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Degradation of IκBα in activated RAW264.7 cells is blocked by the phosphatidylinositol 3-kinase inhibitor, LY294002

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SUMMARY

The mechanism by which LPS or phorbol 12-myristate 13-acetate (PMA) induces production of proinflammatory cytokines in murine macrophages, and a role for phosphatidylinositol 3-kinase (PI3-kinase) had not been well investigated. Activation of the transcription factor, nuclear factors-κB (NF-κB), is initiated by the phosphrylation of the inhibitory subunit, IκB, which targets IκB for degradation and leads to the release of active NF-κB. In this study we demonstrate that 2-(4-morpholinyl)-8-phenylchromone (LY294002) which inhibits PI3-kinase specifically inhibited degradation of IκBα in the RAW264.7 cells stimulated with IFN-γ plus LPS or IFN-γ plus PMA. To elucidate the importance of this activity in RAW 264.7 cells, we examined TNF-α and IL-6 production in the activated cells. Pretreatment of the cells with LY294002 results in the inhibition of TNF-α and IL-6 production in the RAW264.7 cells stimulated with IFN-γ plus LPS or IFN-γ plus PMA. Furthermore, LY294002 inhibited the production of nitric oxide (NO) in the RAW264.7 cells stimulated IFN-γ plus LPS or IFN-γ plus PMA. LY294002 also potentially inhibited inducible NO synthase (iNOS) mRNA expression in the activated RAW264.7 cells. In conclusion, the present results suggest that PI3-kinase is involved in the signal transduction pathway responsible for LPS- or PMA-mediated TNF-α and IL-6 production, and that LY294002 inhibits NO generation through blocking the degradation of IκBα in the activated RAW264.7 cells.

Key words: LY294002, IkBα, RAW264.7 cells

INTRODUCTION

Phosphatidylinositol 3-kinase (PI3-kinase) is a heterodimeric phospholipid kinase composed of an 85 kD (p85) regulatory subunit and a 110 kD (p110) catalytic subunit which specifically phosphorylates the D-3 hydroxyl position of membrane phosphoinositides (Carpenter *et al*., 1990; Whitman *et al*., 1988). PI3-kinase has been shown to associate with several tyrosine kinases such as growth factor receptors and oncogene products (Cantley *et al*., 1991). Although little is known about the downstream effects of 3-phosphorylated phosphoinositides, PI3-kinase has been implicated in a variety of responses in noninflammatory cell types, including membrane ruffling (Wennstrom *et al*., 1994), DNA synthesis (Roche *et al.*, 1994), and the sorting and transport of lysosomal proteins (Brown *et al.*, 1995; Davidson, 1995).

The nuclear transcription factor NF-κB is known to play an important role in immune regulation and inflammation (Baeuerle and Baichwal, 1997). A wide variety of inflammatory stimuli activate NF-κB, including TNF-α, IL-1, LPS (lipopolysaccharide),

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ceramide, phorbol ester, and H_2O_2. NF-κB is present in its inactive state in the cytoplasm. It consists of p50, p65, and IκBα subunits, but when activated it translocates to the nucleus, binds the DNA, and activates genes. The activation involves the phosphorylation, ubiquitination, and degradation of IκBα, leading to the nuclear migration of p50-p65 heterodimer. NF-κB activation regulates a large number of genes involved in inflammation, including cytokines (e.g. TNF-α, IL-1, IL-8, IL-6, granulocyte CSF, granulocyte-macrophage CSF), cell adhesion proteins (e.g. ICAM-1, endothelial leukocyte adhesion molecule-1, and vascular cell adhesion molecule-1), MHC genes, and enzymes (e.g. nitric oxide synthase, cyclo-oxygenase, and magnesium superoxide dismutase) (Baeuerle and Baichwal, 1997).

LPS and PMA have been proposed to mediate the various cytokines synthesis. LPS is a potent activator of macrophage functions, which include the production of TNF-α, IL-1, IL-6, and nitric oxide (NO) (Nathan, 1992). PMA is a well known activator of protein kinase C, and this has been proposed to mediate the induction of IL-6 synthesis by PMA (Sehgal *et al.*, 1987), whereas IL-1 and TNF-α seem to stimulate IL-6 synthesis by a cAMP-dependent pathway (Zhang *et al*., 1988).

