Leptomycin B, a metabolite of *Streptomyces*, inhibits the expression of inducible nitric oxide synthase in BV2 microglial cells

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**Abstract.** Overexpression of inducible nitric oxide synthase (iNOS) and the resultant overproduction of NO has been implicated in neuronal inflammatory diseases. Leptomycin B (LMB), a metabolite of *Streptomyces*, has been identified as a specific inhibitor of CRM1 nuclear export receptor. In this study, we evaluated the effect of LMB on lipopolysaccharide (LPS)-induced iNOS expression in BV2 cells, a murine microglial cell line. LMB strongly inhibited LPS-induced iNOS protein and mRNA expressions in BV2 cells in which 10 ng/ml of LMB (18 nM) was sufficient to greatly down-regulate iNOS by LPS, suggesting the potency of LMB to inhibit iNOS. The data of iNOS promoter-driven luciferase assay further suggested that the LMB inhibitory effect was in part due to inhibition of iNOS transcription. However, LPS-induced activation of various intracellular signaling proteins, such as nuclear factor-κB (NF-κB), extracellular signal-regulated kinases (ERKs), p38s, and c-Jun N-terminal kinases (JNKs), whose activations are known to be important for iNOS expression by LPS in BV2 cells, were not affected in the presence of LMB. Together, these results suggest that LMB inhibits iNOS expression in response to LPS in BV2 microglia, and the inhibition seems to be associated with blockage of CRM1-mediated iNOS mRNA nuclear export and also in part transcriptional down-regulation of iNOS, but not through modulation of NF-κB and the mitogen-activated protein kinase signaling pathways.

**Introduction**

Nitric oxide (NO) is an important signaling molecule involved in vascular homeostasis, neurotransmission, and immune defense against infectious agents (1). Importantly, recent studies have demonstrated that the expression and activity of iNOS, one enzyme producing NO, are abnormally increased in inflammation and cancer, and the resultant NO overproduction plays a causative role in these processes (2,3).

NO is produced from L-arginine and molecular oxygen by the action of NOS (4,5). Three isoforms of NOS (type I, II, and III) have been molecularly identified and cloned in mammals (4-6). The neuronal (type I, nNOS) and endothelial (type III, eNOS) enzymes are constitutively expressed and thought to involve in physiological functions in neuronal and endothelial systems, respectively. On the other hand, the third member of NOS family is an inducible enzyme (iNOS, type II). It has been shown that iNOS expression is largely increased in a variety of cells after the exposure of LPS and interferon-γ (IFN-γ) (7), cytokines (8), or catalase (9).

Regulation of iNOS expression is complex. Primarily, it is regulated at transcription (4,5,10). iNOS transcription in response to LPS or cytokines is greatly dependent on activities of transcription factors, including nuclear factor-xB (NF-xB), activator protein-1, nuclear factor-interleukin 6 (NF-IL6), or IFN-γ responsive factor, which individually acts on its cognate cis-acting element within iNOS promoter (11-13). iNOS expression can also be regulated at mRNA stability or translation (14-16). Furthermore, it is well established that iNOS expression depends on the activity of intracellular signaling proteins, including p38s, c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), phosphatidylinositol 3-kinase (PI3K)/AKT, and p70S6K, which affects iNOS transcription, mRNA stability, and/or protein synthesis (17-20).
Leptomycin B (LMB) was originally discovered as a potent anti-fungal antibiotic from Streptomyces species (21). The cellular target of LMB has been identified as the nuclear export receptor CRM1/exportin 1 (22), which is involved in nuclear trafficking of cellular RNAs or proteins containing the nuclear export sequence (NES) (23). Supporting this, LMB is shown to inhibit nuclear export of RNAs such as e-fos (24) or of the NES-containing proteins such as α-catenin (25) or inhibitor of IkB-α (IkB-α) (26). Of interest, LMB was shown to inhibit mRNA nuclear export of cyclooxygenase-2 (COX-2), thus down-regulating the expression of COX-2, an inflammatory enzyme producing prostaglandins, in MDA-MB-231 cells (27).

