Heparin-Binding Epidermal Growth Factor-Like Growth Factor Inhibits Cytokine-Induced NF-κB Activation and Nitric Oxide Production via Activation of the Phosphatidylinositol 3-Kinase Pathway

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*J Immunol* 2005; 175:1911-1918; doi: 10.4049/jimmunol.175.3.1911

http://www.jimmunol.org/content/175/3/1911
Heparin-Binding Epidermal Growth Factor-Like Growth Factor Inhibits Cytokine-Induced NF-κB Activation and Nitric Oxide Production via Activation of the Phosphatidylinositol 3-Kinase Pathway

Veela B. Mehta* and Gail E. Besner2*†

NO produced by inducible NO synthase (iNOS) has been implicated in various pathophysiological processes including inflammation. Therefore, inhibitors of NO synthesis or iNOS gene expression have been considered as potential anti-inflammatory agents. We have previously demonstrated that heparin-binding epidermal growth factor (HB-EGF)-like growth factor (HB-EGF) decreases proinflammatory cytokine IL-8 and NO production in cytokine-stimulated intestinal epithelial cells by interfering with the NF-κB signaling pathway. However, the upstream signaling mechanisms involved in these responses have not yet been defined. In this report, we show that in intestinal epithelial cells, HB-EGF triggered PI3K-dependent phosphorylation of Akt. Inhibition of PI3K reversed the ability of HB-EGF to block NF-κB activation, expression of iNOS, and NO production. Small interfering RNA of PI3K also reversed the inhibitory effect of HB-EGF on iNOS expression. Alternatively, transient expression of constitutively active PI3K decreased NO production by ~2-fold more than treatment with HB-EGF alone. This PI3K effect was HB-EGF dependent. Thus, activation of PI3K is essential but not sufficient for decreased NO synthesis. PI3K and HB-EGF act synergistically to decrease NO synthesis. Neither overexpression or inhibition of MEK, Ras, or Akt affected HB-EGF-mediated inhibition of NF-κB activation. These data demonstrate that HB-EGF decreases proinflammatory cytokine-stimulated NF-κB activation and NO production via activation of the PI3K signaling pathway. These results also suggest that inhibition of NF-κB and activation of the PI3K-dependent signaling cascade by HB-EGF may represent key signals responsible for the anti-inflammatory effects of HB-EGF. The Journal of Immunology, 2005, 175: 1911–1918.

Cytokines are produced on activation of a number of different cell types and act as potent regulatory molecules of the immune system. Proinflammatory cytokines such as IL-1β, IFN-γ, and TNF-α stimulate cells in the intestine to increase the expression of inducible NO synthase (iNOS). Enhanced expression of iNOS leading to sustained overproduction of NO has been implicated in the pathophysiology of several types of intestinal disease states including the breakdown of intestinal barrier function after intestinal injury and the development of inflammatory bowel disease, including Crohn’s disease and ulcerative colitis (1, 2). Additionally, inflammatory mediators play an important role in the development of necrotizing enterocolitis (3).

Characterization of the intracellular signaling pathways that transduce signals from the cell surface to the nucleus for the induction of iNOS expression is important for the identification of novel targets for therapeutic intervention in NO-mediated inflammatory diseases of the intestine. PI3K and MAPK are key signaling molecules implicated in the regulation of a wide variety of biological responses including cell survival, mitogenesis, and cell migration (4, 5).

PI3K, a dual protein and lipid kinase, is a heterodimer composed of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85). The activated receptor binds PI3K, which then phosphorylates phosphatidylinositol, catalyzing the formation of phosphatidylinositol 3,4,5-triphosphate as the second messenger, using phosphatidylinositol 4,5-bisphosphate as the substrate. This activates downstream signaling molecules such as Akt. Activated Akt then dissociates from the membrane to act on its targets in the cytosol and the nucleus (6, 7).