The biochemical regulation of the production of biologically active molecules by hemopoietic cells is presently not well understood. This is likely to be important, as some of the signaling proteins involved may well be useful targets for therapeutic intervention. However, there are limited data concerning the involvement of PI3-kinase and its putative effector molecules in similar systems. One such example is that histamine secretion in basophils has been demonstrated to be PI3-kinase dependent (Yano *et al*., 1993).

Recent report showed that wortmannin markedly potentiated the LPS-induced NO production and enhanced TNF-α secretion in the initiation stage for activation of macrophages stimulated with LPS (Park *et al.*, 1997). Wortmannin induced the expression of iNOS in LPS- or IL-1 beta-stimulated C6 glial cells and astrocyes without modulating the activation of MAP kinase and NF-κB (Pahan *et al*., 1999). However, the effects on NO production by wortmannin and LY294002 were strikingly different. While both rapamycin and LY294002 resulted in almost complete inhibition of NO production, wortmannin was ineffective (Salh *et al*., 1998). NO is an important biologic molecule with tissue-specific effects that in macrophages include microbicidal and tumoricidal activities as well as apoptosis (Messmer and Brune, 1996).

In this study we provide that in RAW264.7 cells LY294002 inhibited the activation of NF-κB, which explains the mechanism of action of LY294002. LPS- or PMA-induced TNF-α and IL-6 production are inhibited by LY294002. Furthermore, LY294002 inhibits NO generation in activated RAW264.7 cells.

MATERIALS AND METHODS

Materials

Murine rIFN-γ (1×10^5 U/mg), murine rTNF-α (1×10^5 U/ml), rabbit anti-murine TNF-α polyclonal Ab (neutralizing), and hamster anti-murine TNF-α mAb were purchased from Genzyme (Munich, Germany). Mouse rIL-6, rat ant-mouse IL-6 mAb, and biotinylated rat anti-mouse IL-6 mAb were purchased from Pharmingen (San Diego, CA). Phosphatase-labeled anti-rabbit IgG (heavy+light) was purchased from Serotec (Oxford, UK). The rabbit polyclonal ant-IκBα antibody was purchased from Santa Cruz Biotechnology. LPS from Escherichia coli, PMA, LY294002, wortmannin, LiCl, 2,2-azinobis (3-ethylbenzthiazoline sulfonic acid substrate), urea, N-(1-naphtyl)-ethylenediamine dihydrochloride, sodium nitrite, and sulfanilamide were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose, phenol, Moloney murine leukemia virus reverse transcriptase, RNasin, DNA polymerase I, Taq polymerase, and deoxynucleotide triphosphate were purchased from Life Technologies Inc. (Gaithersburg, MD). Dupont MEN (Boston, MA) was the source of [α-^{32}P]dCTP. pBluescript II KS(-) plasmid was purchased from Stratagene (San Diego, CA). All reagents and media for tissue culture experiments were tested for their LPS content with the use of a clorimetric Limulus amoebocyte lysate assay (detection limit 10 pg/ml; Whittaker Bioproducts, Walkerville, MD). Ninety-six-well tissue culture plates and 100-mm diameter petri dishes were purchased from Nunc (Naperville, IL). RPMI1640, Hanks balanced salt solution

(HBSS), fetal calf serum (FCS), and other tissue culture reagents were purchased from Life Technologies.

Cell cultures

RAW 264.7 cells, an Abelson leukemia virus-transformed murine macrophage cell line (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 containing 10% heat-inactivated FCS and antibiotics (complete medium). LY294002 or wortmannin was added to cultures at the time of stimulation with activators unless noted otherwise. Cell viability was routinely monitored by trypan blue exclusion and was unaffected by the concentrations of LY294002 or wortmannin used in this study.

Immunoblotting

Western blot analysis was performed essentially as described previously (Schwenger *et al.*, 1997; Schwenger *et al.*, 1996). Briefly, whole-cell lysates were generated by using a buffer consisting of 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 mM sodium fluride. Equal amounts of lysates were subjected to SDS-PAGE and then transferred to Immobilon-P membrane (Millipore Corp, Bedford, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol). Membranes were first rinsed in Tris-buffered saline (TBS; 10 mM Tris [pH 7.4], 150 mM NaCl) and then blocked overnight at room temperature in TBS-5% non fat dry milk. The anti-IkBα antibody was used at a dilution of 1:200 in TBS-5% non fat dry milk. The blots were washed with TBS/0.05% tween-20, incubated for 1 h in 1 μg/ml horseradish peroxidase-conjungated secondary antibody (Amersham, Arlington Heights, IL), and washed extensively. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). For IκBα blots, equal loading was ascertained by the presence of an ~70 kDa nonspecific band recognized by the anti-IκBα antibody (not shown).

