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Justicidin A Inhibits the Transport of Tumor Necrosis Factor-α to Cell Surface in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

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ABSTRACT

Exposure of macrophages to lipopolysaccharide (LPS) induces release of tumor necrosis factor-α (TNF-α), which is initially synthesized as a 26-kDa pro-TNF-α followed by proteolytic processing to a 17-kDa secreted form. In this study, justicidin A, an arylnaphthalide lignan isolated from *Justicia procumbens*, was found to inhibit LPS-stimulated TNF-α release from RAW 264.7 macrophages in a concentration- and time-dependent manner, and the underlying mechanism was investigated. In the presence of justicidin A, challenge with LPS increased the steady-state level of the 26-kDa membrane-bound form of TNF-α protein, whereas justicidin A had little effect on the expression of TNF-α mRNA and on the synthesis of pro-TNF-α protein. Results of the pulse-chase experiment, revealed that the conversion of pro-TNF-α to mature TNF-α was inhibited by justicidin A. Moreover, justicidin A suppressed the transport of TNF-α to cell surface as analyzed by flow cytometry. The immunofluorescence analysis demonstrated that large amounts of LPS-induced TNF-α accumulated primarily within Golgi complex. These results indicate that justicidin A inhibits TNF-α release at the step of transport of pro-TNF-α to cell surface, and this leads to the accumulation of TNF-α in Golgi complex in RAW 264.7 macrophages.

Tumor necrosis factor α (TNF-α) is an important pro-inflammatory cytokine with a broad range of immune and inflammatory functions. TNF-α is generally considered the principal mediator of septic shock, and its overexpression is associated with autoimmune diseases such as rheumatoid arthritis and Crohn's disease (Beutler, 1999). TNF-α is initially synthesized as a 26-kDa pro-TNF-α, a type II integral membrane protein, and then is proteolytically cleaved to release a mature 17-kDa secreted form (Kriegler et al., 1988; Perez et al., 1990). Both forms of TNF-α are biological active, in which the 26-kDa pro-TNF-α presents at restricted sites and acts by cell-cell contact, whereas the 17-kDa TNF-α exists systemically and acts in paracrine and autocrine modes (Kriegler et al., 1988). Pro-TNF-α is processed by TNF-α converting enzyme (TACE), which is a metalloproteinase ADAM 17 that belong to the ADAM family (Black et al., 1997; Moss et al., 1997). The cleavage of pro-TNF-α occurs primarily at the cell surface (Black et al., 1997; Glaser et al., 1999). On the other hand, there is evidence that the earlier proteolytic processing occurs in Golgi complex (Solomon et al., 1997; Watanabe et al., 1998).

It is well documented that the synthesis of TNF-α is regulated at the stages of transcriptional, post-transcriptional, and translational levels (Han et al., 1990; Biragyn and Nedospasov, 1995; Lewis et al., 1998; Raabe et al., 1998; Anderson, 2000). On exposure to LPS, the steady-state levels of TNF-α mRNA and the translation rate of TNF-α protein increased in activated macrophages. The synthesized TNF-α protein initially accumulates primarily within the Golgi complex and then travels through the secretory pathway to the cell surface (Shurety et al., 2000). A transient appearance of TNF-α on the cell surface (Solomon et al., 1997; Shurety et al., 2000) is followed by either degradation or endocytosis of this protein (Shurety et al., 2001). However, the mechanisms of processing, trafficking, and secretion of TNF-α in cells in response to LPS is far from clear.

Justicidin A, an arylnaphthalide lignan isolated from the acanthaceous plant *Justicia procumbens* (Fukamiya and Lee, 1986), has been shown to have strong antiviral activity (Asano et al., 1996), potent cytotoxic effects (Day et al., 1999, 2002), and enhanced TNF-α generation in LPS-stimulated

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ABBREVIATIONS: TNF, tumor necrosis factor; TACE, TNF-α converting enzyme; ADAM, a disintegrin and metalloprotease; LPS, lipopolysaccharide; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; TBST, Tris-buffered saline-Tween 20; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; RIPA, radioimmunoprecipitation assay.

RAW 264.7 cells at lower concentrations (≤1 μM justicidin A) (Day et al., 2002). Surprisingly, we found that at concentrations greater than 5 μM, justicidin A significantly inhibited LPS-stimulated TNF-α secretion in the present study. This inhibitory effect probably occurs at the post-translational level because justicidin A did not affect the expression of TNF-α mRNA and the synthesis of pro-TNF-α protein. Moreover, justicidin A suppressed the transport of TNF-α to cell surface and resulted in the accumulation of large amounts of LPS-induced TNF-α in Golgi complex.