In this study, we evaluated the effect of LMB on the regulation of iNOS expression in response to LPS, a well-known iNOS inducer, in BV2 cells, a murine microglial cell line and possible inhibitory mechanisms by which LMB regulates iNOS expression.

Materials and methods

Materials. The anti-actin antibody, aprotinin, leupeptin, phenanthroline, and benzamidine-HCl were purchased from Sigma (St. Louis, MO). Anti-rabbit or mouse secondary horseradish peroxidase antibodies and ECL Western detection reagents were bought from Amersham Biosciences (Amersham, UK). Bradford reagent was from Bio-Rad (Hercules, CA). An anti-iNOS polyclonal antibody was obtained from Santa Cruz Company (Santa Cruz, CA). RPMI-1640, Dulbecco’s modified Eagle’s medium (DMEM), and mixtures of penicillin/streptomycin were purchased from Gibco-BRL (Gaithersburg, MD).

Cell culture and preparation of whole cell lysates. BV2 murine microglia were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Raw 264.7 murine macrophages were grown in RPMI supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To prepare whole cell lysates, after stimulation with agents, cells were washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM Na2VO4 and 1 mM NaF, and lysed in a buffer [50 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP40), 1 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was collected, and the protein concentration was determined.

Western blot analysis. Equal amounts of protein (40 μg/lane) were resolved by 10-12% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). The membrane was then washed with Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as iNOS (1:1,000), p-ERKs (1:1,000), p-JNKs (1:1,000), p-p38s (1:1,000), and actin (1:5,000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL western detection reagents.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated with the RNAzol-B (Tel-Test, Friendswood, TX) according to the instructions provided by the manufacturer. Three micrograms of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega) following the manufacturer’ instructions. Single stranded cDNA was then amplified by PCR with specific primers of iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); iNOS sense, 5'-GACAAGCTGCATGTGACATC-3'; iNOS anti-sense, 5'-GGTGAAGGTCGGTTGTGAACG-3'; GAPDH sense, 5'-GGTGAAGGTCGGTTGTAACGC-3'; GAPDH anti-sense, 5'-GGTAGGAAACCGGAAAGGCCA-3'. The following PCR conditions were applied: iNOS, 25 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec; GAPDH, 18 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec. The GAPDH was used as an internal control to evaluate relative expression of iNOS.

Subcellular RNA fractionation. Cells were washed in PBS and successively extracted as previously described (27). Briefly, cells were primarily extracted with buffer A [10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40, 40 U/ml RNasin, 1 mM DTT]. The remaining pellet [rough endoplasmic reticulum (RER) and nucleus] was washed in buffer B [10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 0.5% (v/v) Nonidet P-40, 40 mM EDTA, 40 U/ml RNasin, 1 mM DTT]. The remaining nuclear pellet was washed in buffer B and extracted with buffer C [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 3 mM MgCl2, 0.5% (v/v) Nonidet P-40, 40 U/ml RNasin, 1 mM DTT]. RNA was then purified from each fraction by the RNAzol-B.

Results

Effects of LMB on iNOS protein and mRNA expressions in LPS-stimulated BV2 microglia. Initially, we measured the effect of LMB in different concentrations on the expression
of iNOS protein and mRNA in LPS-stimulated BV2 cells. As shown in Fig. 1, results of RT-PCR and Western analysis demonstrated that LMB concentration-dependently inhibited LPS-induced expression of iNOS protein (Fig. 1A) and iNOS mRNA (Fig. 1B) in BV2 cells. Interestingly, 10 ng/ml of LMB (18 nM) was sufficient to greatly down-regulate the LPS-induced iNOS protein and mRNA expressions, suggesting the potency of LMB in down-regulating iNOS. Collectively, these results demonstrate that LMB potently and strongly inhibits the expression of iNOS protein and mRNA by LPS in BV2 microglia.