Assay of TNF-α Production

TNF-α production was measured by modification of ELISA, as described (Scuderi *et al.*, 1986). The ELISA was sensitive to TNF-α concentrations in medium above 40 pg/ml. The ELISA was devised by coating 96-well plates with 6.25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, coated plates were washed twice PBS containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this were incubated for 1 h at room temperature with coated wells. For standard curve rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to medium, assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and p-nitrophenyl phosphate. OD readings were made within 10 min of addition of the substrate on a Titertek multiscan with a 405 nm filter. Appropriate specificity controls were included.

Assay of IL-6 Production

Rat anti-mouse IL-6 mAb diluted to 2 μg/ml in $NaHCO_3$ (0.1 M; pH8.2) and used to coat 96 well flat bottomed plates (Maxisorp-Nunc), 50 μl per well and incubated overnight at 4°C. Plates were washed (Tris, 50 mM; NaCl, 0.1%; Tween 20, 0.02%) and incubated with 10% FCS in PBS, 200 ml per well for 1 h at room temperature before blotting dry and pipetting 100 μl standard or sample into wells. The plates were thoroughly washed after incubation room temperature 2 h and 50 μl biotinylated rat anti-mouse IL-6 mAb added to wells. Following 1 h incubation at room temperature, plates were washed and 100 μl avidin-peroxidase conjugate was added to wells. After 30 min incubation at room temperature, plates were washed and 100 μl of 2,2'-azino-bis [3-ethylbenzythiazoline-6-sulfonic acid] containing 0.03% H_2O_2 added to the wells, and the absorbance at 405 nm measured after 30 min.

Measurement of nitrite concentration

Experiments were under taken on cells grown either under standard conditions or in the presence of LY294002 with or without stimulants for 24 h. Supernatants in cultured RAW264.7 cells were collected and mixed with an equal volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/2.5%

H_3PO_4) and incubated for 10 min at room temperature (Schwenger *et al*., 1996). Nitrite concentration was determined by measuring the absorbance at 540 nm in a Titertek multiskan (Flow laboratories, North Ryde, Australia). The $NaNO_2$ was used as a standard. Cell-free medium alone contained 5-8 μM of nitrite; this value was determined in each experiment and substrated from the value obtained with cells.

Preparation of probe

To detect iNOS mRNA transcripts, sense and antisense oligonucleotide primers specific for the coding regions of that gene were synthesized by using conventional technology. The following oligonucleotide primers were used: forward primer, 5'-GGCCTTGGCTCCAGCATGTAC-3', 1856 through 1876, reverse primer, 5'-GCTGCCGCTCTCATCCAG-AAC-3', 2395 through 2415. The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence (Beg *et al*., 1993; Miyamoto *et al*., 1994). Total cellular RNA (5 μg) from rIFN-γ+LPS or rIFN-γ+PMA with or without LY294002-stimulated RAW264.7 cells was used as a template and the single-stranded cDNA was synthesized with downstream antisense primers by reverse transcriptase. cDNA was amplified in a 12-μl reaction mixture by using a Dae Han Medical Co. DNA thermal cycler (Seoul, Korea) by using Taq polymerase in 45 cycles of 5 s of denaturation at 94°C, 5 s of annealing at 55°C, and 20 s of synthesis at 72°C. PCR products were examined on 1% agarose gel. For the analysis of DNA sequence, PCR products were gel purified, treated with T4 polynucleodtide kinase, and then with the Klenow fragment of DNA polymerase I. The products were subcloned into the EcoRV site of the pBluescript II KS (-) plasmid. Methods for plasmid DNA preparation were described previously (Maniatis *et al*., 1982). One microgram of plasmid DNA was radiolabeled by random priming with [α-^{32}P]dCTP. The resultant specific activity was approximately 1 ×10^8 cpm/μg and was used at 1×10^7 cpm/blot.