Recently, it has been shown that inhibition of PI3K leads to overexpression of iNOS in macrophages, glial cells, and murine astrocytes stimulated with LPS or proinflammatory cytokines (8–10). The expression of iNOS is regulated by NF-κB (11, 12). The activation of NF-κB by cytokines involves the phosphorylation and degradation of IkBa, leading to the translocation of p50/p65 heterodimeric NF-κB to the nucleus where it regulates a large number of genes involved in the production of inflammatory mediators such as cytokines, iNOS, adhesion molecules, and chemokines (13, 14).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family that was originally identified in the conditioned medium of cultured human macrophages (15). This soluble form of HB-EGF is derived from the processing of a 22-kDa plasma membrane-anchored precursor protein (16). HB-EGF differs from EGF in that it has a high affinity for heparin, which increases its biological...
activity (17). We have recently shown that HB-EGF inhibits activation of NF-κB in intestinal epithelial cells, whereas EGF has been reported to activate NF-κB in some cells (18, 19). HB-EGF is a potent mitogen and chemotactic factor for various cell types including smooth muscle cells, fibroblasts, hepatocytes, keratinocytes, and gastrointestinal epithelial cell lines (15, 17, 20). Many cell types, including epithelial cells, skeletal muscle cells, monocytes, keratinocytes, and lymphoid cells express HB-EGF (21). We have demonstrated that HB-EGF acts as an intestinal cytoprotective agent (22). HB-EGF promotes cell survival of dopaminergic neurons (23), renal epithelial cells (24), and hepatoma cells (25) in addition to intestinal epithelial cells (26).

We have previously shown that HB-EGF pretreatment decreases IL-1β- and IFN-γ-stimulated iNOS expression and production of NO and IL-8 in HT-29 and DLD-1 colonic epithelial cells by interfering with the NF-κB signaling pathway (11, 27). In view of the inhibitory effects exerted by HB-EGF on NF-κB activation, we have now investigated the signaling mechanisms of HB-EGF-induced inhibition of NF-κB activation and NO production. In this report, we describe activation of the PI3K signaling pathway by HB-EGF and demonstrate that inhibition of this pathway reverses the inhibitory effects of HB-EGF on NF-κB activation and NO production. Moreover, transient expression of constitutively active PI3K decreased NO production ~2-fold more than treatment with HB-EGF alone. This PI3K effect was HB-EGF dependent. Thus, HB-EGF and PI3K act synergistically to decrease NO synthesis.

Materials and Methods

Materials

RAW 264.7 mouse macrophage cells and DLD-1 human colonic epithelial cells were obtained from American Type Culture Collection. DMEM was from BioWhittaker. FBS was from Invitrogen. Cell-signaling inhibitors used included the PI3K inhibitors LY294002 (20 μM) and wortmannin (30–300 nM), the MEK inhibitor PD98059 (20 μM), and the EGFR tyrosine kinase inhibitors PD153035 (500 μM) and AG1478 (500 nM). All inhibitors as well as anti-iNOS Ab were purchased from EMD Biosciences. Phospho-stat (serine-727) Ab, IL-1β, and IFN-γ were from BioSource International. Recombinant human HB-EGF corresponding to aa 74–148 of the mature protein was produced in Escherichia coli and purified in our laboratory as previously described (28). Abs to NF-κB (p65), phospho- and total IκBα were from Santa Cruz Biotechnologies. Akt, ERK1/2, and phospho-stat 1 (tyrosine-701) Abs were from Cell Signaling Technology. LPS from Salmonella typhimurium was from EMD Biosciences. β-Actin Ab was from Sigma-Aldrich. Superfect transfection reagent was from Qiagen.

Constitutively active human PI3K construct (p110α, HA-Akt wild type, and HA-Akt-179M (kinase dead) (29)) were kindly provided by Dr. A. Klippen (atugen, Berlin, Germany), Dr. M. Weber (University of Virginia, Charlottesville, VA), Dr. M. Greenberg (Children’s Hospital, Boston, MA). Constitutively active (MEK-R4F) and dominant negative (MEK-SE) MEK constructs and PCEP4 vectors (30) were provided by N. Ahn (University of Colorado, Boulder, CO), and Dr. R. Roth (Stanford University School of Medicine, Stanford, CA). Dr. K. Irani (John Hopkins University, Baltimore, MD) provided CMV-Ras-N17 (inhibitory Ras) and active Ras (RasV12) (31). Small interfering RNAs (siRNA) were purchased from Dharmacon.

Cell culture

DLD-1 cells were cultured in DMEM supplemented with 2 mM t-glutamine, 1 mM sodium pyruvate, and 5% FBS. Cells were maintained at 37°C in an atmosphere of 7.5% CO2. RAW 264.7 mouse macrophage cells were maintained in DMEM supplemented with 2 mM t-glutamine and 5% FBS at 37°C in 5% CO2 humidified air. When cells were treated with inhibitors, the inhibitors were added 30 min before the addition of a stimulant (cytokines and/or HB-EGF) and were maintained for the duration of the experiment without removal.