Materials and Methods

Materials. Justicidin A was isolated and purified as described previously (Fukamiya and Lee, 1986) and dissolved in DMSO. The concentration of DMSO in all experiments was 0.1%. The structure of justicidin A (purity >99%) was characterized by the spectral data of infrared, nuclear magnetic resonance, and mass spectroscopy. Dulbecco's modified Eagle medium, penicillin, streptomycin, and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride membrane was obtained from Millipore (Bedford, MA). TNF-α and IL-6 ELISA kits, and the antibodies against mouse TNF-α and FITC-conjugated anti-mouse TNF-α were obtained from R&D Systems (Minneapolis, MN). ECL Western blotting reagent, Hybond-N nylon membranes, and protein A Sepharose were obtained from Amersham Biosciences (Kent, UK). REzol C&T reagent was obtained from Protech Technology (Taiwan). Express Hyb hybridization solution was obtained from BD Biosciences Clontech (Palo Alto, CA). Random primer fluorescein labeling kit and [^{35}S]methionine were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Mouse TNF-α primer set was obtained from Stratagene (La Jolla, CA). All other reagents and chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Cytokines Assay. The RAW 264.7 murine macrophage-like cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin and maintained in humidified incubator with 5% CO_2. Cells were passaged every 3 to 4 days with 1:10 split ratio, and the passage number never exceeds 10. For TNF-α and IL-6 assay, cells were seeded in 96-well plates at 2×10^5 cells and allowed to adhere overnight. Before stimulation with 1 μg/ml of LPS (*Escherichia coli*, serotype 0111:B4) for 4 h, cells were pretreated with indicated concentration of drug at 37°C for 1 h in a final volume of 200 μl. The cell-free culture medium was collected, and TNF-α and IL-6 content was analyzed using ELISA kit according to the manufacturer's guidelines.

Western Blot Analysis. Cells were washed with PBS twice and harvested in Laemmli SDS sample buffer. Protein extracts were separated by 15% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% nonfat milk. Membranes were washed with TBST buffer and then incubated for 1 h with a polyclonal anti-TNF-α antibody (1:500). After wash with TBST buffer, a horseradish peroxidase labeled anti-goat IgG (1:10,000) was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents. The band intensity was detected by Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

Northern Blot Analysis. Total cellular RNA was isolated using REzol™ C&T reagent (Protech Technology, Taiwan) according to the manufacturer's instructions. For Northern blot analysis, 20 μg of total RNA was denatured in glyoxal/DMSO mixture at 55°C for 1 h, separated by electrophoresis on a 1% agarose gel containing 100 mM sodium phosphate buffer, pH 6.8, and then transferred to Hybond-N nylon membranes. After UV cross-linking, the membranes were prehybridized and then hybridized with TNF-α cDNA probe using ExpressHyb hybridization solution (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. TNF-α cDNA probe was made by RT-PCR amplification using the mouse TNF-α primer set and labeled with random primer fluorescein labeling kit. After hybridization, the membranes were washed and subsequently probed with antifluorescein-HRP conjugate antibody and visualized using nucleic acid chemiluminescence reagent. To control for equal loading of RNA, the GAPDH probe was used as an internal control to normalize the TNF-α mRNA expression.

Metabolic Labeling and Immunoprecipitation. Cells were treated with or without justicidin A for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for another 30 min in methionine-free media. After 15-min labeling with 75 μCi/ml [^{35}S]methionine, cells were lysed in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, and 25 mM Tris-HCl, pH 7.4). The RIPA extracts of cells and the culture media were incubated with TNF-α antibody and protein A Sepharose beads at 4°C for 1 h. Beads were pelleted and washed three times with RIPA buffer, and then boiled in Laemmli SDS sample buffer. Samples were separated on 15% SDS-PAGE. For pulse-chase experiments, cells were labeled for 15 min and chased with fresh media containing unlabeled methionine for the indicated time intervals. The radioactivity of ^{35}S-labeled TNF-α was detected by PhosphorImager (445SI; Amersham Biosciences) using ImageQuant software.

Flow Cytometry. Cells were grown on a six-well plate for 2 days then treated with or without justicidin A for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for another 2 h. After being washed with PBS, cells were stained with FITC-conjugated anti-TNF-α at 4°C for 1 h and counted on a flow cytometer (BD Biosciences).

Immunofluorescence Microscopy. Cells were grown on glass coverslips and then treated with indicated drugs for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for 4 h. Cells were then fixed in 4% paraformaldehyde, permeabilized, and subsequently blocked with blocking buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.2% saponin, 2% fetal calf serum, and 1% bovine serum albumin) for 1 h at room temperature. After being incubated with indicated primary antibodies diluted in blocking buffer at 4°C overnight, the FITC-conjugated or rhodamine-conjugated secondary antibody was added. Coverslips were mounted on glass slides in a solution of 50% glycerol in PBS, then examined using a laser scanning confocal microscope (TCS NT; Leica, Wetzlar, Germany).

Statistical Analysis. Statistical analyses were performed by the Bonferroni *t*-test method after analysis of variance. A p value less than 0.05 was considered significant for all tests. Data are expressed as means ± S.D. of the indicated number of independent experiments.