RNA extraction and northern blot analysis

Total RNA was isolated by modified LiCl-urea method (Shin *et al*., 1997), electrophoresed in 1.2% agarose-formaldehyde gels, and transferred on nylon membranes by capillary action in 20×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). Baked filters were prehybridized at 42°C in a buffer containing 50% formamide, 4×SSC, 0.5 mg/ml sheared salmon sperm DNA, and 1×Denhardt's solution. Hybridization proceeded at 42°C for 15 h in the same buffer containing 1×10^6 cpm/ml of an [α-^{32}P]dCTP labeled probe. The filters were then washed in 2×SSC/0.1% SDS, 1×SSC/0.1% SDS, and 0.2×SSC/0.1% SDS at 55°C for 20 min, dried and examined by autoradiography.

RESULTS

Degradation of IκBα in Activated RAW264.7 Cells Is Blocked by LY294002

LPS or PMA treatment of RAW264.7 cells elicited activation of p70 S6 kinase. The PMA or LPS activation of p70 S6 kinase was also relatively intensive to intervention with wortmannin, yet it was clearly inhibited by LY294002 (Salh *et al*., 1998). To analyze the effect of LY294002 on the degradation of IκBα, RAW264.7 cells were treated for different time periods with the cells stimulated rIFN-γ (5 U/ml) plus LPS (10 ng/ml) in the presence of LY294002. Disappearance of the IκBα protein, as a consequence of its proteolytic degradation

Fig. 1. Effect of LY294002 on the IκBα degradation in RAW264.7 cells. (A) RAW264.7 cells were either untreated or LY294002 (30 μM) in the presence of IFN-γ (5 U/ml) plus LPS (10 ng/ml). They were then either left untreated (*lane 1*) or stimulated with LY294002 for the indicated times: 1 min (*lane 2*), 5 min (*lane 3*), 10 min (*lane 4*), 30 min (*lane 5*), 60 min (*lane 6*), 120 min (*lane 7*). Lysates were blotted with Ab against IkBα. (B) RAW264.7 cells were either untreated or LY294002 (30 μM) in the presence of IFN-γ (5 U/ml) plus PMA (200 nM). They were then either left untreated (*lane 1*) or stimulated with LY294002 for the indicated times: 1 min (*lane 2*), 5 min (*lane 3*), 10 min (*lane 4*), 30 min (*lane 5*), 60 min (*lane 6*), 120 min (*lane 7*). Lysates were blotted with an anti-IκBα Ab.

(Beg *et al.*, 1993; Miyamoto *et al.*, 1994; Raingeaud *et al.*, 1995), was apparent. In agreement of with earlier report (Heitmeier *et al.*, 1998), Treatment with rIFN-γ plus LPS resulted in the disappearance of the IκBα protein (data not shown). Treatment with LY294002 completely inhibited degradation of IκBα in RAW264.7 cells stimulated with rIFN-γ plus LPS (Fig. 1 A). Fig. 1 B shows that LY294002 also inhibited degradation of IκBα in the cells stimulated with rIFN-γ plus PMA. Treatment with rIFN-γ plus PMA resulted in the degradation of IκBα protein (data not shown). Thus, in agreement with earlier findings (Kopp and Ghosh, 1994), our results indicate that LY294002 inhibits IκBα degradation by interfering IκBα phosphorylation, which is require for targeting IκBα for degradation by the ubiquitin-proteosome pathway (Palombella *et al.*, 1994).

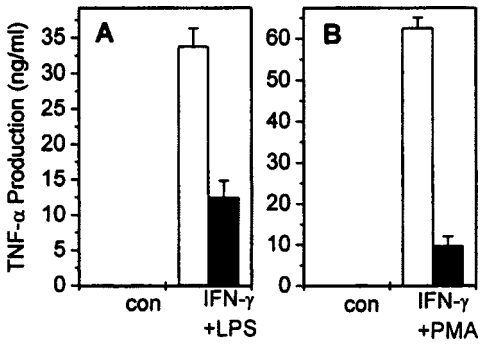

Fig. 3. Effect of LY294002 on the production of TNF-α by IFN-γ plus LPS or IFN-γ plus PMA. (A) RAW264.7 cells (5×10^5) stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml) in the absence (□) or presence (■) of LY294002 (20 μM). The amount of TNF-α production by the cells was measured after 24 h of incubation. (B) RAW264.7 cells (5×10^5) stimulated with IFN-γ plus PMA (200 nM) in the absence (□) or presence (■) of LY294002 (20 μM). The amount of TNF-α production by the cells was measured after 24 h of incubation. Values are the means±SD of six experiments.

