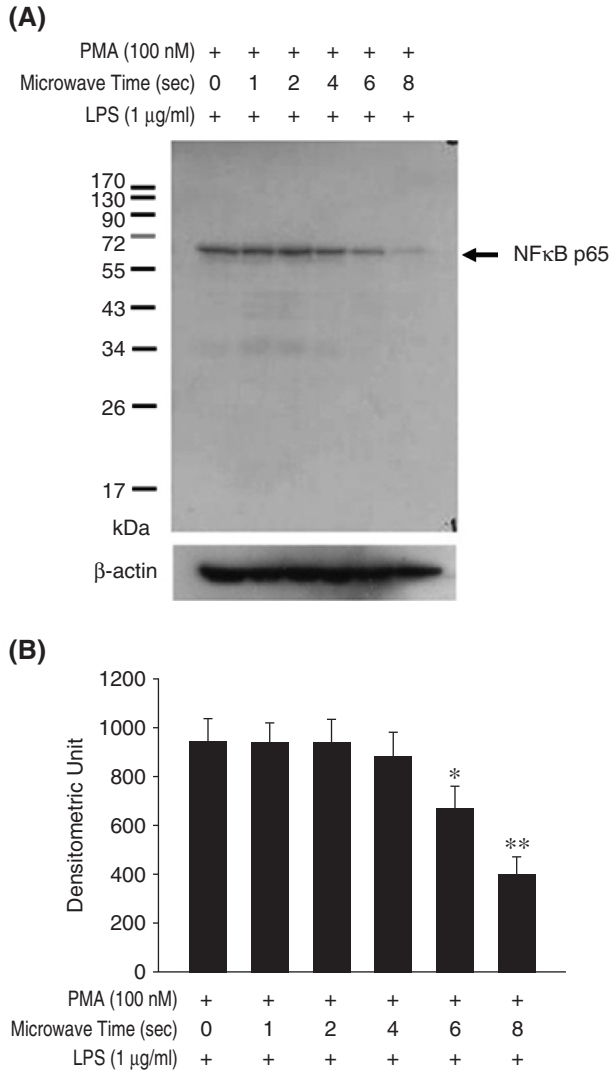

Fig. 4. Effects of microwave radiation on NFκB expression in monocytes. THP-1 monocytes dividing into macrophages were grown in 100 nM PMA for 24 h, and were then exposed to microwave radiation at 2450 MHz for 0, 1, 2, 4, 6 or 8 sec. The treated THP-1 monocytes were then treated with 1 μg/ml LPS for 2 h to observe protein expression. (A) Western blot and (B) densitometric quantification of p65 subunit of NFκB as explained in the legend to Fig. 2. * *P* < 0.05 ***P* < 0.01 *vs*. 100 nM PMA + microwave radiation at 0 sec + 1 μg/ml LPS.

on the levels of NFκB protein became more obvious with increasing time (Fig. 4).

Expression of Induced Cytokine IL-1β when THP-1 Monocytes Were Stimulated with LPS under Microwave Radiations

In the experiments, 100 nM PMA was used to stimulate division of THP-1 monocytes to macrophages for 24 h, and the cells were then exposed to microwave radiations for 0, 1, 2, 4, 6 or 8 sec, respectively. LPS (1 μg/ml) was then added and reaction was allowed to proceed for 2 h. The control group received PMA treatment with LPS as the source of stimulation but the control cells were not subjected to microwave radiations. Results showed that when THP-1 monocytes had been stimulated with PMA and LPS, significant increases in IL-1β expression level were observed. On the other hand, after exposure to microwave radiations, expression levels of cytokine IL-1β decreased as the microwave radiation exposure time increased (Fig. 5). Significant difference was observed in groups

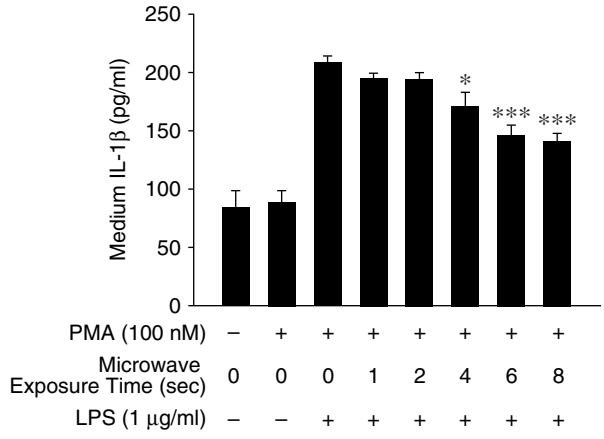

Fig. 5. Microwave radiation inhibited LPS-treated production of interleukin-1β in THP-1 monocytes differentiated to macrophages. THP-1 monocytes dividing into macrophages were grown in 100 nM PMA for 24 h, and were then exposed to microwave radiation at 2450 MHz at 0, 1, 2, 4, 6 or 8 sec. The treated THP-1 cells were then treated with 1 μg/ml LPS for 2 h before IL-1β in the media was measure. *$P < 0.05$ and ***$P < 0.001$ when compared with LPS-treated THP-1.

that had been exposed to microwave radiations for 4 sec ($P < 0.05$), and in groups that had been exposed to for 6 or 8 sec ($P < 0.001$) compared to the control group.

