Inhibition of Extracellular Signal-Regulated Kinase Suppresses Endotoxin-Induced Nitric Oxide Synthesis in Mouse Macrophages and in Human Colon Epithelial Cells

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ABSTRACT

Macrophages produce large amounts of nitric oxide (NO) in response to proinflammatory cytokines and lipopolysaccharide (LPS) by expressing inducible isof orm of NO synthase (iNOS). We examined the role of extracellular signal-regulated kinase (p42/44 MAPK) (Erk1/2) in signal transduction pathways leading to induction of NO synthesis in response to LPS in J774 mouse macrophages and T-84 human colon epithelial cells. LPS activated Erk1/2 and induced iNOS and subsequent NO production. Erk1/2 activation was inhibited by PD 98059, a specific inhibitor of mitogen-activated protein kinase kinase (Mek) that is an upstream activator of Erk1/2. At corresponding concentrations PD 98059 reduced LPS-induced NO formation by 40 to 50% by inhibiting iNOS expression in J774 and T-84 cells. Inhibition of iNOS expression was not mediated by nuclear factor-κB because PD 98059 had no effect on nuclear factor-κB activity in J774 macrophages. In addition, PD 98059 reduced LPS-induced L-arginine transport into the cells as measured in J774 macrophages, whereas the availability of tetrahydrobiopterin was not a limiting factor in NO production after PD 98059. Our results indicate that Erk1/2 activation mediates up-regulation but is not essential for LPS-induced iNOS expression.

Nitric oxide (NO) is a short-lived, highly reactive gas that acts as a signaling molecule in various physiological and pathophysiological processes in the human body. In infection and inflammation NO serves as a cytotoxic and cytostatic agent and modulates immune response (Moilanen et al., 1999). NO is generated from L-arginine by a family of NO synthase (NOS) enzymes (Knowles and Moncada, 1994). In macrophages and other inflammatory cells NO is generated in high amounts for a prolonged time by inducible NOS (iNOS) in response to lipopolysaccharide (LPS) and various cytokines (MacMicking et al., 1997).

LPS activates monocytes and macrophages by forming a complex with serum LPS-binding protein, which binds to membrane glycosphatidilinositol-anchored protein CD14 (Wright et al., 1990). Activation of monocytes and macrophages by LPS leads to activation of protein tyrosine kinases (Stefanova et al., 1993), protein kinase C (PKC) (Paul et al., 1995), and subsequent signaling cascades, resulting in expression of various inflammatory genes.

Extracellular signal-regulated kinase p42/p44 (Erk 2 and 1, respectively) belongs to a group of serine/threonine-specific mitogen-activated protein kinases (MAPKs). Erk1/2 is activated by various extracellular stimuli, including LPS through kinase cascade Raf-1 → mitogen-activated protein kinase kinase-1 (Mek-1) → Erk1/2. This kinase cascade connects the extracellular signal to intracellular transcription factors and other regulatory proteins, thus participating in regulation of gene expression (Su and Karin, 1996). LPS-induced activation of Raf-1 → Mek-1 → Erk1/2 pathway has been shown to be tyrosine kinase-dependent (Weinstein et al., 1992; Reimann et al., 1994), Ras-independent, and to involve PKC activation (Buscher et al., 1995). Tyrosine kinases and PKC also are involved in the LPS-induced signal transduction pathways leading to iNOS expression and NO production in macrophages (Akarasereenont et al., 1994; Fujihara et al., 1994; Paul et al., 1995). Therefore, we hypothesized that Erk1/2 could be a part of the signaling mechanisms involved in LPS-induced iNOS expression and NO
synthesis. We used the specific inhibitor of Mek-1 activation, PD 98059 (Alessi et al., 1995; Dudley et al., 1995), to test this hypothesis. The results show that Erk1/2 augments LPS-induced NO-production by enhancing iNOS expression and l-arginine transport.

Experimental Procedures

Materials. Reagents were obtained as follows: PD 98059 (Calbiochem, La Jolla, CA), ammonium pyrrolidinedithiocarbamate (PDTc) (Tocris Cooks, Bristol, UK), rabbit polyclonal mouse and human iNOS and goat anti-rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and PhosphoPlus p44/42 MAP kinase (Thr-202/Tyr-204) antibody kit (New England Biolabs Inc., Beverly, MA). Sepiapterin was from Alexis Corporation (Lülfelfingen, Switzerland). N-monomethyl-l-arginine (l-NMMA) was purchased from Clinalfa (Lülfelfingen, Switzerland). All other reagents were from Sigma (St. Louis, MO).