Western blot analysis

Cells were lysed in buffer containing 50 mM Tris-Cl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 2 μg/ml protease inhibitors. Clarified cytosolic extracts were subjected to SDS-PAGE followed by Western blot analysis using specific Abs. Protein bands were detected with ECL detection reagents (Amersham Biosciences). To confirm equal protein loading, membranes were stripped and probed with either a 1/3000 dilution of total ERK 1/2 or total Akt, or a 1/10,000 dilution of anti-β-actin Ab.

Preparation of nuclear lysates and EMSA

Nuclear extracts were prepared as described (11) using the method of Dignam et al. (32). Briefly, serum starved cells were stimulated for 30 min and lysed in buffer A (10 mM HEPES (pH 7.9), 0.2 mM EDTA, 10 mM KCl, 2 mM MgCl2, 0.5 mM DTT, and 5 μg/ml protease inhibitors) containing 0.2% Nonidet P-40. Cytoplasmic proteins were removed by centrifugation, and nuclear pellets were suspended in buffer A containing 0.35 M NaCl, incubated on ice for 30 min, and clarified at 14,000 x g for 15 min. NF-κB DNA-binding activity was determined by EMSA using the Promega gel shift assay system. DNA–protein complexes were resolved on 4% non-denaturing polyacrylamide gels. The gels were dried and subjected to autoradiography.

Assay for NO

Cells were cultured and stimulated with cytokines as described (11). Briefly, 24 h after plating, some wells received HB-EGF (50 ng/ml) for 48 h. Cells were then treated with HB-EGF alone, IL-1β (20 ng/ml), and IFN-γ (10 ng/ml) alone, or a combination of IL-1β and IFN-γ plus HB-EGF in the presence or absence of inhibitors as indicated, and incubated for an additional 24 h. Culture supernatants were collected for NO measurements. Synthesis of NO was measured as the accumulation of nitrite/nitrate in the medium using a Sievers Model 280 nitric oxide analyzer. NO concentrations were calculated from a standard curve derived from NaNO3.

Immunochemistry for NF-κB localization

DLD-1 cells were stained with anti-p65 Ab (NF-κB subunit) as previously described (11). Briefly, DLD-1 cells were fixed in 3% parafomaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 2% BSA and 2% goat serum in PBS for 1 h. Cells were incubated in a 1/600 dilution of anti-p65 Ab (NF-κB subunit) followed by biotinylated secondary Ab (1:200 dilution; Vector Laboratories) for 1 h at room temperature. Cells were incubated with Rhodamine Red-X-conjugated streptavidin for 15 min, washed, mounted, and viewed with a Zeiss LSM 510 META confocal imaging system.

Plasmids and transfections

DLD-1 cells were transiently transfected with plasmids (1 μg) or siRNA (50 nM) in 24-well plates using the Superfect reagent according to the manufacturer’s instructions. Cells were allowed to recover for 24 h after transfection and were then starved for 24 h before cytokine stimulation. Where indicated, HB-EGF was added during starvation and was present during the rest of the experimental period.

Statistical analysis

Statistical analyses were performed using Student’s paired t test, with p < 0.05 considered significant. Data are expressed as the means ± SE.

Results

HB-EGF activates both PI3K- and ERK1/2-signaling pathways in DLD-1 cells

We initially identified the signaling pathways that are activated by HB-EGF in DLD-1 cells. We examined activation of Akt, because this pathway has been implicated in cell survival and inhibition of iNOS expression. HB-EGF addition led to rapid phosphorylation of Akt (Fig. 1A, lane 2), which was inhibited by the PI3K inhibitor LY294002 (Fig. 1A, lane 5) but not by the MEK inhibitor PD98059 (Fig. 1A, lane 8), suggesting that HB-EGF-induced activation of Akt is PI3K dependent. A combination of IL-1β and IFN-γ did not activate Akt (Fig. 1A, lane 3). Wortmannin, another PI3K inhibitor, showed effects similar to those of LY294002 (data not shown).

Addition of HB-EGF rapidly led to activation of ERK1/2 (Fig. 1B, lane 2). Increased phosphorylation of ERK1/2 was evident 2 min after addition of HB-EGF, with maximum phosphorylation.
ERK1/2 by cytokines does not involve EGFR- or PI3K-mediated inhibitors (Fig. 1), suggesting that activation of Akt (C, lane 3) but was not affected by either LY294002 or EGFR inhibitor, AG1478 (A, lane 10), demonstrating that HB-EGF-mediated activation of Akt and ERK1/2 occurs via interaction of HB-EGF with the EGFR.