Discussion

THP-1 monocytes and macrophages form the mechanism of innate immunity, and lymphocytes form the mechanism of adaptive immunity. Inflammatory cytokines released from monocyte-derived macrophages can induce immune responses (2). Various information regarding host defense reactions and inflammatory cytokines, used LPS as stimulator (10, 21, 22, 28). The NFκB has been implicated in the transcriptional activation of genes encoding inflammatory cytokines, in response to different kinds of stimuli such as PMA, LPS (11, 13). Differentiation of the human THP-1 monocytes into macrophage-like cells was induced by exposure of the cells to PMA. (11). Our data in Fig. 2 showed that PMA-induced differentiation of THP-1 monocytes expressed the basal level of NFκB and were treated with different doses of LPS that cells expressed the high levels of NFκB. We identified the ability of the differentiated THP-1 cells to respond to LPS stimulation that enhanced high levels of NFκB expression. We also had found the optimal system for detecting the damages of microwave radiation exposure. The optimal detection system was the PMA-induced differentiation of THP-1 monocytes co-treated with 1 μg/ml LPS for 2 h. In order to refine and reduce *in vivo* testing, THP-1 monocytes model alternative was used in the microwave radiation setting. This alternative would provide a substantial contribution towards the replacement of animal model testing.

Illnesses induced by increasing immunological disorders have been observed, and most causative agents contributing to immunological disorders are environmental, including microwave radiations (12). Results of a previous report have shown that microwave radiations can affect the immunological avidity of macrophages (8, 23) and have a close relationship with many inflammatory responses of chronic diseases (3). We had established *in vitro* studies using cultured THP-1 monocytes model testing. *In vitro* studies showed that the influences of microwave radiation exposure could be ranked similarly for cell deaths and cellular function changes. Our results (Fig. 1) showed that THP-1 monocytes were directly exposed to microwave radiation in 8 seconds under the circumstances that cell death was prevented, and it was observed that cell viability did not decrease significantly. Simultaneously, our results (Fig. 4) showed that the transcription factor NFκB was triggered when THP-1 monocytes were stimulated by LPS, but NFκB expression was inhibited by microwave radiation. In addition, the activity of monocytes induced LPS could be prevented by microwave radiation. Although the mechanism of cellular regulatory in this event is still unknown, further studies are needed to understand the relationship between possible activities in inflammation.

When immune systems are stimulated by foreign microorganisms, Toll-like receptor 4 (TLR4) is the major activation pathway for monocytes infected with bacteria (30). The stimulation of TLR4 by LPS induces the release of inflammatory cytokines such as IL-1β and TNF-α that are necessary to active potent immune responses (6, 19). Our data showed that the transcription factor NFκB could be triggered when THP-1 monocytes were stimulated by LPS, but NFκB expression could be inhibited by microwave radiation. Known inducers of NFκB activity are highly variable and include LPS, IL-1β and TNF-α (15). The effects of NFκB expression on microwave radiation maybe involved in regulating TLR and interleukin-1 (IL-1) signaling pathways. Our results (Fig. 5) showed that when THP-1 monocytes had been stimulated with PMA and LPS, significant increased in IL-1β expression level was observed. On the other hand, after exposure to microwave radiations, expression levels of cytokine IL-1β decreased as the microwave radiation exposure time increased. When the PMA-treated THP-1 monocytes were exposed in the short time of microwave radiation, the expression amounts of NFκB and IL-1β both decreased in these cells. Regarding the exposure time of microwave radiation, we found the facts that the expression amount of

NFκB decreased significantly at 6,8 seconds, but the expression amount of IL-1β decreased significantly at 4, 6 or 8 seconds. In the beginning of decreased expression amount, IL-1β happened earlier than NFκB. Our inference was that NFκB could firstly trigger IL-1β expression, and then the amount of expressed IL-1β secreted into medium to the feedback regulation of NFκB expression level in the cell. Therefore, microwave radiation directly affected the expression level of IL-1β that secreted to outside of cells and influenced on the expression of NFκB. In our opinion, IL-1β regulated NFκB *via* the IL-1 signaling pathway.

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