Cell Culture. J774 macrophages and human T-84 colon epithelial cells were cultured at 37°C, 5% CO₂ atmosphere, in Dulbecco’s modified Eagle’s medium with glutamax-I containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Gibco BRL, Paisley, UK). Cells were seeded in 24-well plates for nitrite and cytokine measurement, and 250 ng/ml amphotericin B (all from Gibco BRL, Paisley, MA). Sepiapterin was from Alexis Corporation (Lülfelfingen, Switzerland). N-monomethyl-l-arginine (l-NMMA) was purchased from Clinalfa (Lülfelfingen, Switzerland). All other reagents were from Sigma (St. Louis, MO).

Preparation of Cell Lysates. At indicated time points the culture medium was collected for nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1% naphthylethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% H₃PO₄) and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

Preparation of Nuclear Extracts. J774 macrophages were seeded on 10-cm dishes and grown for 72 h to confluency before experiments. Cells were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1mM EGTA, 1 mM Na₂VO₄, 1 mM NaF) and incubated for 20 min on ice. The nuclei were vortexed 30 s and nuclei extracts were obtained by centrifugation at 4°C and 15,000 rpm for 10 min. Nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.1mM EGTA, 1 mM Na₂VO₄, 1 mM NaF) and incubated for 20 min on ice. Nuclei were vortexed 30 s and nuclear extracts were obtained by centrifugation at 4°C and 15,000 rpm for 2 min. Protein content of the nuclear extracts were measured by Coomassie blue method (Bradford, 1976).

Electrophoretic Mobility Shift Assay. Single-stranded oligonucleotides (5'-AGTTAGGGGACTTTCCAGGC-3', 3'-TCAACTC-CCCTGAAAGGGTGCG-5'; Amersham Pharmacia Biotech, Piscataway, NJ) containing the nuclear factor-κB (NF-κB)-binding sequences were annealed and 5'-32P-end labeled with DNA 5'-End Labeling kit (Boehringer Mannheim, Indianapolis, IN). For binding reactions 10 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl, and 10% glycerol for 20 min at room temperature. Then 0.2 ng of 32P-labeled oligonucleotide probe was added and the reaction mixture was incubated for 10 min. Protein/DNA complexes were separated from DNA probe by electrophoresis on a native 4% polyacrylamide gel. Gel was dried and autoradiographed with intensifying screen at ~70°C.

Statistics. Data are expressed as mean ± S.E. When indicated, statistical significance was calculated by ANOVAs supported by Dunnett’s adjusted significance levels. Differences were considered significant at P < .05.

Results

LPS Activates Erk1/2 and Induces Expression of iNOS in J774 Macrophages. The ability of LPS to activate Erk1/2 was studied by Western blot analysis with an antibody directed against Thr-202/Tyr-204-phosphorylated p42/44MAPK. Erk1/2 activation was seen 10 min after addition of LPS (Fig. 1A). The activation peaked at 15 min and reduced thereafter. LPS activated Erk1/2 in a concentration-dependent manner, the activation being detectable at an LPS concentration of 10 ng/ml (Fig. 1B). Western blot analysis of iNOS protein was carried out to investigate whether LPS was able to induce expression of iNOS. Figure 2 shows that LPS (10 ng/ml)-induced iNOS expression that could be detected 4 h after stimulation. iNOS expression was maximal after 12 h and remained at a high level up to 24 h.

