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Induction of Nitric Oxide Production by Bafilomycin A_1 in Mouse Leukemic Monocyte Cell Line

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Abstract – In the mouse leukemic monocyte cell line RAW 264.7, the vacuolar-type (H^+)-ATPase (V-ATPase) inhibitor bafilomycin A_1 at 10 and 100 nM decreased cell growth and survival as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in a concentration-dependent manner. At such concentrations, bafilomycin A_1 induced nitric oxide (NO) production through the expression of inducible nitric oxide synthase (iNOS). The bafilomycin A_1-induced NO production was inhibited by the NOS inhibitor N^G-monomethyl-L-arginine acetate (L-NMMA). Our findings suggest that the V-ATPase inhibitor bafilomycin A_1 induces NO production through the expression of iNOS protein.

Key words □ RAW 264.7 cells, Bafilomycin A_1, Nitric oxide, V-ATPase inhibitor

INTRODUCTION

Previously, we have suggested that apicularen A, a cytostatic macrolide isolated from a variety of strains of the myxobacterial genus *Chondromyces* (i.e. *C. apiculatus, C. lanuginosus, C. pediculatus, and C. robustus*) (Kunze *et al.*, 1998), inhibits V-ATPase in the human promyelocytic leukemia cell line HL-60 as revealed by the vital staining of intracellular organelles with acridine orange (Hong *et al.*, 2005a), and have recently demonstrated that apicularen A inhibits the ATP-dependent proton transport into inside-out microsome vesicles and inhibits the V-ATPase inhibitor bafilomycin A_1-sensitive ATP hydrolysis in mouse peritoneal macrophages, indicating that apicularen A inhibits V-ATPase (Hong *et al.*, 2006).

In addition, we have clarified that apicularen A induces production of nitric oxide (NO) and the newly produced NO partially participates in apicularen A-induced apoptosis in the mouse leukemic monocyte cell line RAW 264.7 (Hong *et al.*, 2005b). In this study, we intended to clarify whether the commercially available V-ATPase inhibitor bafilomycin A_1 also has an activity to induce NO production in RAW 264.7 cells.

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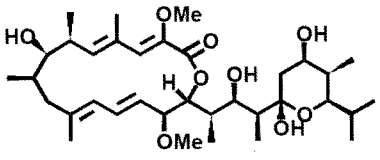
MATERIALS AND METHODS

Reagents

Bafilomycin A_1 (Fig. 1), lipopolysaccharide (LPS) and the non-specific inhibitor of nitric oxide synthase (NOS), N^G-monomethyl-L-arginine acetate (L-NMMA), were purchased from Wako Pure Chemicals (Osaka, Japan). Bafilomycin A_1 was dissolved in dimethyl sulfoxide (DMSO). LPS and L-NMMA were dissolved in the medium. An aliquot of each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to 0.1%. The control medium contained the same amount of the vehicle.



Bafilomycin A_1

Fig. 1. Chemical structure of bafilomycin A_1.

Cell culture

RAW 264.7 cells were obtained from RIKEN Gene Bank (Tsukuba, Japan) and cultured at 37°C under 5% CO_2-95% air in Eagle's minimal essential medium (Nissui, Tokyo, Japan) containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Sigma), 1% non-essential amino acid solution (Sigma), penicillin G potassium (18 μg/ml) and streptomycin sulfate (50 μg/ml) (Meiji Seika, Tokyo, Japan). The cells at passage number 10 or lower were used for experiments.

Measurement of cell growth and survival

RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were washed three times with medium, and further incubated for 20 h at 37°C in 0.5 ml of medium containing various drugs. Then, 10 μl of phosphate-buffered saline containing 3-(4,5-dimethyl(thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (5 mg/ml) was added, and the cells were further incubated for 4 h at 37°C. After the removal of the medium, 100 μl of DMSO was added, and the absorbance at 595 nm was measured (Mosmann, 1983).

Measurement of nitrite

RAW 264.7 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with medium, and further incubated for the specified period at 37°C in 0.5 ml of medium in the presence or absence of drugs. After incubation, nitrite levels in the conditioned medium were determined using Griess reagent (Green *et al.*, 1982).