LPS- or PMA-induced TNF-α Production is Inhibited by LY294002

Since the induction of TNF-α synthesis in the cells by LPS is dependent upon NF-κB activation, we next assumed that effect of LY294002 on inhibition of NF-κB activation is dependent on the inhibition of IκBα degradation. We determined whether inhibition of IκBα degradation was correlated with TNF-α production. RAW 264.7 cells were incubated for 1 h with medium alone or with medium that contained various concentrations of LY294002. The cells were treated with LPS (10 ng/ml) and cultured for 23 h. We assessed quantitatively the amount of TNF-α production from activated RAW 264.7 cells. LY294002 dose-dependently inhibited LPS- or PMA-induced TNF-α production (Fig. 2). LY294002 also potently inhibited IFN-γ plus LPS- or IFN-γ plus PMA-induced TNF-α production (Fig. 3 A and B). This inhibitory effect on TNF-α production by LY294002 was not due to killing of the cells by an apoptosis-promoting effect, as cell viability, assessed by trypan blue exclusion, at 24 h was unchanged among the various treatment groups (data not shown). The results implicate that the inhibition of TNF-α production by LY294002 may be mediated by NF-κB inactivation.

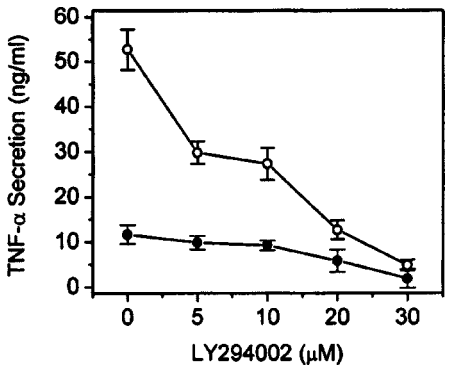

Fig. 2. Effect of LY294002 on the production of TNF-α by LPS (●)- or PMA (○)-stimulated RAW 264.7 cells. The cells (5×10^5) were cultured with LPS (10 ng/ml) or PMA (200 nM) in the absence or presence of LY294002. The amount of TNF-α production by the cells was measured after 24 h of incubation. Values are the means±SD of six experiments.

IL-6 Production is Inhibited by LY294002 in the Activated RAW 264.7 cells

In order to determine whether LY294002 is involved in LPS- or PMA-induced IL-6 production, the effect of LY294002 on the production of IL-6 was tested. The cells were treated with LY294002 before the stimulation with LPS. PKC is involved in LPS-induced secretion of central pro-inflammatory mediators (Shapira *et al.*, 1994; Novotney *et al.*, 1991). To determine whether LY294002 can also modulate PMA-induced production of IL-6, the cells were incubated for 30 min with LY294002 and

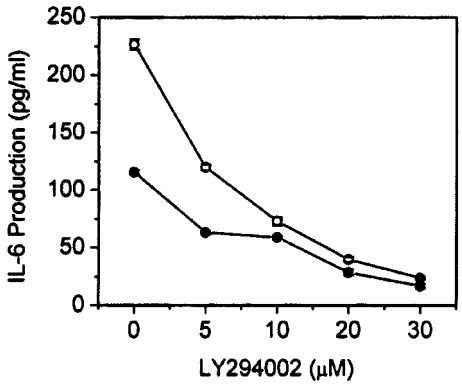

Fig. 4. Effect of LY294002 on the production of IL-6 by LPS (●)- or PMA (○)-stimulated RAW 264.7 cells. The cells (5×10^5) were cultured with LPS (10 ng/ml) or PMA (200 nM) in the absence or presence of LY294002. The amount of IL-6 production by the cells was measured after 24 h of incubation. Values are the means±SD of six experiments.