PD 98059 Inhibits LPS-Induced Erk1/2 Activation and Reduces NO Production. PD 98059 is a specific inhibitor of Mek-1 activation, with Mek-1 being an upstream activator of Erk1/2 (Alessi et al., 1995; Dudley et al., 1995). The concentration of PD 98059 required for Mek-1 inhibition is dependent on the strength of stimulation (Alessi et al.,
The highest concentration of PD 98059 (10 μM) used in the following experiments completely inhibited the activation of Mek-1, even when stimulated maximally by LPS (Fig. 3), but is low enough to exclude the possibility of unspecific inhibition of other kinases (Alessi et al., 1995; Dudley et al., 1995). To investigate the effect of PD 98059 on LPS-induced NO production, J774 macrophages were incubated with or without PD 98059 and stimulated with LPS (10 ng/ml). PD 98059 reduced NO production in a concentration-dependent manner, maximal reduction being 50% with the highest concentration used (10 μM; Fig. 4A). The inhibitory effect was present but the percentage inhibition was lower when cells were stimulated with increasing concentrations of LPS (Fig. 4B), and was 30% when LPS was given at a concentration of 100 ng/ml.

**PD 98059 Inhibits iNOS Expression.** To further evaluate the mechanism by which PD 98059 affects NO generation, the compound was added to J774 cells before or 6 h after LPS. In contrast to the inhibitory effect seen when added before stimulation, PD 98059 did not inhibit NO production when added 6 h after LPS (Fig. 5), a time point at which iNOS protein was already present in J774 cells but no detectable amounts of nitrite had been produced (n = 6; data not shown). Nitrite did not accumulate in the growth medium of cells treated with L-NMMA, cycloheximide, or PDTC (Fig. 5), indicating that nitrite was derived from NO generated by NOS and that the induction of NOS required de novo protein synthesis and involved activation of transcription factor NF-κB. These are features of iNOS (Nathan and Xie, 1994).
Fig. 5. Effect of PD 98059 on nitrite accumulation when added before or after stimulation with LPS. J774 macrophages were incubated with cycloheximide (1 μg/ml), PDTC (100 μM), or L-NMMA (2 mM) 30 min before stimulation with LPS (10 ng/ml). PD 98059 (10 μM) was added in growth medium 30 min before or 6 h after stimulation with LPS (10 ng/ml). After 24 h of incubation nitrite accumulation was measured from growth medium. Values are mean ± S.E. (n = 6). **P < .01 and ***P > .05 compared with respective control.

Furthermore, unstimulated cells did not produce detectable amounts of nitrite. Thus, the results suggest that PD 98059 reduces NO production by inhibiting expression of iNOS or by having an effect on another inducible element of NO production. To test this hypothesis we continued by investigating the effect of PD 98059 on iNOS expression. Western blot analysis showed that PD 98059 (10 μM) reduced iNOS expression (Fig. 6A). Densitometric evaluation of Western blots of five different cell preparations showed a mean of 30% reduction in the intensity of iNOS band (Fig. 6B). Cycloheximide and PDTC inhibited iNOS expression completely.

Effect of PD 98059 on NO Production Did Not Result from Reduced Availability of Tetrahydrobiopterin (BH4). NOS requires BH4 as a cofactor in NO synthesis (Tayeh and Marletta, 1989). GTP cyclohydrolase I is the first enzyme in the pathway for de novo synthesis of BH4 and is induced by cytokines and LPS in many cell types, including macrophages (Werner et al., 1989; Simmons et al., 1996). Sepiapterin was added to growth medium of J774 macrophages to investigate whether the reduction in NO synthesis by PD 98059 was a result of reduced BH4 availability. Sepiapterin is converted to BH4 by the pterin salvage pathway, to increase cellular BH4 levels (Werner et al., 1989). Addition of sepiapterin slightly increased LPS-induced nitrite accumulation in growth medium (Fig. 7). However, addition of sepiapterin did not reverse the PD 98059-induced inhibition of NO production. Thus, the results suggest that the effect of PD 98059 is not due to inhibition of BH4 synthesis.

