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Triggering of the macrophage cell line RAW 264.7 with LPS promotes a transient activation of phosphatidylinositol 3-kinase (PI3-kinase). Incubation of activated macrophages with wortmannin and LY294002, two inhibitors of PI3-kinase, increased the amount of inducible nitric oxide synthase (iNOS) and the synthesis of nitric oxide. Treatment with wortmannin promoted a prolonged activation of NF-κB in LPS-treated cells as well as an increase in the promoter activity of the iNOS gene as deduced from transfection experiments using a 1.7-kb fragment of the 5′ flanking region of the iNOS gene. Cotransfection of cells with a catalytically active p110 subunit of PI3-kinase impaired the responsiveness of the iNOS promoter to LPS stimulation, whereas transfection with a kinase-deficient mutant of p110 maintained the up-regulation in response to wortmannin. These results indicate that PI3-kinase plays a negative role in the process of macrophage activation and suggest that this enzyme might participate in the mechanism of action of antiinflammatory cytokines. *The Journal of Immunology,* 1999, 162: 6184–6190.

Macrophage activation constitutes a key component of the immune response and several proinflammatory cytokines and bacterial products (among them LPS and lipoproteins) participate actively in the triggering of this process (1–3). The activation elicited by LPS induces in turn the synthesis of additional cytokines such as TNF-α, IL-1β, and IL-6, which favor the amplification of the original response. LPS interacts with the macrophage through CD14, a glycophasphatidylinositol-anchored molecule (4). Activation through this receptor promotes the stimulation of several protein tyrosine kinases of the Src family favoring the association of p53/p56lck to the receptor (5, 6). The activation of this kinase facilitates the association with PI3-kinase as well as the involvement of several serine/threonine protein kinases, among them several members of protein kinase C family (6–8).

Activated macrophages release oxygen and nitrogen radicals that are important bactericidal and cytotoxic molecules (1, 2, 9). However, massive production of these mediators can exert detrimental effects in the organism as occurs during septic shock or persistent local inflammatory processes (10, 11). For this reason, the study of the mechanism of action of antiinflammatory cytokines and drugs has constituted a subject of current interest (10–14).

One of the aspects most studied in stimulated macrophages is the induction of the inducible type of NO synthase (iNOS) and the increase of NO synthesis by these cells (1, 15, 16). It is well known that iNOS expression is regulated mainly at the transcription level due to the activation of several transcription factors that bind to the promoter region of the iNOS gene, such as NF-κB, STAT1 and IRF-1 (15–17). The activation of NF-κB depends on the degrada-
tion of the corresponding inhibitory proteins κBz and κBβ that retain inactive the NF-κB complex in the cytosol (18, 19). Several data point to NF-κB activation as a critical event in the expression of iNOS (17, 20, 21), and most studies focused on the analysis of antiinflammatory mechanisms suggested a prominent role for the inhibition of this transcription factor in their mode of action (22, 23).

More recently, several groups have shown that in the course of macrophage activation various inhibitory mechanisms are engaged favoring a controlled regulation of the process to avoid the harmful effects of an exacerbated activation. Regarding iNOS expression, a negative regulation by NO has been described (24), as well as a competition between type I and type II IFNs in the synergistic action with LPS (14). Moreover, it has been shown that LPS increases the levels of PPARγ and this nuclear factor exerts an inhibitory effect on macrophage activation, including the inhibition of iNOS and gelatinase B transcription (12, 25).

In this work, we show that activation with LPS of the macrophage cell line RAW 264.7 activates PI3-kinase. However, inhibition of this kinase by wortmannin and LY294002 (5, 7, 26) results in an up-regulation of iNOS expression, mainly through a mechanism that involves a sustained activation of NF-κB. Moreover, expression of a constitutively active p110 subunit of the PI3-kinase attenuates the promoter activity of cells cotransfected with a plasmid containing a 1.7-kb fragment of the 5′ flanking region of the murine iNOS gene. These results indicate that PI3-kinase plays a negative role in the expression of iNOS and might contribute to understanding the mechanism of action of antiinflammatory cytokines (IL-13 and IL-10) that activate PI3-kinase in the course of their intracellular signaling (26, 27).