Western blotting analysis for iNOS

RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37 °C in 2 ml of medium. The cells were then washed three times with medium, and further incubated at 37°C for 24 h for the detection of iNOS in 2 ml of medium in the presence or absence of bafilomycin A_1. After incubation, the cells were washed three times with PBS, dipped in 150 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1% Triton-X 100, 10% glycerol, 1 M sodium fluoride, 2.5 mM *p*-nitrophenylene phosphate, 10 μg/ml of phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 5 μg/ml of leupeptin, and 1 mM EDTA) for 15 min, and disrupted with a Handy Sonic Disruptor (UR-20P, Tomy, Tokyo, Japan). The lysis buffer containing the disrupted cells was centrifuged at 13,000×*g* and 4°C for 20 min. The supernatant obtained was boiled for 5 min in 3× sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg/ml of bromophenol blue) at a ratio of 2:1 (v/v), loaded on an acrylamide gel (8% or 10%) and subjected to electrophoresis (150 min at 125 V). The antibody for iNOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Western blotting was carried out as described previously (Hong *et al.*, 2005b). The levels of iNOS protein were quantified by scanning densitometry, and the individual band density value for each point was expressed as the relative density signal.

Statistical analysis

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

RESULTS

Suppression of cell growth and survival by bafilomycin A_1

RAW 264.7 cells were incubated at 37°C for 24 h in medium containing various concentrations of bafilomycin A_1. Significant inhibition of cell growth and survival as determined by MTT assay was induced by bafilomycin A_1 at 10 and 100 nM in a concentration-dependent manner (Fig. 2). LPS at 0.1 μg/ml also inhibited the MTT response to almost the same level as 100 nM of bafilomycin A_1 (Fig. 2).

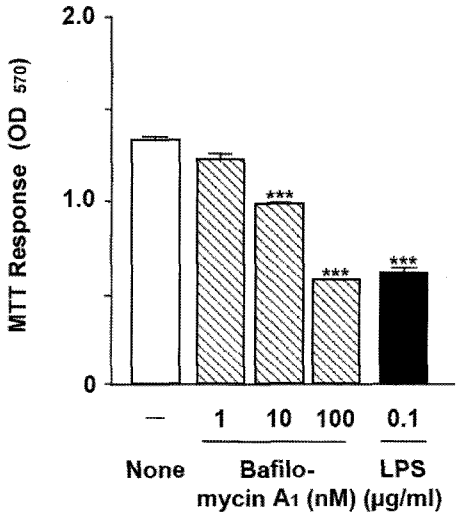


Fig. 2. Effects of bafilomycin A_1 and LPS on cell growth and survival. RAW 264 cells (5.0×10^4 cells) were incubated for 24 h at 37°C in 0.1 ml of medium. The cells were then washed three times with PBS, and further incubated for 24 h at 37°C in 0.1 ml of medium containing the indicated concentrations of bafilomycin A_1 or 0.1 μg/ml of LPS. Cell growth and survival were assessed using the MTT assay. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: *** $P<0.001$ versus the control.

Induction of nitrite production by bafilomycin A_1

RAW 264.7 cells were incubated for 24 h in the presence of various concentrations of bafilomycin A_1. A significant increase in nitrite production was induced by bafilomycin A_1 at 10 and 100 nM (Fig. 3A). LPS at 0.1 μg/ml also induced nitrite production at 24 h (Fig. 3A). Analysis of the time-course of changes in nitrite production revealed that no significant increase was induced at 6 h by 100 nM of bafilomycin A_1 although LPS at 0.1 μg/ml significantly induced nitrite production at 6 h (Fig. 3B). Nitrite production induced by bafilomycin A_1 increased time dependently from 6 to 18 h, and reached a plateau at 18 to 24 h (Fig. 3B).