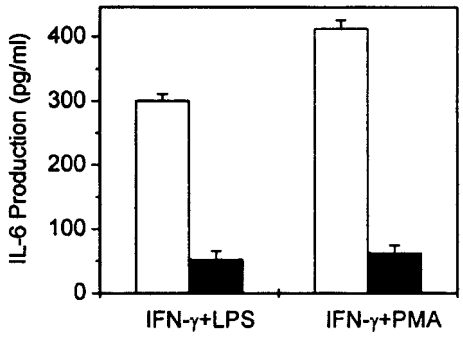

Fig. 5. Effect of LY294002 on the production of IL-6 by IFN-γ plus LPS or IFN-γ plus PMA. RAW 264.7 cells (5×10^5) stimulated with IFN-γ (5 U/ml) plus LPS (10 ng/ml) or IFN-γ plus PMA (200 nM) in the absence (□) or presence (■) of LY294002 (20 μM). The amount of IL-6 production by the cells was measured after 24 h of incubation. Values are the means±SD of six experiments.

stimulated by PMA. In agreement with earlier finding (Dentener *et al*., 1993), LPS induced production of IL-6. LY294002 inhibited LPS-induced IL-6 release in a dose dependent manner (Fig. 4). LY294002 alone failed to induce IL-6 production. In addition, when the cells were treated with LY294002 30 min before the stimulation with IFN-γ plus LPS or IFN-γ plus PMA, LY294002 potently inhibited IL-6 production (Fig. 5).

rIFN-γ plus LPS/PMA-induced NO Production is Inhibited by LY294002

Having demonstrated that biochemical activation of signaling molecules under investigation does

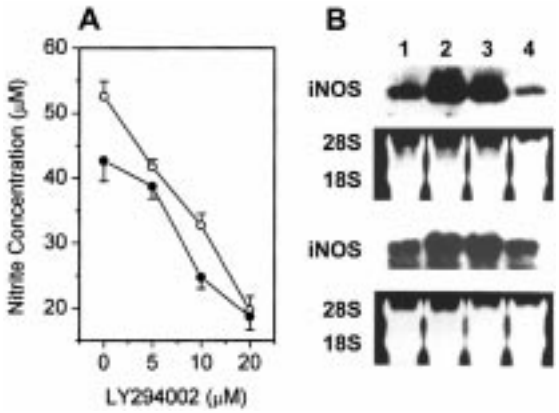

Fig. 6. Effects of LY294002 on the NO production and iNOS expression in RAW264.7 cells. (A) Dose-dependent effect of LY294002 on NO production in the cells (10^6 cells/ml) treated with IFN-γ (5 U/ml) plus LPS (10 ng/ml) or IFN-γ plus PMA (200 nM). The cells were cultured with IFN-γ plus LPS (○) or IFN-γ plus PMA (●) in the absence or presence of LY294002. After 24 h, NO production was measured by the method of Griess (nitrite). Values are the means±SD of six experiments. (B) Induction of expression of iNOS genes. The cells were cultured with IFN-γ plus LPS (*upper panel*) or IFN-γ plus PMA (*lower panel*) in the absence or presence of LY294002. After 24 h, the cells were collected. *Upper panel*, control, (*Lane 1*); IFN-γ plus LPS, (*lane 2*); LY294002 (1 μM) plus IFN-γ plus LPS, (*lane 3*); LY294002 (20 μM) plus IFN-γ plus LPS, (*lane 4*). *Lower panel*, control, (*lane 1*); IFN-γ plus PMA, (*lane 2*); LY294002 (1 μM) plus IFN-γ plusPMA, (*lane 3*); LY294002 (20 μM) plus IFN-γ plus PMA, (*lane 4*). Total RNA was prepared and iNOS mRNA were analyzed by Northern hybridization. Blots were hybridized with the indicated [α-^{32}P]dCTP-radiolabeled cDNA and exposed to x-ray film for 8 h.

indeed occur in macrophage in response to LPS/PMA, and that their regulation occurred somewhat differently from the accepted model, the next step was to investigate the effects of LY294002 on NO production. NO is a functionally important molecule that can be deleterious in certain situations when produced in excess. RAW264.7 cells were treated with different concentrations of LY294002 for 30 min before the addition of rIFN-γ plus LPS/PMA. Then the cells were cultured for 24 h, and NO production was measured by using the method of the Griess (nitrite). As Fig. 6 A shows, LY294002 inhibited nitrite production from activated RAW 264.7 cells in a dose dependent manner. This result is not in agreement with previous report that another PI3-kiase inhibitor, wortmannin, markedly

potentiated the LPS-induced NO production in a dose dependent manner (Park *et al*., 1997). To determine whether the reduced NO production is correlated with iNOS mRNA expression, we analyzed iNOS mRNA expression by Northern hybridization with radiolabeled cDNA that encoded iNOS gene. On the basis of the Northern hybridization data, expressed iNOS mRNA levels were associated with NO production (Fig. 6B). Our data indicate that inhibition of NO production by LY294002 was correlated with iNOS mRNA expression.