FIGURE 1. HB-EGF independently activates Akt and ERK1/2 in intestinal epithelial cells. A, PI3K-dependent activation of Akt. Serum-starved DLD-1 cells were left untreated or were treated with LY294002 (a PI3K inhibitor (inhib)), PD98059 (a MEK inhibitor), or AG1478 (a specific inhibitor of EGFR phosphorylation) for 30 min. Cells were then stimulated as indicated. Cytosolic extracts were subjected to immunoblot analysis using phospho-specific Akt Ab (p-Akt). Membranes were stripped and blotted with anti-total Akt (T-Akt) Ab. Lanes 1–5 were calculated for each time point and averaged for each treatment. Average values, shown as percent of total, are shown at the bottom of B and C. EGFR inhibitor, AG1478; nd, not determined. Achieved in 10 min. Phosphorylation of ERK1/2 was not affected by LY294002 (average of ~97% of total; Fig. 1B, lane 5). This indicates that the activation of ERK1/2 by HB-EGF is independent of the PI3K pathway. Specific inhibitors of EGFR tyrosine kinase phosphorylation (AG1478) inhibited HB-EGF-mediated phosphorylation of Akt and ERK1/2 (Fig. 1, A, lane 10, and B, lane 4, respectively), demonstrating that HB-EGF-mediated activation of Akt and ERK1/2 occurs via interaction of HB-EGF with the EGFR.

Treatment of cells with a combination of IL-1β/IFN-γ also increased phosphorylation of ERK1/2, with maximum phosphorylation observed at 25 min (Fig. 1C, lane 2). IL-1β/IFN-γ-stimulated phosphorylation was inhibited by PD98059, a MEK inhibitor (Fig. 1C, lane 3) but was not affected by either LY294002 or EGFR inhibitors (Fig. 1C, lanes 4–5), suggesting that activation of ERK1/2 by cytokines does not involve EGFR- or PI3K-mediated signaling pathways.

FIGURE 2. HB-EGF-mediated inhibition of NF-κB DNA-binding activity requires activation of the PI3K pathway. A, PI3K inhibition reverses the inhibitory effect of HB-EGF on NF-κB DNA-binding activity (left). Serum-starved DLD-1 cells were stimulated for 30 min as indicated, and nuclear lysates were prepared as described in Materials and Methods. NF-κB DNA-binding activity was determined by EMSA. Lane 1 contains nuclear extract from cells treated with vehicle as a control. The specificity of NF-κB complexes was determined by supershift assay using anti-p50 Ab. A representative EMSA from three separate experiments is shown. NS, nonspecific binding; competitor, nonradioactive κB oligonucleotides. The effect of HB-EGF on NF-κB DNA binding activity requires interaction with EGFR (right). DLD-1 cells were treated with the EGFR phosphorylation-specific inhibitor PD153035, and nuclear lysates were used for EMSA. EGFR inhibitor, PD153035. B, ERK1/2 does not modulate the inhibitory effect of HB-EGF on NF-κB DNA-binding activity. Cells were transfected with vector alone, catalytically active MEK (CA-MEK), or dominant negative MEK (DN-MEK) cDNA constructs using Superfect transfection reagent. After 48 h, serum-starved cells were treated with the indicated stimuli, and EMSA was performed on nuclear lysates. A representative EMSA from three independent experiments is shown. lane 1 contains nuclear extract from cells treated with vehicle as a control. C, PI3K inhibition reverses the inhibitory effect of HB-EGF on nuclear translocation of NF-κB. DLD-1 cells were stimulated as indicated. Where indicated, serum-starved cells were treated with the PI3K inhibitor LY294002 30 min before stimulation. The cellular distribution of NF-κB was detected using an Ab to the p65 subunit of NF-κB. The confocal images shown represent typical microscopic fields of cells from at least three independent experiments.
A

**FIGURE 3.** PI3K inhibition reverses the ability of HB-EGF to suppress cytokine-induced IκBα phosphorylation and degradation. DLD-1 cells were stimulated as indicated with or without the PI3K inhibitor LY294002 or the MEK inhibitor PD98059. Endogenous levels of phosphorylated IκBα (p-IκBα) and total IκBα (T-IκBα) were determined by Western blot analysis of two separate SDS-PAGE gels ran in parallel. The total IκBα blot was stripped and immunoblotted with anti-β-actin Ab to confirm equal protein loading in all lanes. Data are representative of three independent experiments. Densitometric scans of phosphorylated IκBα and total IκBα blots were quantitated using AlphaEaseFC software, with data presented as percent of total. Arbitrary values obtained for cytokine-stimulated cells in the phosphorylated IκBα blot (lane 5) and in unstimulated cells in the total IκBα blot (lane 1) were set at 100%.