PD 98059 Reduces α-Arginine Transport in J774 Macrophages. LPS and cytokine stimulation facilitates arginine transport into many cell types and the rate of arginine transport can be a rate-limiting factor in NO synthesis (Bogle et al., 1992). Facilitated arginine transport has been shown to result from increased synthesis of cationic amino acid transporter molecules (Simmons et al., 1996). We investigated whether the effect of PD 98059 on NO production could be explained by reduced rate of arginine transport in J774 macrophages. Stimulation by LPS increased the rate of arginine transport in macrophages, the rate of transport being 50% higher in cells treated with 1 μg/ml LPS compared with unstimulated cells 10 h after addition of LPS (Fig. 8A). As measured 10 h after addition of LPS, PD 98059 reduced arginine transport slightly in cells stimulated with 1 μg/ml LPS, but no difference could be seen in cells stimulated with 10 ng/ml LPS (Fig. 8B). At 24 h PD 98059 reduced arginine transport by 12 to 18% both in cells stimulated with low (10 ng/ml) and high (1 μg/ml) LPS. For comparison, cycloheximide reduced arginine transport by 60%, indicating requirement for de novo protein synthesis to sustain normal levels of arginine transport (data not shown). Thus, PD 98059 inhibits arginine transport into J774 macrophages and reduces the availability of α-arginine, the precursor of NO production.

PD 98059 Has No Effect on NF-κB DNA-Binding Activity. NF-κB is an essential transcription factor in LPS-

Fig. 6. Effect of PD 98059 on LPS-induced iNOS expression. A, J774 macrophages were incubated with PD 98059 (10 μM), cycloheximide (1 μg/ml), or PDTC (100 μM) 30 min before stimulation with LPS (10 ng/ml). Incubations were terminated after 12 h. Immunoblots were run with antibody against mouse iNOS. B, densitometric measurements were made from immunoblots of five different cell preparations. Values are expressed as percentage from control, mean ± S.E. *P < .05 compared with control without PD 98059.

Fig. 7. Effect of sepiapterin on nitrite accumulation in LPS-stimulated J774 macrophages in the presence or absence of PD 98059. J774 macrophages were incubated with PD 98059 (10 μM) 30 min before stimulation with LPS (10 ng/ml) in the presence or absence of sepiapterin (100 μM). After 24 h of incubation accumulated nitrite was measured from growth medium. Values are mean ± S.E. (n = 6). **P < .01 and ***P > .05 compared with the respective control without PD 98059.
induced iNOS expression (Kim et al., 1997). Therefore, we investigated whether the inhibitory effect of PD 98059 on iNOS expression is due to reduction of NF-κB activity. As studied by gel shift assays with an oligonucleotide probe containing a specific NF-κB-binding sequence, LPS caused a marked increase in NF-κB-binding activity compared with untreated cells (Fig. 9). PD 98059 treatment (10 μM) had no effect on LPS-induced NF-κB activity, whereas PDTC (100 μM) reduced LPS-induced NF-κB activity almost to control levels. These results suggest that PD 98059 does not inhibit iNOS expression at the level of NF-κB activation and DNA-binding activity.

PD 98059 Reduced LPS-Induced NO Production and iNOS Expression in T-84 Human Colon Epithelial Cells. We used T-84 human colon epithelial cells to test whether the results obtained in J774 macrophage also are applicable to human cells. LPS (1 μg/ml) induced iNOS expression and subsequent NO production in T-84 cells, which could be inhibited by cycloheximide (1 μg/ml; Fig. 10). PD 98059 (10 μM) caused a marked inhibition in iNOS expression (Fig. 10B), which resulted in 40% inhibition in NO production (Fig. 10A).

**Fig. 8.** Effect of PD 98059 on arginine transport in J774 macrophages. A, cells were stimulated with 10 or 1000 ng/ml LPS. At indicated time points arginine transport was measured by uptake of L-[14C]arginine as described under Experimental Procedures. B, cells were incubated with PD 98059 (10 μM) 30 min before stimulation with 10 ng/ml (●) or 1000 ng/ml (□) LPS. At indicated time points arginine transport was measured as described under Experimental Procedures. CPM; measured radioactivity from cell lysates. Values are mean ± S.E. (n = 6). **P < .01, *P < .05, and nsP > .05 compared with the respective control.

Fig. 9. Effect of PD 98059 on LPS-induced NF-κB activation. J774 macrophages were incubated with PD 98059 (10 μM) or PDTC (100 μM) 30 min before stimulation with LPS (10 ng/ml). Incubations were terminated 30 min after addition of LPS. NF-κB DNA-binding activity was analyzed by electrophoretic mobility shift assay. The data are representative of two separate experiments that gave similar results.

**Discussion**

Erk1/2 is involved in the regulation of cell cycle and differentiation in various cell types. These data extend the function of Erk1/2 to the regulation of iNOS expression and NO production in activated macrophages and colon epithelial cells.