**Materials and Methods**

**Chemicals**

Biochemicals and reagents were from Sigma (St Louis, MO) or from Boehringer Mannheim (Mannheim, Germany). Wortmannin and LY294002 were from BioMol (Plymouth Meeting, PA). Electrophoresis equipment and reagents were from Bio-Rad (Richmond, CA) or from Amersham (Amersham Bucks., U.K.). Serum and media were from Bio-Whittaker (Walkersville, MD).
**Cell culture**

RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and were seeded at 0.8–1 × 10^7/cm^2 in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS, and antibiotics (50 μg/ml of penicillin, streptomycin, and gentamicin). After 2 days in culture, the cell layers were washed with PBS and the culture medium was replaced by phenol red-free RPMI 1640 containing 0.5 mM arginine and 5% FCS.

**Plasmid construction and preparation**

The 1749-bp HincII fragment corresponding to the 5' flanking region of iNOS (14–16) fused to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene (pNOS.CAT) was a generous gift from Drs. Q.-w. Xie and C. Nathan (Cornell University, Ithaca, NY). A (XbaI)Con.A.CAT plasmid construct, which contains three copies of the XbaI motif from the HIV long terminal repeat enhancer with the conalbumin promoter, was used to measure XbaB transactivation capacity (21). A Con.A.CAT vector lacking the XbaI tandem was used as control. rCD2p110, which encodes a constitutively active molecule, including the extracellular and transmembrane domains of the rat CD2 cell surface Ag and the p110α catalytic subunit of PI3-kinase, rCD2p110kd, a kinase-deficient mutant, and p85d, which is unable to bind p110 and therefore inhibits the recruitment of p110 to 2% formaldehyde, the RNA was transferred to a Nytran membrane (Amersham) and the intensity of the bands was measured by laser densitometry (Molecular Dynamics, Sunnyvale, CA). Hybridization with an 18S ribosomal probe was used as an internal standard.

**Charaterization of proteins by Western blot**

A modified procedure based on the method of Diaz-Guerra et al. (21) Schreiber et al. (32) was used. Cultured RAW 264.7 cells (3–4 × 10^5) were washed twice with PBS and collected by centrifugation. Cell pellets were homogenized with 100 μl of buffer A. After 10 min at 4°C, NP-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8,000 × g for 15 min. The supernatants were stored at −80°C (cytosolic extracts) and the pellets were resuspended in 50 μl of buffer A supplemented with 20% glycerol, 0.4 M KCl, and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 × g for 15 min, and aliquots of the supernatant were stored at −80°C. Protein content was assayed using the Bio-Rad protein reagent. All steps of cell fractionation were carried out at 4°C.

**Oligonucleotides**

Oligonucleotides were synthesized in a Pharmacia (Piscataway, NJ) oligonucleotide synthesizer and the sequence 5'-TGCTAGGGGAGCTCTCTGAG-3' corresponding to the consensus NF-κB-binding site (8) (−87 to −95) of the murine iNOS promoter was used (15). Boldface type corresponds to the binding sequence. Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0; 50 mM NaCl, 10 mM MgCl_2, and 1 mM DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme fragment in the presence of 50 μCi of [γ-32P]ATP and the other unlabeled dNTPs in a final volume of 50 μl. A total of 5 × 10^6 dpm of the DNA probe were used for each binding assay of nuclear extracts as follows: 3 μg of protein were incubated for 15 min at 4°C with the DNA and 2 μg of poly(dI:dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM MgCl_2, 1 mM DTT, and 10 mM Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.3% Tris-borate-EDTA buffer (21). Supershift assays were carried out after incubation of the nuclear extracts with the Ab (0.5 μg) for 1 h at 4°C, followed by EMSA (not shown). Anti-p50 (human) and anti-κ-B (human) were a generous gift of Dr N. R. Rice (National Cancer Institute, Frederick, MD); anti-p65 (murine) Ab was from Santa Cruz.

**Data analysis**

The number of experiments analyzed is indicated in the corresponding figure. Statistical differences (p < 0.05) between mean values were determined by one-way analysis of the variance followed by Student’s t test. In experiments using x-ray films (Hyperfilm, Amersham), different exposure times were used to ensure that bands were not saturated.