**FIGURE 4.** PI3K inhibition abolishes the inhibitory effect of HB-EGF on iNOS expression and NO production. A, The PI3K inhibitor LY294002 reverses the ability of HB-EGF to decrease NO production. DLD-1 cells were incubated for 24 h with the indicated stimuli, and synthesis of NO was measured in cell supernatants using a Sievers NO analyzer. Results show the mean ± SE of duplicate measurements of four independent experiments. *p < 0.05 compared with cytokines alone (column 4 compared with column 3); **p < 0.05 compared with HB-EGF + cytokines (column 8 compared with column 4). B, LY294002 abolishes the ability of HB-EGF to decrease iNOS protein expression. Cells were exposed to various stimuli as described above, and cell lysates containing 10 μg of protein were subjected to Western blot analysis using anti-iNOS Ab. The blot was stripped and immunoblotted with anti-β-actin Ab to demonstrate equal protein loading in all lanes. EGFR inhibitor, AG1478. C, Quantitative analysis of B. Autoradiographs from three independent experiments were scanned and quantitated using AlphaEaseFC analysis software. Data are presented as percent of total. An arbitrary value obtained for cytokine-stimulated cells was set at 100% (B, column 3).

HB-EGF inhibits cytokine-stimulated NF-κB DNA-binding activity via activation of the PI3K pathway

We previously demonstrated that HB-EGF pretreatment blocks cytokine-activated NF-κB DNA-binding activity in DLD-1 and HT-29 cells (11). We therefore tested whether the inhibition of NF-κB activation by HB-EGF is mediated via PI3K. HB-EGF pretreatment inhibited IL-1β/IFN-γ-stimulated NF-κB DNA binding activity in DLD-1 cells (Fig. 2A, compare lanes 3 and 6), and this inhibition was reversed by the addition of LY294002 (Fig. 2A, lane 9). These results suggest that the ability of HB-EGF to inhibit NF-κB activation is mediated by the PI3K pathway. In contrast, PD98059 (Fig. 2A, lanes 10 and 11), or the transient expression of catalytically active MEK or dominant negative MEK (Fig. 2B, lanes 6–10) had no effect on either cytokine-activated NF-κB activation or HB-EGF-mediated inhibition of NF-κB activation, suggesting that the ERK1/2 pathway is not critical for HB-EGF-mediated suppression of NF-κB activation. The EGFR tyrosine kinase inhibitor PD153035 also reversed the inhibitory effect of HB-EGF on NF-κB activation (Fig. 2A, lanes 12–14), indicating requirement of receptor engagement for HB-EGF function.

PI3K inhibition reverses the ability of HB-EGF to block nuclear translocation of NF-κB

We next examined the effect of LY294002 on HB-EGF-mediated inhibition of nuclear translocation of NF-κB. As shown in Fig. 2C, HB-EGF blocked IL-1β/IFN-γ-induced nuclear translocation of NF-κB (Fig. 2C, compare two middle panels) and LY294002 inhibited this HB-EGF effect (Fig. 2C, right panel). Thus, activation of PI3K is essential for HB-EGF-mediated inhibition of nuclear translocation of NF-κB.

PI3K inhibition blocks the ability of HB-EGF to inhibit cytokine-stimulated IκBα phosphorylation and degradation