Results

Justicidin A Inhibits LPS-Induced TNF-α Secretion. To assess the effect of justicidin A on LPS-stimulated TNF-α secretion, RAW 264.7 cells were treated with various concentrations of justicidin A for 1 h before stimulation with LPS for 4 h. The levels of TNF-α secreted into the culture medium were determined by ELISA (Fig. 1A). Justicidin A at 1 and 3 μM had no significant enhancement of TNF-α secretion. However, justicidin A inhibited TNF-α release at concentrations ≥5 μM. A significant inhibition by justicidin A was observed at concentrations ≥15 μM (60.6 ± 13.8% inhibition at 30 μM justicidin A). In addition, justicidin A inhibition of LPS-induced TNF-α secretion in a time course study was also preformed (Fig. 1B). Significant reduction of TNF-α secretion was observed as early as 2 h, and persisted for at least 8 h.

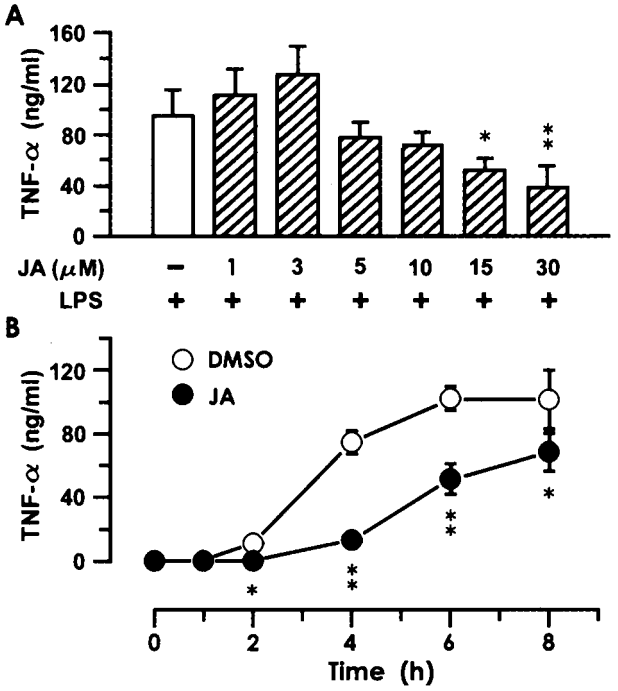

Fig. 1. Effect of justicidin A on LPS-stimulated TNF-α secretion. A, cells were pretreated with vehicle (as control) or the indicated concentrations of justicidin A (JA) for 1 h before stimulation with 1 μg/ml of LPS for 4 h. TNF-α in the culture medium was measured by ELISA. Values are expressed as means ± S.D. from four independent experiments, each done in duplicate. *, $p < 0.05$; **$p < 0.01$ compared with the control value. B, cells were pretreated with vehicle (as control) or 30 μM justicidin A for 1 h before stimulation with 1 μg/ml of LPS for the indicated time intervals. TNF-α in the culture medium was measured by ELISA. Values are expressed as means ± S.D. from five independent experiments, each done in duplicate. *, $p < 0.05$; **, $p < 0.01$ compared with the corresponding control values.

Cell viability was always >90% at concentrations tested as assessed by trypan blue exclusion and lactate dehydrogenase release assay (compared with 0.1% Triton X-100-treated value).

Justicidin A Inhibits LPS-Induced TNF-α Production. We next examined the effect of justicidin A on cell-associated TNF-α protein by Western blot analysis. Justicidin A concentration-dependently enhanced LPS-induced 26-kDa pro-TNF-α and 17-kDa TNF-α production in cells within the range of 0.3 to 3 μM and reached maximal level over 5 to 30 μM (Fig. 2A). The effect of justicidin A on TNF-α protein production after LPS stimulation for various time intervals was also examined. A marked increase in production of 26-kDa pro-TNF-α was observed in cells at 1 h after LPS stimulation, followed by a decrease. Justicidin A greatly enhanced 26-kDa pro-TNF-α production at 1 h after exposure to LPS, and the levels of this protein continued to rise for at least another 7 h (Fig. 2B).

Effect of Justicidin A on TNF-α mRNA Expression. Besides the translation level, the production of TNF-α is also regulated at the level of transcription processing (Han et al., 1990; Biragyn and Nedospasov, 1995; Raabe et al., 1998). LPS treatment induced the expression of TNF-α mRNA based on Northern blot analysis. The optimal activation of TNF-α mRNA expression was observed after 1 h LPS stimulation. However, this response was not significantly changed by justicidin A at tested range of concentrations (Fig. 3). Justicidin A alone had no effect on cellular TNF-α mRNA level (data not shown).