**Results**

**Wortmannin and LY294002 increase NO synthesis in LPS-activated macrophages**

Cultured RAW 264.7 cells were incubated with wortmannin (100 nM) and LY294002 (10 μM) followed by stimulation with LPS,
IFN-γ, IL-1β, TNF-α, or combinations of these, and the accumulation of nitrite in the culture medium was determined. As Fig. 1A shows, in the presence of wortmannin or LY294002 a 4- and 2.7-fold increase in the concentration of nitrite was measured in cells treated with 200 ng/ml of LPS. The effect of these drugs was less important when cells were activated with TNF-α, IL-1β, IFN-γ, or IFN-γ plus LPS acting in concert. Agreement was observed between the synthesis of nitrite and the levels of iNOS. Higher amounts of iNOS were observed when cells were treated with LPS plus these inhibitors (Fig. 1B). These results indicated that the maximal effectiveness of wortmannin on iNOS expression was observed in cells stimulated with LPS. As Fig. 2A shows, low doses of LPS fail to induce NO synthesis. However, when wortmannin was present, a dose-dependent increase of nitrite accumulation was measured. This potentiation of NO synthesis was still evident in cells treated with LPS and IFN-γ, and the apparent Ki value for wortmannin was similar in both cases (20 nM). The dose-dependent curve for LPS is shown in Fig. 2B, and saturation in the presence of 100 nM wortmannin was obtained at concentrations of LPS higher than 200 ng/ml. Moreover, to establish the optimal period of wortmannin treatment to increase NO synthesis, the drug was added at several times with respect to LPS challenge. As Fig. 2C shows, an almost linear fall in the response was observed after LPS challenge, with 50% of the effect obtained when added at 2.5 h. This restricted effect of wortmannin to early times of LPS activation is compatible with a main effect of this molecule at the transcription level. Since wortmannin appears to be unstable, sequential additions (1-h periods) were performed although this did not modify the pattern of response (not shown). Indeed, when the iNOS mRNA levels were determined at 6 h after LPS stimulation, wortmannin notably increased iNOS expression, exhibiting an inhibition at concentrations higher than 200 nM (Fig. 3).

Wortmannin potentiates NF-κB activation in LPS-treated macrophages

To further study the mechanism of wortmannin enhancement of iNOS expression, we investigated the effect of this substance on NF-κB activity. As Fig. 4A and B show, in LPS-treated cells wortmannin did not significantly increase this activity at 30 min but promoted a time-sustained activation. Both p50 dimers and p50/p65 complexes persisted up to 4 h after LPS stimulation, whereas in cells treated without wortmannin the fall of both complexes was evidenced after 2 h. To have more accurate information about the effect of wortmannin on NF-κB activity, cells were transfected with a (κB)3ConA.CAT plasmid and the activity of the reporter was measured after 18 h. As Fig. 4C shows, wortmannin did not affect the basal CAT activity; however, in LPS-treated cells, wortmannin increased 3.9-fold the reporter activity with respect to the LPS condition. The effect of LPS and wortmannin on CAT activity were specific since in cells transfected with a ConA.CAT plasmid and the activity of the reporter was measured after 18 h. As Fig. 4C shows, wortmannin did not affect the basal CAT activity; however, in LPS-treated cells, wortmannin increased 3.9-fold the reporter activity with respect to the LPS condition. The effect of LPS and wortmannin on CAT activity were specific since in cells transfected with a ConA.CAT plasmid these stimuli failed to promote a significant transcription of the reporter gene (not shown). Because of these results on NF-κB activity, the effect of both wortmannin and LY294006 on IκBα and IκBβ levels, the inhibitory subunits that retain NF-κB inactive in the cytosol, were measured. As Fig. 4D shows, after 1 h of stimulation of cells with LPS, both LY294002 and wortmannin

FIGURE 1. Wortmannin and LY294002 increase nitrite synthesis and iNOS protein levels in activated RAW 264.7 cells. Macrophages were stimulated for 18 h with LPS (200 ng/ml), IFN-γ (20 U/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), LY294002 (10 μM), wortmannin (100 nM), or combinations of these. The amount of nitrite released to the medium was determined (A). Cells incubated for 18 h with LPS (20 or 200 nM) and IFN-γ (5 U/ml) were homogenized and the amount of iNOS was evaluated by Western blot following the appearance of a 130-kDa band. Results show the mean ± SEM of three experiments. *, p < 0.05; **, p < 0.001 vs the corresponding condition in the absence of wortmannin or LY294002.

FIGURE 2. Dose-dependent potentiation of NO synthesis by wortmannin. Macrophages (2 × 10⁵ cells) were incubated for 18 h with the indicated concentrations of wortmannin and LPS (50 ng/ml) or LPS plus IFN-γ (5 U/ml) (A), or wortmannin (100 nM) and LPS (B), and the amount of nitrite released was determined. C shows the effect on the synthesis of nitrite of wortmannin added at different moments with respect to LPS challenge. Results are the mean ± SEM of three experiments.
exhibited low levels of IκBα and IκBβ in the cytosol, suggesting a potentiation of the effect of LPS on IκB degradation.