Next, we determined whether PI3K inhibition could block the ability of HB-EGF to inhibit cytokine-stimulated IκBα phosphorylation and degradation. Treatment of cells with IL-1β/IFN-γ resulted in phosphorylation and degradation of IκBα (Fig. 3, lane 3), whereas HB-EGF pretreatment inhibited cytokine-stimulated phosphorylation (Fig. 3, lane 4, p-IκBα blot) and degradation (Fig. 3, lane 4, T-IκBα blot) (by ~90%) of IκBα. Addition of LY294002 suppressed the inhibitory action of HB-EGF (Fig. 3, lane 6), whereas the MEK inhibitor PD98059 had no effect (Fig. 3, lane 8). These results indicate that LY294002 interferes with the inhibitory effects of HB-EGF on cytokine-activated IκBα.
HB-EGF decreases iNOS expression and NO production in DLD-1 cells via activation of the PI3K signaling pathway

iNOS activity is mainly regulated at the transcriptional level by activation of NF-κB (33, 34). We have previously shown that pre-treatment of DLD-1 cells with HB-EGF inhibited IKK activation and the subsequent NF-κB signaling cascade, leading to decreased NO production (11). To determine the relevance of the PI3K signaling pathway on the ability of HB-EGF to decrease NO synthesis in response to IL-1β/IFN-γ challenge, cells were treated with LY294002 in the presence or absence of HB-EGF. Treatment of cells with LY294002 did not alter the basal level of NO synthesis (Fig. 4A, column 5) or iNOS expression (Fig. 4, B and C; compare lanes 3 and 7) or NO synthesis (Fig. 4A, compare columns 3 and 7). However, LY294002 abolished the inhibitory effect of HB-EGF on iNOS and NO synthesis (Fig. 4, B and C; compare lanes 4 and 8; and Fig. 4A, compare columns 4 and 8). Wortmannin (300 nM) also reversed the inhibitory effect of HB-EGF on NO production (data not shown).

The EGFR inhibitor AG1478 blocked the inhibitory effect of HB-EGF on iNOS protein expression (Fig. 4B, compare lanes 4 and 16) but had no effect on cytokine-induced iNOS protein expression (Fig. 4B, lane 15). These results suggest that the inhibitory effect of HB-EGF on NO production is due to activation of the PI3K signaling pathway.

Transient expression of catalytically active PI3K suppresses iNOS expression and NO production in an HB-EGF-dependent manner

Having established that activation of PI3K is required for the inhibitory effects of HB-EGF on NF-κB activation, iNOS expression and NO synthesis, we next tested whether expression of activated PI3K alone can mimic these HB-EGF effects. The effect of PI3K on cytokine-stimulated iNOS expression and NO synthesis was evaluated using transient expression of a catalytically active subunit of PI3K (p110α*). In addition, a pool of four siRNA duplexes for p110α were transfected to decrease PI3K expression. To determine the efficiency of transfection of cDNA constructs and siRNA duplexes, Western blot analysis of p110α was conducted. There was an ~2- to 3-fold increase in total p110α protein levels in cells transiently expressing the catalytically active p110α* cDNA plasmid construct compared with cells transfected with vector alone (Fig. 5C; compare lanes 3 and 4 with lane 2). There was an ~50–60% decrease in p110α protein level in cells transfected with a pool of siRNA duplexes for p110α compared with mock transfected cells or cells expressing a nontargeted siRNA pool (Fig. 5C; compare lanes 6–9 with lanes 5 and 10).

As shown in Fig. 5, overexpression of activated PI3K failed to modulate cytokine-activated iNOS protein expression (Fig. 5, A and B; compare lanes 3 and 12) or NO synthesis (Fig. 6; compare columns 3 and 4). However, its expression decreased cytokine-activated NO synthesis in the presence of HB-EGF (~2-fold (43–55%) more than addition of HB-EGF alone (Fig. 6; column 6 compared with column 5), demonstrating that PI3K-mediated NO inhibition is HB-EGF dependent. The decrease in NO production by p110α* paralleled a decrease in expression of iNOS (Fig. 5A; lane 4). Additionally, siRNA of p110α blocked the ability of HB-EGF to decrease iNOS expression (Fig. 5A; lane 8 compared with lane 13). Thus, activation of PI3K is necessary but not sufficient for inhibition of cytokine-induced NO synthesis.
HB-EGF inhibits NO production via PI3K

Activation of Akt and ERK1/2 pathways are not necessary for the inhibitory effects of HB-EGF on NO synthesis

The serine-threonine protein kinase Akt is one of the downstream substrates of PI3K and is a critical mediator of growth factor-induced survival of many cell types. To determine whether Akt might be involved in the effects of HB-EGF, DLD-1 cells were transfected with catalytically active (HA-Akt) and inactive mutant (HA-K179M) forms of Akt. Altered Akt expression had no effect on cytokine-activated NO synthesis or HB-EGF-mediated inhibition of NO production (Table I). siRNA of Akt also had no significant effect on HB-EGF-mediated inhibition of iNOS protein expression (data not shown), even though the transfection of siRNA duplexes for Akt decreased the expression and phosphorylation of Akt by ~70% (Fig. 5, D and E), and the expression of dominant negative Akt decreased phosphorylation of Akt by ~60% (Fig. 5F, lane 4).