Effect of Justicidin A on TNF-α Protein Synthesis. Because justicidin A did not change the steady-state level of TNF-α mRNA, it is likely that justicidin A exerts action on a post-transcriptional level. Therefore, we examined the effect of justicidin A on TNF-α protein synthesis by metabolically labeling with [^{35}S]methionine for 15 min and performing immunoprecipitation of cell lysates and culture media using anti-TNF-α antibody. The results demonstrated that the amount of radiolabeled cell associated-TNF-α was not reduced, but the production of 17-kDa TNF-α in the culture medium was inhibited by justicidin A (Fig. 4). Monensin, which blocks Golgi apparatus function, had a similar result. Thus, justicidin A had no effect on TNF-α protein synthesis

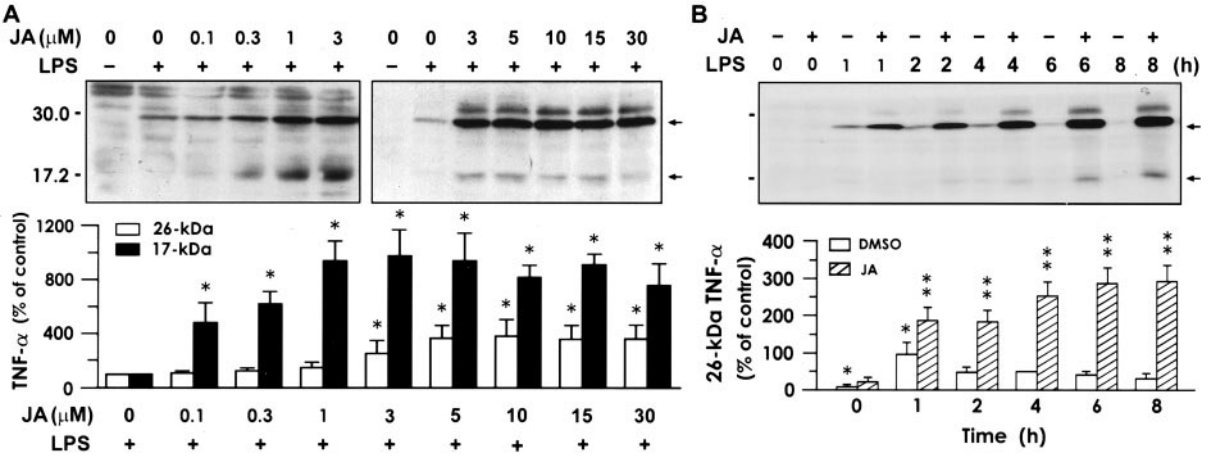

Fig. 2. Effect of justicidin A on LPS-stimulated TNF-α protein levels. A, cells were pretreated with vehicle or the indicated concentrations of justicidin A (JA) for 1 h before stimulation with 1 μg/ml of LPS for 4 h, and then lysates were subjected to Western blotting using anti-TNF-α antibody. Top, results of Western blot analysis. The arrows indicate the 26-kDa pro-TNF-α and 17-kDa TNF-α, respectively. Bottom, percentage response of the control (the vehicle-treated and LPS-stimulated groups) as means ± S.D. from three to four independent experiments. *, $p < 0.01$ compared with the control value. B, cells were pretreated with vehicle or 30 μM justicidin A for 1 h before stimulation with 1 μg/ml of LPS for the indicated time intervals, and then lysates were subjected to Western blotting using anti-TNF-α antibody. Top, results of Western blot analysis. The arrows indicate the 26-kDa Pro-TNF-α and 17-kDa TNF-α, respectively. Bottom, percentage response of the control (vehicle-treated and LPS stimulation for 4 h) as means ± S.D. from five independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the control value.

at translational level, but probably affected TNF-α synthesis at the steps of post-translational processing.

The pulse-chase-labeling experiments were performed to assess the effect of justicidin A on the rate of pro-TNF-α processing. Figure 5 shows that the 26-kDa pro-TNF-α level began to decrease after chasing in LPS-stimulated cells and was undetectable at $\geq$60 min. In the presence of 30 μM justicidin A, the onset of the decrease in pro-TNF-α was greatly delayed. The loss of pro-TNF-α in cells is coincident with the appearance of 17-kDa mature protein in the culture medium. The rate of pro-TNF-α conversion to 17-kDa TNF-α was markedly reduced. This was determined by measuring the half-life of the labeled 26-kDa pro-TNF-α ($T_{1/2}$ 28.5 $\pm$ 9.3 versus 8.8 $\pm$ 0.7 min for control, $p < 0.05$). In justicidin A-treated cells, mature TNF-α appeared after a lag of approximately 30 min.