Effects of LMB on iNOS transcription and iNOS mRNA stability in LPS-stimulated BV2 microglia. It has been well documented that induction of iNOS by extracellular stimuli such as LPS or cytokines correlates with increase in iNOS transcription and mRNA stability (11-13). This led us to test the effect of LMB on iNOS transcription and iNOS mRNA stability in LPS-stimulated BV2 cells using luciferase transfection assay with iNOS promoter/luciferase DNA constructs and actinomycin D chase experiments, respectively. As shown in Fig. 2A, LMB concentration-dependently reduced the expression of luciferase in LPS-stimulated BV2 cells. Treatment of LMB at 10 ng/ml concentration was found to inhibit about 50% iNOS transcription in LPS-stimulated BV2 cells. Data of actinomycin D chase experiments also demonstrated that presence of LMB accelerated the destabilization of iNOS mRNA in LPS-stimulated BV2 cells. Taken together, these data suggest that LMB may act at the levels of iNOS transcription and iNOS mRNA stability in response to LPS in BV2 cells.

Effects of LMB on nuclear export of iNOS mRNA in LPS-stimulated in BV2 microglia. LMB is a specific inhibitor of CRM1 (22), which mediates the delivery of cellular proteins or RNAs from the nucleus to the cytoplasm (23). Given that LMB inhibits LPS-induced iNOS expression in BV2 cells (Fig. 1), we hypothesized that in BV2 cells LPS might induce CRM1-dependent nuclear export of iNOS mRNA. Thus, we tested if it was inhibited by LMB. BV2 cells were initially pre-treated with LMB for 1 h and then exposed to LPS for 4 h. Cytoplasmic and nuclear RNA pools were prepared and used for RT-PCR analysis to measure subcellular distribution of iNOS mRNA. Unfortunately, we were not able to measure iNOS mRNA levels under these experimental conditions, because of a very low expression of iNOS mRNA in either total or subcellular RNA pools (data not shown). This is explained with the data of Fig. 1B wherein LMB pre-treatment almost completely inhibited iNOS mRNA expression by LPS, which might be correlated with iNOS transcriptional suppression by LMB as shown in Fig. 2A. This led us to perform an alternative experiment in which BV2 cells were primarily treated with LPS for 4 h to highly induce iNOS mRNA levels, following 4-h post-treatment with LMB and preparation of subcellular RNA fractions. Under this condition, in BV2 cells, LPS induced high expression of total iNOS mRNA (Fig. 3A, lane 3), cytoplasmic localization of a majority...
of iNOS mRNA in BV2 cells (Fig. 3B, lane 3), suggesting that iNOS mRNA transcribed following LPS stimulation is exported from the nucleus to the cytoplasm where translation occurs. Unlike the inhibitory effect of LMB pre-treatment on iNOS mRNA expression by LPS (Fig. 1B), however, 4-h post-treatment of LMB did not affect total amounts of iNOS mRNA that was induced by 4-h LPS treatment (Fig. 3A, lane 4). Of interest, LPS-induced subcellular distribution of iNOS mRNA was changed by 4-h post-treatment of LMB, which effectively blocked LPS-induced cytoplasmic localization of iNOS mRNA, retaining a majority of iNOS mRNA in the nuclear compartment (Fig. 3B, lane 4). Under same experimental conditions, cellular distribution of GAPDH mRNA was not affected by 4-h post-treatment of LMB, suggesting the specificity of LMB to inhibit nuclear export of iNOS mRNA in response to LPS. Collectively, these results suggest that LMB may inhibit LPS-induced iNOS expression in BV2 cells, not by ERKs, JNKs, p38 MAPK, and NF-κB signaling pathways activated by LPS.

Effects of LMB on activation of MAPKs and NF-κB by other iNOS inducer or in other type of cells. We further evaluated the effect of LMB on iNOS expression by LPS in Raw 264.7 murine macrophages (Raw 264.7 cells). As shown in Fig. 5A, LMB concentration-dependently inhibited the expression of iNOS by LPS in Raw 264.7 cells. In particular, 2.5 ng/ml of LMB mRNA in the nuclear compartment (Fig. 3B, lane 4). Under same experimental conditions, cellular distribution of GAPDH mRNA was not affected by 4-h post-treatment of LMB, suggesting the specificity of LMB to inhibit nuclear export of iNOS mRNA in response to LPS. Collectively, these results suggest that LMB may inhibit LPS-induced iNOS expression in BV2 cells, in part, by blockage of nuclear export of iNOS mRNA which may occur in a CRM1-dependent pathway.