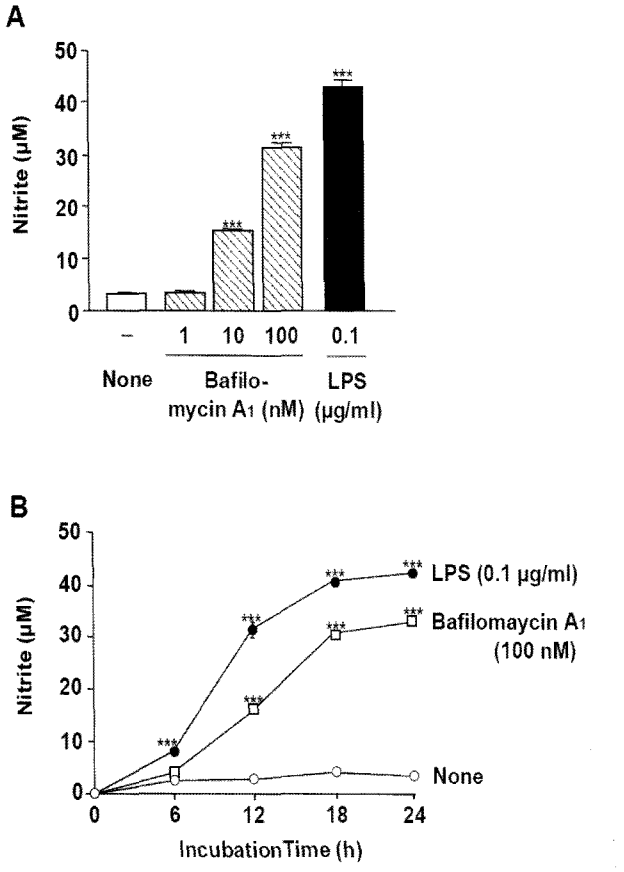


Fig. 3. Effects of bafilomycin A_1 and LPS on nitrite production. RAW 264 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with PBS, and further incubated for 24 h at 37°C in 0.5 ml of medium containing the indicated concentrations of bafilomycin A_1 or 0.1 μg/ml of LPS (A), and for the periods indicated at 37°C in 0.5 ml of medium containing bafilomycin A_1 (100 nM) or LPS (0.1 μg/ml) (B). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: *** $P < 0.001$ versus the control.

Expression of iNOS protein by bafilomycin A_1

RAW 264.7 cells were incubated at 37°C for 24 h in medium containing various concentrations of bafilomycin A_1, and protein levels of iNOS were determined by Western blotting. Upon treatment with bafilomycin A_1 at 10 and 100 nM, iNOS protein levels increased in a concentration-dependent manner (Fig. 4). LPS at 0.1 μg/ml also increased the iNOS protein level to that achieved by 100 nM bafilomycin A_1 (Fig. 4).

Inhibition by L-NMMA of bafilomycin A_1-induced nitrite production

RWA 264.7 cells were incubated at 37°C for 24 h in medium containing bafilomycin A_1 (100 nM) in the presence or absence of the nitric oxide synthase (NOS) inhibitor L-NMMA. Nitrite production at 24 h induced by bafilomycin A_1 was suppressed by L-NMMA at 10 and 100 nM (Fig. 5), indicating that NOS is responsible for the nitrite production induced by bafilomycin A_1. The LPS (0.1 μg/ml)-induced nitrite production was also suppressed by L-NMMA at 10 and 100 μM (Fig. 5).

DISCUSSION

It was reported that the V-ATPase inhibitor bafilomycin A_1 (Bowman *et al.*, 1988) induces apoptosis in the human pancreatic cancer cell line Capan-1 (Ohta *et al.*, 1998) and in RAW 264.7 cells (Xu *et al.*, 2003). The bafilomycin A_1-induced

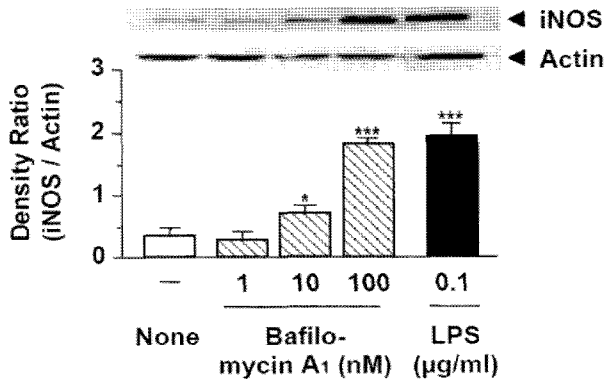


Fig. 4. Effects of bafilomycin A_1 and LPS on the levels of iNOS protein. RAW 264 cells (1.0×10^6 cells) were incubated for 24 h at 37°C in 2.0 ml of medium. The cells were then washed three times with PBS, and further incubated for 24 h at 37°C in 2.0 ml of medium containing the indicated concentrations of bafilomycin A_1 or 0.1 μg/ml of LPS. The protein levels of iNOS and actin were determined by Western blotting. The density ratios of iNOS to actin were calculated. Statistical significance: * $P<0.05$, *** $P<0.001$ versus the control.

decrease in cell growth and survival determined with the MTT assay (Fig. 2) might be a reflection of apoptosis, because the V-ATPase inhibitor apicularen A also decreased the cell growth and survival and induced apoptosis as evidenced by the condensation of chromatin, the formation of DNA ladder, an increase in the percentage of annexin V-positive and propidium iodide-negative cells, and the activation of caspase (Hong *et al.*, 2003). However, the mechanism underlying the V-ATPase inhibitor-induced apoptosis still remains to be elucidated. Recently, we have reported that apicularen A induces NO production in RAW 264.7 cells, and suggested that the newly produced NO participates in the apicularen A-induced apoptosis, because the NOS inhibitor L-NMMA and the NO scavenger carboxy-PTIO partially alleviated the apicularen A-induced apoptosis (Hong *et al.*, 2005b). In this study, to analyze the mechanism of the V-ATPase inhibitor-induced apoptosis in RAW 264.7 cells, we intended to clarify whether bafilomycin A_1 also induces NO production.