DISCUSSION

We have found that LY294002 blocked IκBα degradation in activated RAW264.7 cells. LY294002 and wortmannin are PI3-kinase inhibitors. However, the explanation for the differential response to LY294002 and wortmannin is not immediately clear. p70 S6 kinase activation is only partially abolished by wortmannin in response to both LPS and PMA, whereas LY294002 completely inhibited this activation with agonists (Salh *et al*., 1998). Although the question of wortmannin stability is an important one, several lines of evidence in different cells systems indicate that this may have been overemphasized. A study in chicken macrophages clearly showed an inhibitory effect of wortmannin on LPS-induced NO production (Yang *et al*., 1996). In an intestinal cell line, it was clearly able to attenuate the inhibitory effect of IL-13 on TBF/IFN-γ/IL-1-mediated NO production (Wright *et al*., 1997). In agreement with previous reports (Salh *et al*., 1998; Yang *et al*., 1996; Wright *et al*., 1997), wortmannin did not inhibit IkBα degradation in activated RAW264.7 cells (data not shown). NF-κB activation regulates a large number of genes involved in inflammation, including cytokines, cell adhesion proteins, and enzymes (Baeuerle and Baltimore, 1996). It is possible that the effects of LY294002 are due to inhibition of expression of some these genes through inhibition of NF-κB activation. How LY294002 blocks NF-κB activation is not clear and will not be until we have full understanding of the pathway leading to NF-κB activation. LY294002, however, blocked IκBα degradation induced by PMA or LPS, indicating that a common step in the pathway for these agents is blocked by LY294002. LPS and PMA both elicit activation of S6 kinase. The FRAP inhibitor rapamycin and the PI3-kinase inhibitor LY294002 inhibit LPS- or PMA-induced the phosphorylation and the activation of S6 kinase (Salh *et al*., 1998). It is possible that LY294002 may block IκBα degradation through FRAP.

A role for PKC in the regulation of LPS-inducible events in macrophages has been suggested from the observations that exposure of macrophages to LPS activates PKC (Novotney *et al*., 1991; Fujihara *et al*., 1994; Aderem *et al*., 1988) and that pretreatment of macrophages with either PKC inhibitors or phorbol esters inhibits LPS-induced TNF-α and NO production (Novotney *et al*., 1991; Kovacs *et al*., 1988; Tremblay *et al*., 1995). LPS activates the Raf-1/MAP kinase pathway in macrophages and evidence was provided that Raf-1 may participate in the induction of IL-1 and TNF-α gene expression (Hambleton *et al*., 1995; Reimann *et al*., 1994). The overexpression of Raf-1 (Reimann *et al*., 1994; Li and Sedivy, 1993) or p42 MAP kinase (Park and Levitt, 1993) results in enhanced expression of variety of cytokine genes in T cells and macrophages (Reimann *et al*., 1994; Park and Levitt, 1993), the inactivation of IκB (Li and Sedivy, 1993), and the enhanced binding activity of cytokine transcription factors such as NF-κB (Park and Levitt, 1993). Our results indicated that LY294002 inhibited LPS- or PMA-induced TNF-α release, indicating that p70 S6 kinase may be involved in Raf-1/MAP kinase pathway in RAW264.7 cells stimulated with LPS or PMA. Since LPS-induced IκBα phosphorylation and degradation and NF-κB activation take place in the cells, it is likely that the effect of LY294002 may be involved in this pathway through Raf-1/MAP kinase. Considering that NF-κB plays an important role in the transcriptional activation of TNF-α gene expression (Sweet and Hume, 1996), this suggestion would be consistent with the effect of LY294002 on LPS-induced TNF-α expression. However, another PI3-kinase inhibitor, wortmannin, potentiates the LPS-induced TNF-α production in macrophages (Park *et al*., 1997). On the basis of these results, we suggest that p70 S6 kinase may be involved in LPS-induced TNF-α production through NF-κB activation. In this regards, further studies are thus required to elucidate the identity of the

LPS-induced pathway(s) and transcription factors regulated by LY294002.