Effects of LMB on activation by other iNOS inducer or in other type of cells. We further evaluated the effect of LMB on iNOS expression by LPS in Raw 264.7 murine macrophages (Raw 264.7 cells). As shown in Fig. 5A, LMB concentration-dependently inhibited the expression of iNOS by LPS in Raw 264.7 cells. In particular, 2.5 ng/ml of LMB mRNA in the nuclear compartment (Fig. 3B, lane 4). Under same experimental conditions, cellular distribution of GAPDH mRNA was not affected by 4-h post-treatment of LMB, suggesting the specificity of LMB to inhibit nuclear export of iNOS mRNA in response to LPS. Collectively, these results suggest that LMB may inhibit LPS-induced iNOS expression in BV2 cells, in part, by blockage of nuclear export of iNOS mRNA which may occur in a CRM1-dependent pathway.

Effects of LMB on activation of MAPKs and NF-κB in LPS-stimulated BV2 microglia. It is well known that LPS induces activation of various intracellular signaling proteins including NF-κB transcription factor, ERKs, JNKs, and p38 MAPK whose activation is critical for LPS-induced iNOS expression (17-20). This led us to test the effect of LMB on LPS-triggered activation of these signaling pathways. In this study, the degree of phosphorylation of MAPKs (ERKs, JNKs, and p38 MAPK) and of IκBα degradation, respectively, in response to LPS stimulation was assessed as an indicator of the activation of each MAPK and NF-κB. As anticipated, LPS treatment led to activation of ERKs, JNKs, and p38 MAPK and proteolytic degradation of IκBα in BV2 cells (Fig. 4A and B, lane 3). Notably, LMB treatment had no effect on LPS-induced activation of ERKs, JNKs, and p38 MAPK and proteolytic degradation of IκBα in BV2 cells (Fig. 4A and B, lane 4). These results indicate that LMB exert its inhibitory effect on LPS-induced iNOS expression in BV2 cells, not by ERKs, JNKs, p38 MAPK, and NF-κB signaling pathways activated by LPS.
was sufficient to almost completely down-regulate iNOS expression by LPS in Raw 264.7 cells (Fig. 5A, lane 5). Recently, we have reported that iNOS expression is highly increased by the exposure of catalase, an antioxidant enzyme, into Raw 264.7 cells (9). This led us to further test if LMB inhibits iNOS expression by catalase. As shown in Fig. 5B, LMB potently inhibited the expression of iNOS by catalase in Raw 264.7 cells. Together, these data strongly indicate that LMB inhibition of iNOS expression is not limited to LPS and BV2 cells.

Discussion

Excessive iNOS expression is associated with a variety of brain diseases, such as neuronal inflammation. Any compound that affects iNOS expression has the potential to be clinically useful against iNOS-associated neuronal diseases. In this study, we have evaluated the anti-inflammatory effect of LMB, an inhibitor of CRM1 nuclear export receptor, on the context of regulation of iNOS expression in response to LPS in BV2 microglial cells. Here we demonstrate, for the first time, that LMB potently inhibits LPS-induced iNOS expression in BV2 cells and the inhibition is, in part at least, associated with the ability of LMB to inhibit iNOS mRNA nuclear export, mRNA stability, and transcription.