**LPS increases PI3-kinase activity**

Wortmannin and LY294002 are known inhibitors of PI3-kinase activity (7, 33). To determine whether this enzyme is activated after LPS stimulation of macrophages, PI3-kinase was immunoprecipitated, and after resuspension, the lipid kinase activity was measured in vitro. As Fig. 5 shows, treatment of cells with LPS rapidly increased the phosphatidylinositol kinase activity in the immunoprecipitated extracts. However, this response was absent in LPS-treated cells incubated with 200 nM of wortmannin.

**PI3-kinase activity decreases iNOS transcription**

Incubation of macrophages with wortmannin had no effect on the CAT activity of cells transfected with p1NOS.CAT, a vector that contains a 1.7-kb fragment of the murine iNOS promoter. However, this drug increased the reporter activity when cells were stimulated with LPS, but not when activation was performed with LPS and IFN-γ (Fig. 6). To gain insight into the mechanism of action of wortmannin in this system, cells were cotransfected with p1NOS.CAT and either a plasmid encoding a p110 catalytically active PI3-kinase subunit (rCD2p110), a kinase-deficient mutant (rCD2p110kd), or a dominant negative form of p85 (p85d). As Fig. 7 shows, cotransfection with rCD2p110 resulted in a decrease of the reporter activity with respect to the p1NOS.CAT alone (Fig. 6) or when compared with the effect of transfection with rCD2p110kd. Indeed, in cells cotransfected with rCD2p110 plus p1NOS.CAT, wortmannin was unable to increase CAT activity as occurred in cells transfected with p1NOS.CAT or with the p110 kinase-deficient mutant and stimulated with LPS. Interestingly, these plasmids encoding p110 had only minimal effects on the...
reporter activity measured in cells stimulated with IFN-γ and LPS acting synergistically. Cotransfection of p1NOS.CAT with a vector encoding a dominant negative form of p85 had profound effects on the reporter activity, suggesting that p85 might be involved at different levels in the signaling pathway triggered by LPS. Divergent responses to dominant negative p85 and p110 active subunits have been observed in other experimental systems (29), and probably indicate that p85 has additional adapter functions for other proteins different from p110.

Discussion

The identification of pathways involved in the negative control of monocyte/macrophage activation constitutes a field of growing interest in order to understand the physiologically occurring antiinflammatory mechanisms and to take advantage of this information for the design of new pharmacological strategies (11, 12, 25). The observation that wortmannin and, to a lesser extent, LY294002, two inhibitors of PI3-kinase (33, 34), increased NO synthesis in peritoneal macrophages and in RAW 264.7 cells stimulated with LPS is compatible with the proposal of a negative role for PI3-kinase in the expression of iNOS (Ref. 35; this work). However, these inhibitors are not specific for PI3-kinase, although at the concentrations used, the effect of wortmannin fits well with the reported \( K_i \) values for PI3-kinase (34, 36). With respect to LY294002, this inhibitor exhibited an important toxicity when used in RAW 264.7 cells at concentrations higher than 10–20 \( \mu M \), hence limiting its use at about this concentration. In addition to these inhibitors, transfection of cells with different subunits of PI3-kinase (28, 29, 34) contributed to establish the role for this enzyme in the expression of iNOS in activated macrophages.

Stimulation of RAW 264.7 cells with LPS fails to significantly induce iNOS. In these macrophages, LPS promotes a transient activation of PI3-kinase as determined by the increase of phosphatidylinositol (PI) phosphorylation using immunoprecipitated enzyme. Therefore, the mechanism by which PI3-kinase inhibitors enhanced iNOS transcription and NO synthesis after LPS-stimulation implies the suppression of a LPS-dependent negative signaling. The effect of PI3-kinase inhibitors in RAW 264.7 cells appears to include a sustained activation of NF-κB that extends for a longer period of time than if cells are treated with LPS alone. It is possible that this persistent activation of NF-κB might contribute to favor the signaling by autocrine factors released in response to LPS challenge (TNF-α and several proinflammatory ILs). In this regard, it should be noted that the maximal efficiency of wortmannin was obtained when added immediately following LPS stimulation, suggesting an effect over early responses activated by LPS. The observation of lower levels of I-κB proteins in the cytosol of cells treated with LPS and PI3-kinase inhibitors is compatible with the sustained activation of NF-κB, and suggests a possible role for wortmannin in the regulation of IκB-kinase or proteasome activities (18, 19). The abrogation of the effect of wortmannin on NO synthesis when cells are activated with LPS and IFN-γ acting synergistically suggests that the negative effect of PI3-kinase on this process can be suppressed by other stimuli.