Consistent with the previous findings with other macrophage cell lines (Weinstein et al., 1992; Reimann et al., 1994; Buscher et al., 1995) our results show that LPS stimulation leads to a rapid activation of Erk1/2 in J774 macrophages and these events are followed by iNOS expression and NO production. Inhibition of Erk1/2 activation by PD98059 resulted in a reduction in the amount of NO produced. However, when PD 98059 was added 6 h after LPS, it did not affect NO production. This suggested that PD 98059 did not inhibit iNOS activity, but rather reduced the induction of iNOS expression or some other inducible element in NO synthesis. Western blot analysis showed that PD 98059 reduced LPS-induced iNOS expression. Other inducible components needed for the production of NO include the cofactor BH₄ and L-arginine transporter molecules (Simmons et al., 1996). Our results indicate that PD 98059 does not reduce de novo synthesis and availability of BH₄ to a significant degree. On the contrary, PD98059 reduced l-arginine transport by 12 to 18%. This finding is supported by previous findings (Caivano, 1998) on the inhibitory effect of PD98059 on arginine transport. The rate of l-arginine transport via inducible cationic amino acid transporter molecules may be a rate-limiting factor in the production of NO (Shibazaki et al., 1996). Together, the results suggest that Erk1/2 is involved in the up-regulation of both iNOS protein and the rate of l-arginine transport in LPS-stimulated macrophages.
Activation of NF-κB is critical for iNOS induction (MacMicking et al., 1997) and also in these experiments PDTC (an inhibitor of NF-κB) totally inhibited iNOS expression and NO production. Our results show that PD 98059 did not have any effect on the activation and DNA-binding activity of NF-κB, even though some interactions between Mek kinase-1 and I-κB kinases have been previously documented (Yin et al., 1998). Two other transcription factors in iNOS promoter region, activator protein-1 and C/EBPβ (CCAAT/enhancer binding protein β, NF-IL-6), have been reported to be controlled by Erk1/2. Erk1/2 activates expression of c-fos, a component of heterodimer activator protein-1 (Su and Karin, 1996) and Erk1/2 tyrosine phosphorylates C/EBPβ, increasing its transactivating activity (Nakajima et al., 1993). Splenic macrophages from C/EBPβ-deficient mice have impaired NO production (Screpanti et al., 1995), suggesting a functional role for C/EBPβ in iNOS induction. In addition to regulation of transcription there is also evidence that Erk1/2 might be involved in the regulation of translation. PHAS-I is a protein that binds to translation initiation factor 4E (eIF-4E) and inhibits its function, thus limiting translation (Pause et al., 1994). Erk1/2 phosphorylates PHAS-I, which inhibits the binding of PHAS-I to translation initiation factor 4E (Lin et al., 1994). Inhibition of Erk1/2 by PD 98059 may thus suppress either iNOS transcription or translation. Newly discovered toll-like receptors, which mediate some responses of LPS, offer another possible explanation to our results at the level of LPS binding because it has been shown recently that PD 98059 inhibits expression of toll-like receptor 2 in T lymphocytes (Matsuguchi et al., 2000).

Although PD 98059 is regarded to be a specific inhibitor of Mek-1 activation, it has been reported to also inhibit aryl hydrocarbon receptor (AhR) (Reiners et al., 1998) independently of inhibition of Erk1/2. According to our results 2,3,7,8-tetrachlorodibenzo-p-dioxin, which activates AhR, did not induce NO production in J774 macrophages and it also had no effect on LPS-induced NO production (A. Lahti, unpublished data). Therefore, it is unlikely that inhibition of NO production by PD 98059 results from inhibition of AhR.

In conclusion, we have shown in this study that Erk1/2 pathway mediates up-regulation but is not essential for LPS-induced iNOS expression and subsequent NO production in J774 macrophages and T-84 epithelial cells. Furthermore, Erk1/2 has a minor regulatory role in inducible t-arginine transport in J774 macrophages, which may contribute to the suppressive effect on NO production by Erk1/2 inhibition. Both of these findings suggest that Raf-1 → Mek-1 → Erk1/2 is an important signaling pathway in LPS-induced cell activation.

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