It has been reported that the primary cellular target of LMB is CRM1, which mediates delivery of cellular RNAs or proteins containing NES (22). Supporting this, recent studies have shown that LMB interferes with nuclear export of RNAs, such as c-fos (24) or COX-2 (27), and of NES-containing proteins, such as α-catenin (25) or IκB-α (26). Given that CRM1 mediates nuclear trafficking of cellular RNAs and/or proteins and LMB inactivates CRM1, it is believed that LMB may be a useful experimental tool to understand regulation of gene expression in the context of RNA nuclear export. Timely nuclear export of RNA upon transcription into the cytoplasm where translation occurs is critical for gene expression (28). Presently, there is no report as to the relationship between LMB and iNOS mRNA nuclear export as well as iNOS expression. In this study, through RNA fractionation experiments, we have demonstrated that LPS induces cytoplasmic localization of iNOS mRNA in BV2 cells, but LMB inhibits the process, rather causing nuclear retention of iNOS mRNA. Given that LMB is an inhibitor of CRM1 (22), it is likely that iNOS mRNA nuclear export induced by LPS in BV2 cells is CRM1-dependent. To our knowledge, this is the first report showing LMB regulation of iNOS expression at level of iNOS mRNA nuclear export, a novel post-transcriptional regulation of iNOS expression by LMB. It has been shown that LMB inhibition of CRM1-mediated export pathway affects the expression levels of <2% of detectable mRNAs in Drosophila Schneider (S2) cells (29). Thus, our present data showing that LMB did not affect nuclear export and subcellular distribution of GAPDH mRNA in response to LPS in BV2 cells may suggest the specificity of LMB to block iNOS mRNA nuclear export in response to LPS in BV2 cells.

Besides the LMB inhibitory effect on iNOS mRNA nuclear export, in this study, we have shown that LMB partially inhibits iNOS transcription by LPS in BV2 cells. It is well established that iNOS transcription largely depends on the activities of transcription factors, including NF-κB, activator protein-1, NF-IL6, or IFN-γ responsive factor, which individually binds its cognate cis-acting elements within the iNOS promoter (11-13). Among these, activation of NF-κB by LPS is linked to proteolytic degradation of IκB-α, an inhibitor of NF-κB (30). Interestingly, IκB-α is reported to have NES moieties and thus able to shuttle between the cytoplasm and the nucleus (31). Indeed, it has been demonstrated that LMB causes nuclear retention of IκB-α, which protects pro-inflammatory cytokines IL-1β- or TNF-α-induced degradation of IκB-α and inhibits the signal-induced NF-κB activation in HeLa cells (32). However, in this study, we observed that LMB has no effect on subcellular distribution of IκB-α (data not shown) and LPS-induced degradation of IκB-α in BV2 cells, suggesting that LMB inhibition of LPS-induced iNOS transcription may be unrelated to NF-κB. Thus it is likely that LMB may affect other transcriptional activators or repressors whose activities or nuclear exporting are necessary for iNOS transcription. We also showed that iNOS mRNA induced by LPS in BV2 cells is unstable in the presence of LMB, as determined by actinomycin D chase experiments. These data suggest that LMB may exert its inhibitory effect on iNOS expression at the levels of transcription and post-transcription such as mRNA stability and nuclear export.

Accordingly, iNOS expression is shown to be strongly related with activation of PI3K, AKT, Ras, Raf, ERKs, p38s, and/or JNKs (17-20,33). Timely localization of some cytoplasmic signaling proteins or transcription factors into the nucleus where gene transcription occurs is a pivotal step...
in the activation of gene expression in response to extracellular signals (34). ERKs, which locate to the cytoplasm in quiescent cells, translocate to the nucleus upon treatment with extracellular stimuli (35). LMB was shown to inhibit the nuclear export of ERKs (35) and MEK-1/2, the upstream activator of ERKs (36). Inconsistent with this, we found that LMB does not affect nuclear localization (data not shown) and activation of ERKs, p38s, or JNKs induced by LPS in BV2 cells. It is, therefore, unlikely that LMB may exert its inhibitory effect on LPS-induced iNOS expression in BV2 cells through modulation of MAPKs.

An interesting result in the present study is that with similar potency LMB can suppress iNOS expression by LPS or catalase, an inducer of iNOS (9), also in Raw 264.7 cells, indicating that LMB inhibition of iNOS expression is not limited to LPS and BV2 cells.

In conclusion, we demonstrate the potency and strength of LMB to inhibit the expression of iNOS induced by LPS in BV2 microglia. LMB may exert its inhibitory effect on LPS-induced iNOS expression in BV2 microglia, at least in part, by down-regulating iNOS transcription, reducing LPS-induced iNOS expression in BV2 cells. It is, therefore, unlikely that LMB may exert its inhibitory effect on LPS-induced iNOS expression in BV2 cells through modulation of MAPKs.

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