Additionally, it is conceivable that ERK1/2 can directly suppress IL-1β/IFN-γ stimulated NO synthesis. As shown in Table I, transient expression of either dominant negative or catalytically active MEK had no effect on either cytokine-stimulated NO synthesis or on the inhibitory effect of HB-EGF on NO synthesis. Similarly, Ras transfection had no effect. Thus, the PI3K pathway, but not the Akt or Ras-ERK1/2 pathways, is required for HB-EGF to produce its anti-inflammatory effects. The lack of MEK effect on NO production was not due to poor transfection efficiency, because the Western blot analysis of pERK1/2 showed that transient expression of dominant negative MEK-8E significantly decreased HB-EGF-stimulated phosphorylation of ERK1/2 (Fig. 5G, lane 4).

We also investigated the effect of HB-EGF, PI3K inhibitors and MEK inhibitors on IL-1β/IFN-γ induced phosphorylation of STAT1 at tyrosine-701 and serine-727. HB-EGF did not alter the phosphorylation state of STAT1. The PI3-kinase inhibitors LY294002 and wortmannin and the MEK inhibitor PD98059 also had no effect on IFN-γ-induced phosphorylation of STAT1 at tyrosine-701 or serine-727 (data not shown). Thus, HB-EGF decreases the expression of iNOS and NO production in cytokine-stimulated cells without modulating the activation of the JAK/STAT pathway.

It has been reported that the PI3K inhibitor wortmannin induces iNOS expression in Raw 264.7 mouse macrophage cells exposed to LPS (10, 35). As described above, HB-EGF inhibits iNOS expression in intestinal epithelial cells via activation of the PI3K pathway. To show that this effect of HB-EGF was not unique to one particular cell type, we next examined whether HB-EGF could inhibit LPS-induced expression of iNOS in Raw 264.7 macrophage cells. As shown in Fig. 7, as little as 1 ng/ml LPS induced iNOS protein expression in macrophages, and addition of small doses of wortmannin (30–50 nM) increased iNOS protein level ~2-fold compared with LPS alone (Fig. 7, lanes 3 and 4). Importantly, HB-EGF pretreatment decreased iNOS protein levels by ~2-fold compared with treatment with LPS alone (Fig. 7, lanes 2 and 5). The demonstration that HB-EGF can decrease iNOS production in both intestinal epithelial cells and macrophages further supports an anti-inflammatory role for this growth factor.

Discussion

Characterization of a consensus sequence for an NF-κB binding site in the iNOS promoter (12), as well as inhibition of iNOS expression by inhibitors of NF-κB activation, have established an essential role of NF-κB in the induction of iNOS (12, 14, 36–38). Once iNOS is induced in cells, the generation of NO can continue for days, leading to significant intestinal barrier dysfunction (39). This can lead to bacterial translocation followed by severe sequelae, progressing to multiple organ dysfunction syndrome.

We have previously demonstrated that HB-EGF down-regulates cytokine-induced activation of iNOS and NO production in intestinal epithelial cells pretreated with HB-EGF. Additionally, HB-EGF substantially suppressed cytokine-induced NF-κB activity and IL-8 release. HB-EGF blocked NF-κB activation by inhibiting

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**Table I. Effect of Akt, Ras, and MEK expression on NO synthesis***

<table>
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<tr>
<th>Treatments</th>
<th>NO (μM)</th>
<th>Akt</th>
<th>Ras</th>
<th>Mek</th>
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<tr>
<td>No addition†</td>
<td>6.34 ± 2.07</td>
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<tr>
<td>HB-EGF†</td>
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<td>IL-1β + IFN-γ†</td>
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<td>IL-1β + IFN-γ + HB-EGF†</td>
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<td>40.80 ± 3.40</td>
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<tr>
<td>IL-1β + IFN-γ + vector</td>
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* DLD-1 cells were transiently transfected with vector, catalytically active (CA) or dominant negative (DN) cDNA constructs as indicated and then stimulated with IL-1β + IFN-γ or IL-1β + IFN-γ plus HB-EGF as indicated. Supernatants were harvested for NO measurements 24 h later. To determine the expression levels and phosphorylation status of MEK and Akt in transfected cells, Western blot analysis was performed, with results shown in Figure 5, D-G.