PI3-kinase is a complex enzyme that includes different isotypes (7, 33, 37, 38). At least three isoenzymes of PI3-kinase have been identified, including the “classic” p85/p110 heterodimer, as well as a G protein-coupled PI3-kinase-γ, and a PI3-kinase with narrow specificity for PI (37–39). This diversity in the isoforms might
contribute to the regulation of specific responses (7). Since PI3-
kinase is involved in early steps of intracellular signaling, we
investigated other downstream PI3-kinase targets (7). PI3-kinase sig-
naling pathways include the activation of the serine/threonine
kinase, PKB, which activates p70 S6-kinase through a mechanism
controlled by the rapamycin target (mTOR) (7, 40). Treatment of
RAW cells with rapamycin (from 10 to 200 nM) did not affect NO
synthesis, which suggests that the PKB/mTOR pathway is not re-
ponsible for these effects. Moreover, in human monocytes and in
other cell types, LPS activates PI3-kinase, which in turn stimulates
protein kinase C-ζ (6). However, the effect of PI3-kinase on iNOS
expression cannot be attributed to protein kinase C-ζ activation
since in preliminary experiments of cotransfection of RAW 264.7
cells with a plasmid encoding a constitutively active protein kinase
C-ζ and a plasmid containing the iNOS promoter an important
increase of the iNOS promoter activity was observed. These data
suggest the existence of a compensatory mechanism of the PI3-
kinase inhibition downstream from the pathway (work in progress).
Indeed, overexpression in these cells of protein kinase C-ζ, an isoenzyme activated by PI3-kinase-derived lipids, also po-
tentiates iNOS transcription (41). Taken together, these results
suggest that PI3-kinase might exert a modulatory role on early
steps of the LPS-dependent macrophage activation.

As previously discussed, wortmannin is not a PI3-kinase-spe-
cific inhibitor, and at the low doses used it inhibits phospholipase
A2 (42) and, therefore, the synthesis of arachidonic acid-derived
metabolites (for example, PGs). Since some PGs synthesized by
cyclooxygenase, such as 15-deoxy-PG J2, could bind to the
PPARγ and inhibit iNOS expression (25), we investigated this
possibility by adding exogenous arachidonate to the cells. How-
ever, NO synthesis was not affected under these conditions (not
shown), suggesting that this mechanism is not relevant for the
action of wortmannin in RAW 264.7 cells.

Different inhibitory pathways have been described for the reg-
ulation of iNOS transcription: NO and glucocorticoids decrease
NF-κB activation mainly through an up-regulation of IκB levels
(22–24); PPARγ ligands seem to block the transactivating activity
of different transcription factors including NF-κB, AP-1, and
STAT1, without affecting their binding capacity (25). Antiinflam-
matory cytokines such as IL-10 or IL-13 appear to activate PI3-
kinase (26, 43, 44). Moreover, the inhibition of iNOS expression
after RON receptor engagement was abolished in macrophages
treated with PI3-kinase inhibitors (45).

Regarding the possible physiological relevance of the results
described, we can speculate that in LPS-activated cells PI3-kinase
constitutes a switch of macrophage activation by LPS, favoring the
cooperation of other stimuli to initiate iNOS transcription. In line
with this, it has been shown that LPS stimulation of macrophages
increases the levels of PPARγ, and this nuclear factor exerts an
important inhibition of the activation process, including a de-
creased iNOS expression (25). Additionally, it has been proposed
that PI3-kinase participates in the process of monocyte/macro-
phage proliferation in the case of activation by oxidized low den-
sity lipoprotein (46). In this sense, the PI3-kinase inhibition of
iNOS expression might protect from the high output release of NO
that, because of its cytostatic and cytotoxic effects, may preclude
the expansion of the precursors. The analysis of this LPS-depend-
ent PI3-kinase activation in other cells might contribute to better
understanding of the contribution of PI3-kinase to the regulation
of iNOS expression. Finally, this work increases the number of in-
hibitory mechanisms engaged in the process of macrophage acti-
vation, and is intended to avoid the harmful effects of an exacer-
bated activation.

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FIGURE 7. Effect of PI3-kinase subunits on the iNOS promoter activity. Macrophages (2 × 106) were transfected with equal amounts of the indicated
vectors (2 µg of p1NOS.CAT and 4 µg of the PI3-kinase subunit), and after stimulation for 24 h as indicated in Fig. 6, CAT activity was measured.
Expression of the CD2 constructs was assessed by flow cytometry (data not shown). Results show the mean CAT activity ± SEM of three experiments.
* p < 0.01 vs the condition in the absence of wortmannin.