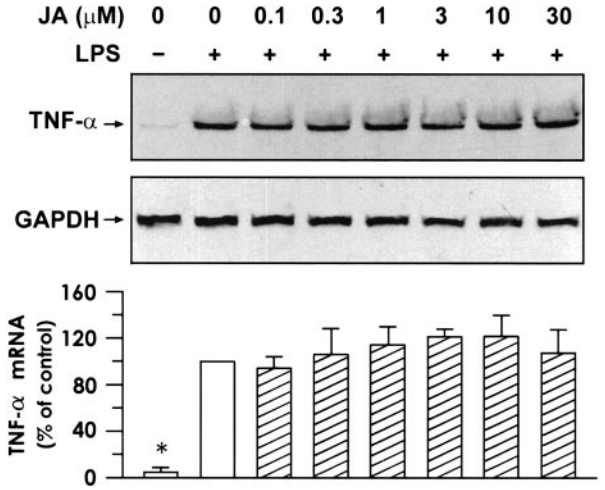

Fig. 3. Effect of justicidin A on LPS-stimulated TNF-α mRNA steady-state levels. Cells were pretreated with vehicle or the indicated concentrations of justicidin (JA) for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for 1 h. Expression of TNF-α mRNA was analyzed by Northern blot. GAPDH was used as an internal control. The lower shows the percentage response of the control (the vehicle-treated and LPS-stimulated groups) as means $\pm$ S.D. from four independent experiments. *, $p < 0.01$ compared with the control value.

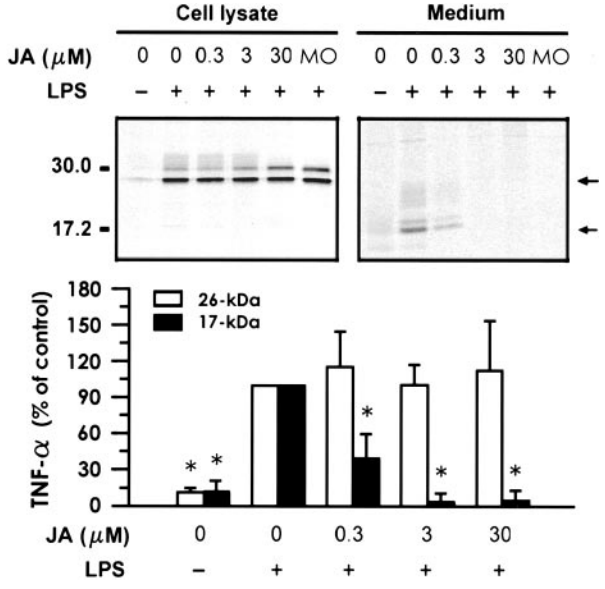

Fig. 4. Effect of justicidin A on the synthesis of cell-associated and secreted TNF-α. Cells were pretreated with vehicle or the indicated concentrations of justicidin (JA) or 3 μM monensin (MO) for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for 30 min. After being labeled with [^{35}S]methionine for 15 min, cell lysates, and media were immunoprecipitated with anti-TNF-α antibody. Samples were separated by SDS-PAGE and visualized by autoradiography. The arrows indicate the 26-kDa pro-TNF-α and 17-kDa TNF-α, respectively. Bottom, percentage response of the control (the vehicle-treated and LPS-stimulated groups) as means $\pm$ S.D. from three independent experiments. *, $p < 0.01$ compared with the control value.

Effect of Justicidin A on TNF-α Production in Cells Prestimulated with LPS. We next assessed the effect of justicidin A on the post-translation process of TNF-α production. Justicidin A significantly inhibited LPS-induced TNF-α secretion whether this compound was added into the cell culture 1 h before, together with, or 1 or 2 h after LPS addition (Fig. 6A). A significant increase in the production of 26-kDa pro-TNF-α was also observed in cells under the same justicidin A treatment (Fig. 6B).

Justicidin A Inhibits the Transport of TNF-α to Cell Surface. We next assessed the effects of justicidin A on the transport of pro-TNF-α to cell surface in LPS-stimulated RAW 264.7 cells. The expression of cell surface TNF-α was assayed by flow cytometry analysis using anti-TNF-α antibody in cells that were not permeabilized. Upon LPS stimulation, a significant increase in TNF-α expression in cell surface (mean fluorescence intensity, 47.8 $\pm$ 15.7 versus 14.4 $\pm$ 2.9 for control, $p < 0.01$) was observed (Fig. 7). However, the expression of membrane TNF-α in response to LPS was diminished by 30 μM justicidin A (mean fluorescence intensity, 24.8 $\pm$ 7.9, $p = 0.22$ compared with control values).