† Mock transfected.
IKK activation and IκBα phosphorylation and degradation, thus interfering with NF-κB nuclear translocation, DNA-binding activity, and NF-κB-dependent transcriptional activity (11). The ability of HB-EGF to inhibit NF-κB activation and NO production induced by proinflammatory cytokines appears to be an important anti-inflammatory function of HB-EGF. However, the signaling cascades that mediate the effects of HB-EGF on NF-κB and NO production have not been previously characterized. The present study was undertaken to investigate whether these effects of HB-EGF were mediated via PI3K or ERK1/2 signaling pathways. We have used the PI3K inhibitor LY294002 to demonstrate that HB-EGF-mediated activation of PI3K is necessary for HB-EGF-induced suppression of NF-κB activation and NO production induced by a combination of proinflammatory cytokines. The present demonstration that PI3K inhibition prevents the effects of HB-EGF on NF-κB activation and NO production is reminiscent of the report demonstrating that endothelin-1-induced inhibition of iNOS expression in adipocytes is dependent on PI3K activation (40). Additionally, inhibition of PI3K induced the expression of iNOS in LPS-stimulated microglial cells (8, 9). These reports further strengthen our findings and demonstrate an important role for PI3K in the regulation of iNOS and NO production in various cell types.

In the presence of HB-EGF, expression of a catalytically active p110α subunit of PI3K inhibited cytokine-induced expression of iNOS and NO synthesis 2-fold more than HB-EGF alone. However, in the absence of HB-EGF, expression of p110α had no effect on cytokine-activated NO synthesis. It is possible that p110α alone is not able to inhibit cytokine-stimulated activation of NF-κB, suggesting that inhibition of iNOS expression and NO synthesis by p110α requires the inhibition of NF-κB activation provided by HB-EGF pretreatment. Thus, HB-EGF and PI3K act synergistically to decrease iNOS expression and NO production.

More importantly, the suppression of NO production by HB-EGF in cells expressing a catalytically active PI3K further supports the conclusion that activation of PI3K provides a necessary signal for the inhibition of cytokine-stimulated NO production by HB-EGF. HB-EGF appears to differ from EGF in its NO-inhibitory effects in intestinal epithelial cells in that EGF and IL-1β have been found to synergistically stimulate iNOS expression and NO production in intestinal epithelial cells (41).

Neither overexpression of constitutively active or dominant negative Akt, Ras, or MEK affected HB-EGF-mediated inhibition of NF-κB activation or NO production. This indicates that activation of the PI3K pathway, but not activation of Akt or ERK1/2, is required for HB-EGF to exert its inhibitory effects. Additionally, LY294002 had no effect on the activation of ERK1/2, suggesting that LY294002 inhibits the effects of HB-EGF without modulating the activation of ERK1/2; thus, the activation of ERK1/2 is independent of PI3K activation.

This report provides the first evidence demonstrating that PI3K activation is an important signal in HB-EGF-mediated inhibition of NF-κB activation and NO production in response to inflammatory cytokines in intestinal epithelial cells. Thus, the PI3K pathway is one of the signaling pathways by which HB-EGF exerts its intestinal cytoprotective effects. These findings further our knowledge regarding the pathways used by HB-EGF in exerting its protective effects and support our belief that HB-EGF may hold future clinical promise in the development of novel therapeutic strategies for the treatment of inflammatory-based intestinal diseases.

Disclosures

The authors have no financial conflict of interest.

References


FIGURE 7. HB-EGF decreases LPS-induced iNOS protein production in mouse macrophage cells. A, Inhibition of iNOS expression in macrophages by wortmannin (Wort) and HB-EGF. Raw 264.7 cells were stimulated with LPS (1 ng/ml) for 24 h in the presence or absence of HB-EGF (50 ng/ml) or wortmannin (30 and 50 nM) as indicated. iNOS protein levels were determined by Western blot analysis using anti-iNOS Ab. The blot was stripped and blotted with anti-β-actin Ab to demonstrate equal protein loading in all lanes. Wort, Wortmannin; *p < 0.0005 compared with LPS-stimulated cells (lane 2). B, Quantitative analysis of A. The autoradiographs from three independent experiments were scanned and quantitated using AlphaEaseFC software. Data are presented as percentage of total. An arbitrary value obtained for LPS-stimulated cells was set as 100% (A, lane 2).

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