As shown in Fig. 3, it was clearly demonstrated that bafilomycin A_1 induces NO production at 10 and 100 nM. The time-course of changes in NO production (Fig. 3B) indicated that a longer time-lag is necessary for the bafilomycin A_1-induced NO production than for the LPS-induced NO production. Therefore, the possibility remained that bafilomycin A_1 induces some cytokine such as interleukin-1β which in turn induces NO production. Bafilomycin A_1 induced iNOS protein expression (Fig. 4), and the bafilomycin A_1-induced NO production was inhibited by the NOS inhibitor L-NMMA (Fig. 5). These findings indicated that the bafilomycin A_1-induced NO production is due to the expression of the *iNOS* gene. The promoter region of the *iNOS* gene contains the binding sites for NF-κB, AP-1 and C/EBP, and the component essential for the expression of the *iNOS* gene is NF-κB (Xie *et al.*, 1994). We have reported that apicularen A induces NO production by increasing the level of iNOS mRNA and expression of iNOS protein through the activation of NF-κB and AP-1 in RAW 264.7 cells (Hong *et al.*, 2005b). Therefore, it is possible that bafilomycin A_1 also activates NF-κB and AP-1, and thus induces NO production. In mouse peritoneal macrophages, it is reported that NF-κB is activated by the V-ATPase inhibitor bafilomycin A_1 (Conboy *et al.*, 1999).

NO, a radical produced from L-arginine by NOS, plays a significant role as a cellular second messenger (Palmer *et al.*, 1988), and iNOS produces a high level of NO, while constitutively expressed NOS generates a low level of physiologically active NO (Kubes, 2000). It is possible that a high level of NO

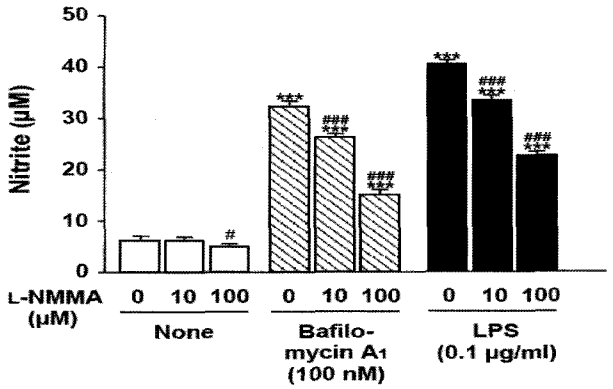


Fig. 5. Effects of L-NMMA on bafilomycin A_1- and LPS-induced nitrite production. RAW 264 cells (2.5×10^5 cells) were incubated for 24 h at 37°C in 0.5 ml of medium. The cells were then washed three times with PBS, and further incubated for 24 h at 37°C in 0.5 ml of medium containing the indicated concentration of L-NMMA in the presence or absence of bafilomycin A_1 (100 nM) or LPS (0.1 μg/ml). Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: $^{***}P<0.001$ versus the control and $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ versus the corresponding control.

is associated with apoptosis, anti-cancer, bactericidal and anti-parasitic effects probably due to the formation of reactive radicals including peroxynitrite (Szabo and Ohshima, 1997). However, NO seems to be a bifunctional regulator of apoptosis. It exerts an anti-apoptotic action in splenocytes (Genaro *et al.*, 1995), vascular endothelial cells (Dimmeler *et al.*, 1997), hepatocytes (Kim *et al.*, 1997), and eosinophils (Hebestreit *et al.*, 1998). On the other hand, it induces apoptosis in macrophages (Albina *et al.*, 1993; Shimaoka *et al.*, 1995), pancreatic islet cells (Heller *et al.*, 1995), thymocytes (Fehsel *et al.*, 1995), vascular endothelial cells (Lincoln *et al.*, 1996), and neurons (Leist *et al.*, 1997). In RAW 264.7 cells, it is also reported that NO induces apoptosis (Messmer *et al.*, 1995; Jun *et al.*, 1999; Gotoh *et al.*, 2002). Therefore, it is possible that NO produced by bafilomycin A_1 also participates in bafilomycin A_1-induced apoptosis in RAW 264.7 cells. On the other hand, depending on surrounding cell environment, cells undergo oxidative stress, which induces apoptosis in a variety of cells, when levels of reactive oxygen species exceed the counter-regulatory antioxidant capacity of the cell (Martindale and Holbrook, 2002). Therefore, participation of reactive oxygen species in the bafilomycin A_1-induced apoptosis in RAW 264.7 cells remains to be elucidated.

In this study, we indicated that the V-ATPase inhibitor bafilo-

mycin A_1 also induces NO production as the V-ATPase inhibitor apicularen A. It is necessary to clarify whether other V-ATPase inhibitors such as concanamycin A also induce NO production and clarify the role of newly produced NO.

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