The production of IL-6 has been found to play a central role in the regulation of defense mechanism, haematopoiesis, and acute phase reaction (Gauldie *et al*., 1987; Gauldie *et al*., 1992). Previous report demonstrate that the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) molecules inhibit the production and mRNA expression of cytokines (IL-6, IL-8, GM-CSF) induced by proinflammatory stimuli (TNF-α, LPS) in human endothelial cells (Munoz *et al*., 1996). The activation of NF-κB is indispensable for IL-6 and IL-8 genes activation in any type of cell examined (Shimizu *et al*., 1990; Zhang *et al*., 1990; Libermann and Baltimore, 1990). In addition, molecular analysis of IL-6 by different agents such as glucocorticoids, estrogens, cyclosporin, or FK506 showed that all of them affected the activity of NF-κB, thereby suppressing the transcription of this cytokine (Mukaida *et al*., 1994; Okamoto *et al*., 1994; Stein and Yang, 1995). Recent reports in LPS-stimulated RAW264.7 cells have demonstrated a regulatory effect of p42/44 (ERK) MAPK on TNF-α transcription, and a significant role of the MEKK1/MEK4/JNK cascade on TNF-α translation (Swantek *et al*., 1997). p38 MAPK inhibitor, SB203580, completely inhibited TNF−α induced synthesis of IL-6 (Beyaert *et al*., 1996). LY294002 inhibited the phosphorylation of p38 MAPK in mast cell line (our unpublished data). Our results indicate that LY294002 inhibits LPS- or PMA-induced IL-6 release, suggesting that LY294002 blocks IL-6 release through inactivation of IκB. However, it is impossible to rule out that LY294002 blocks autocrine effect of LPS-induced TNF-α production and then reduced TNF-α decreases IL-6 production.

In macrophages, NO is a cytotoxic mediator and contributes to the antimicrobial and tumoricidal activity of these cells (Moncada *et al*., 1991; Nathan and Hibbs, 1991). In addition, NO has been implicated as a mediator of many pathophysiological conditions such as septic shock, rheumatoid arthritis, and multiple sclerosis (Liu *et al*., 1993; Sakurai *et al*., 1995; Bo *et al*., 1994). Despite a large number of data describing biological activities of NO, precise molecular mechanisms of macrophage activation containing NO synthesis remain to be defined. In the present study, we showed that LY294002 blocked the NO release in the RAW264.7 cells treated IFN-γ plus LPS or IFN-γ plus PMA. However, recent report showed that another PI-3 kinase inhibitor, wortmannin, markedly pontentiated the LPS-induced NO production in a dose-dependent manner (Park *et al*., 1997). A strong case is made for the involvement of FRAP in the LPS-mediated production of NO. A differential sensitivity of FRAP to LY294002 and wortmannin in this particular system could be an explanation for the lack of a functional effect for the latter. This is possible, as one study clearly demonstrated that FRAP kinase activity was wortmannin insensitive (Brown *et al*., 1995). Inhibition of iNOS expression and cytokines by antioxidants and tyrosine kinase inhibitors suggests the possible involvement of reactive oxygen species and tyrosine phosphorylation in the signaling (Pahan *et al*., 1997; Nishiya *et al*., 1995). Several evidences clearly indicate that LPS induces iNOS via activation of NF-κB activation (Xie *et al*., 1994; Kwon *et al*., 1995; Pahan *et al*., 1997). Recent reports have identified the activation of Ras/Raf/MEK/MAP kinase cascade after LPS stimulation (Reimann *et al*., 1994). Although the direct involvement of this cascade in the induction of iNOS is not established so far, activation of NF-κB by Raf (Li and Sedivy, 1993), the presence of NF-κB binding site in the promoter region of iNOS, and activation of NF-κB in LPS-induced iNOS induction, suggests a role of Raf/MAP kinase cascade and NF-κB activation in the induction of iNOS. Inhibition of IκB degradation and iNOS mRNA expression by LY294002 indicates that the observed inhibition of iNOS expression is due to inhibition of NF-κB activation.

We demonstrated that LY294002 inhibited degradation of IκBα, TNF-α, IL-6, and NO production in activated RAW264.7 cells. These results suggest that LY294002 blocks TNF-α, IL-6, NO production through inhibition of IκB degradation and that differential effects of LY294002 and wortmannin result in different function and signaling pathway(s) in RAW264.7 cells.

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