Intracellular Localization of TNF-α. The intracellular localization of TNF-α was assessed by immunofluorescence staining. In unstimulated cells, faint diffuse staining was detected (Fig. 8A). After exposure to LPS for 4 h, intense immunoreactivity localized in a characteristic perinuclear compartment appeared in the Golgi region (Fig. 8B), which was consistent with the finding of a previous report (Shurety

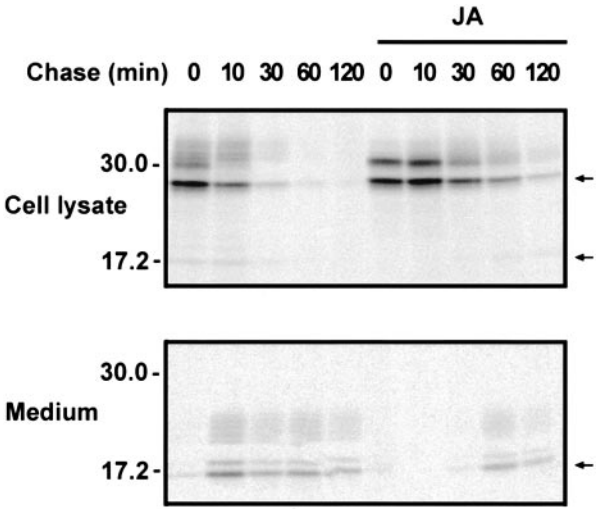

Fig. 5. Pulse-chase analysis of TNF-α in LPS-stimulated cells pretreated with justicidin A. Cells were pretreated with or without 30 μM justicidin A (JA) for 1 h before stimulation with 1 μg/ml of LPS for 30 min. Cells were then pulse-labeled for 15 min and subsequently chased with a medium containing unlabeled methionine. Cell lysates and media were harvested at the indicated chase time points and immunoprecipitated with anti-TNF-α antibody. Top, arrows indicate the 26-kDa pro-TNF-α and 17-kDa TNF-α. Bottom, arrow indicates the 17-kDa TNF-α. Similar results were obtained from three independent experiments.

et al., 2000). Treatment with 30 μM justicidin A resulted in intense immunoreactivity of TNF-α accumulated in the perinuclear region (Fig. 8C). In contrast, treatment with brefeldin A, a Golgi-disturbing agent (André and Berger, 1998), caused no perinuclear Golgi staining, but a strong immunofluorescent signal was observed throughout the cell (Fig. 8D). This observation is consistent with that of a previous report in which fused Golgi-endoplasmic reticulum membranes in the presence of brefeldin A were noted (Lippincott-Schwartz et al., 1991).

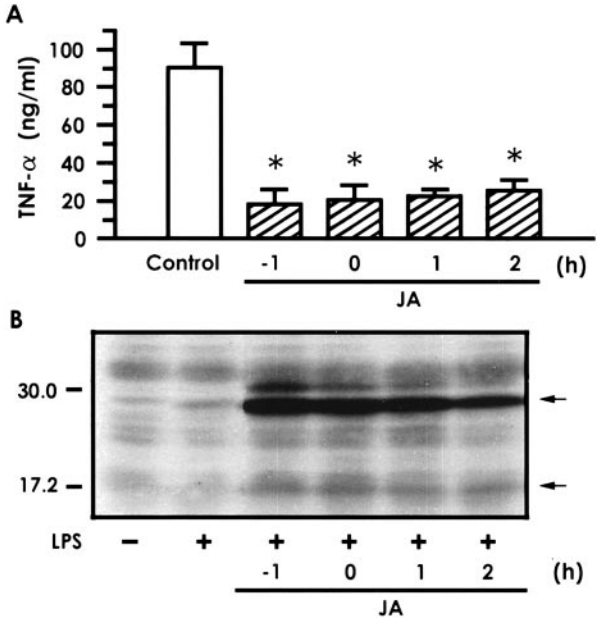

Fig. 6. The time course of justicidin A affects TNF-α production. Cells were treated with 30 μM justicidin A (JA) 1 h before, together with, at 1 or 2 h after LPS stimulation, or treated with DMSO for 1 h before stimulation (as control) or nonstimulation with 1 μg/ml of LPS for 4 h. A, TNF-α in the culture medium was measured by ELISA. Values are expressed as means ± S.D. of four independent experiments, each done in duplicate. *, $p < 0.01$ compared with the control value. B, cell lysates were subjected to Western blotting using anti-TNF-α antibody. The *arrows* indicate the 26-kDa pro-TNF-α and 17-kDa TNF-α, respectively. Similar results were obtained from four independent experiments.

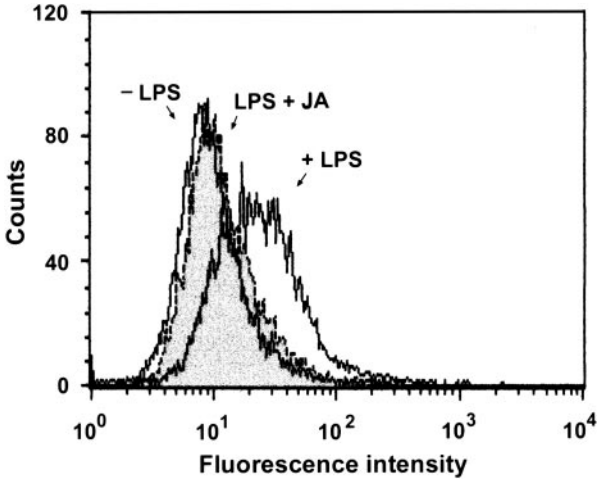

Fig. 7. Effect of justicidin A on the expression of cell surface TNF-α. Cells were pretreated with or without 30 μM justicidin A (JA) for 1 h before stimulation or nonstimulation with 1 μg/ml of LPS for 2 h. Surface TNF-α was determined by FACS analysis using FITC-conjugated anti-TNF-α antibody in cells that were not permeabilized. Similar results were obtained from seven independent experiments.

To further evaluate the localization of LPS-stimulated TNF-α in cells pretreated with justicidin A, we used confocal microscopy employing antibodies to TNF-α and the *cis*-Golgi peripheral membrane protein GM130. LPS-induced TNF-α staining in perinuclear region, corresponded to the Golgi apparatus, as evidenced by its colocalization with the *cis*-Golgi marker (Fig. 9, A–C). Justicidin A caused the accumulation of TNF-α in Golgi complex (Fig. 9, D–F).

Justicidin A Inhibits IL-6 Release. To confirm that justicidin A inhibit LPS-induced TNF-a secretion is caused by blockade of Golgi complex function and/or normal cellular trafficking from Golgi complex to cell surface, we tested another Golgi-related secretory cytokine, IL-6. LPS significantly induced IL-6 release at 8 h (1264.8 ± 367.5 ng/ml) by measuring cell culture media with ELISA assay. Justicidin A at 30 μM inhibits IL-6 secretion (494.8 ± 100.2 ng/ml, $p < 0.01$, $n = 4$).

Discussion

In this present study, we found that justicidin A suppressed LPS-induced TNF-α secretion from RAW 264.7 cells at concentration ≥5 μM. This compound significantly increased the amounts of membrane-bound 26-kDa TNF-α but had no effect on the expression of TNF-α mRNA and the synthesis of pro-TNF-α protein. Based on the pulse-chase experiment, the conversion of 26-kDa pro-TNF-α to 17-kDa mature TNF-α was attenuated. Moreover, the transport of

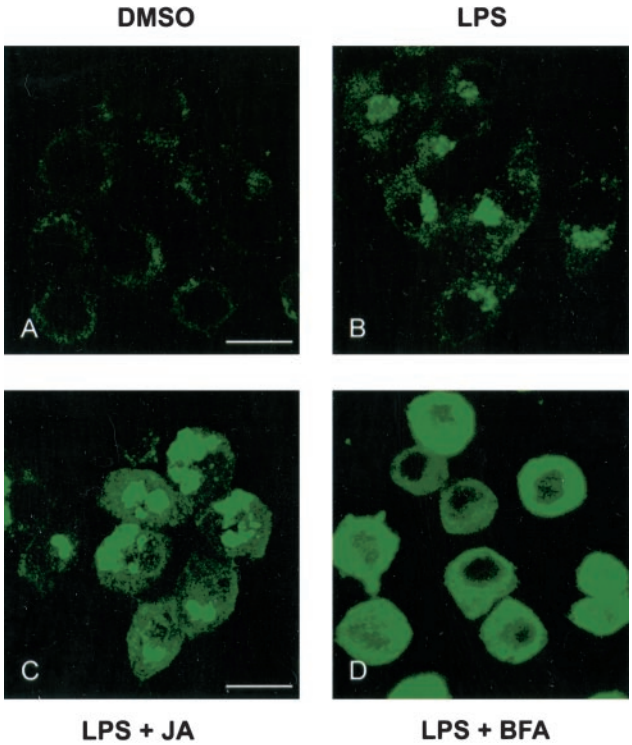

Fig. 8. Immunolocalization of TNF-α in LPS-stimulated cells pretreated with justicidin A. Cells, grown on coverslips, were pretreated with DMSO (A and B), 30 μM justicidin A (JA) (C), or 10 μg/ml of brefeldin A (BFA) (D) for 1 h before stimulation (B–D) or nonstimulation (A) with 1 μg/ml of LPS for 4 h. Cells were then fixed and prepared for indirected double immunofluorescence confocal microscopy (see *Materials and Methods*) using TNF-α antibody. Images are from projected Z-series images. Scale bar at left (A and C), 10 μm. Similar results were obtained from three independent experiments.

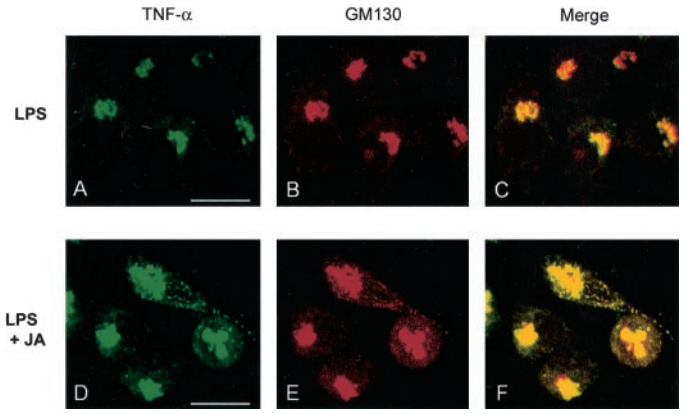

Fig. 9. Colocalization of TNF-α with Golgi marker in LPS-stimulated cells pretreated with justicidin A. Cells, grown on coverslip, were pretreated with DMSO (A–C) or 30 μM justicidin A (JA) (D–F) for 1 h before stimulation with 1 μg/ml of LPS for 4 h. After incubation, the cells were fixed and prepared for confocal microscopy using the antibody to TNF-α and GM130 (Golgi marker). TNF-α was visualized using FITC-conjugated anti-goat IgG, and GM130 was localized using a rhodamine-conjugated anti-mouse IgG. To demonstrate overlap of TNF-α and GM130, FITC (green) and rhodamine (red) images were merged (C and F). Images are from projected Z-series images. Scale bar at left (A and D), 10 μm. Similar results were obtained from three independent experiments.

TNF-α to cell surface analyzed by flow cytometry was suppressed. These results indicate that the site of inhibitory action of justicidin A appears neither at the transcriptional nor at the translational level but probably at the post-translational level. By using immunofluorescence analysis, justicidin A was demonstrated to suppress the traffic of TNF-α to cell surface, which resulted in accumulation of large amounts of TNF-α in Golgi complex. Inhibition of IL-6 secretion, another Golgi-related secretory cytokine, by justicidin A further confirm the conclusion.

TNF-α is synthesized initially as a 26-kDa transmembrane precursor, which is then processed to release a mature molecule of 17 kDa. The isoform that migrates directly above the major 17-kDa mature form has been identified as a product of cleavage of the transmembrane precursor at an alternate site (Cseh and Beutler, 1989). This isoform can be visualized in cells radiolabeled with ^{35}S followed by immunoprecipitation experiments as shown in Figs. 4 and 5. There are a number of species of higher molecular mass that appear in the regions of 26-kDa transmembrane precursor and 17-kDa mature TNF-α, and these isoforms are probably results of differential glycosylation of TNF-α (Watts et al., 1997). These slower-migrating bands, corresponding to glycosylated forms of TNF-α in cell lysates, were reduced in the presence of justicidin A, suggesting the interference of Golgi function by justicidin A. Monensin, a Golgi-disturbing agent (André and Berger, 1998), had the same result as justicidin A.

Pro-TNF-α is processed by a membrane-associated metalloprotease that has been identified as TACE ADAM 17 (Black et al., 1997; Moss et al., 1997). The release of soluble TNF-α can be effectively inhibited by metalloprotease inhibitors (Gearing et al., 1994; McGeehan et al., 1994; Black et al., 1997; Moss et al., 1997). In the presence of metalloprotease inhibitors, the amount of uncleaved TNF-α on the activated cell surface is increased (Gearing et al., 1994; McGeehan et al., 1994; Solomon et al., 1997; Shurety et al., 2001). In this present study, justicidin A inhibited LPS-induced processing of TNF-α by pulse-chase analysis. However, the attenuation of TNF-α expression on cell surface and the presence of 17-kDa mature TNF-α in justicidin A-treated cells preclude the involvement of metalloprotease inhibition.

Because the cleavage of pro-TNF-α by TACE occurs primarily at the cell surface (Black et al., 1997; Glaser et al., 1999; Shurety et al., 2001), the observed delaying effect of justicidin A on the rate of pro-TNF-α conversion to mature protein is probably caused by the blockade of transport of TNF-α to cell surface. It has been reported that brefeldin A inhibits ADP-ribosylation factor activation by stabilizing the inactive ADP-ribosylation factor-guanine nucleotide exchange factor complex resulting in retrograde transport of Golgi content to the endoplasmic reticulum (Peyroche et al., 1999). However, the features of immunolocalization of TNF-α in cells treated with justicidin A distinct from that with brefeldin A excludes the involvement of this possibility. Monensin induces the *trans*-membrane exchange of Na^+ for protons, which lead to the neutralization of acidic intracellular compartments and disruption of *trans*-Golgi apparatus cisternae (Mollenhauer et al., 1990). Whether the inhibition of TNF-α secretion by justicidin A via the interference of Golgi function might be mediated by the disruption of *trans*-Golgi apparatus cisternae requires further investigation.

In summary, our findings demonstrate that justicidin A interfered with LPS-stimulated TNF-α secretion. The blockade of Golgi complex function and/or normal cellular trafficking from Golgi complex to cell surface, which results in the accumulation of TNF-α in the Golgi complex, might be the cellular mechanism underlying the justicidin A inhibition of TNF-α secretion. Justicidin A may have the potential to be a pharmacological agent for studying the Golgi apparatus.

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