Epidermal growth factor and interleukin-1β synergistically stimulate the production of nitric oxide in rat intestinal epithelial cells

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Epidermal growth factor and interleukin-1β synergistically stimulate the production of nitric oxide in rat intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 287: G1188–G1193, 2004. First published July 22, 2004; doi:10.1152/ajpgi.00254.2004.—Epidermal growth factor (EGF) is one of the trophic factors for intestinal adaptation after small bowel transplantation (SBT). A recent report indicates that nitric oxide (NO) has cytoprotective effects on bacterial translocation (BT) after SBT. We hypothesized that EGF stimulates the expression of the inducible NO synthase (iNOS) gene in the graft and that NO prevents increased BT, although it induces an apoptotic effect. We reported that EGF stimulates the intestinal structure and function in a rat model of isograft (16) and allograft (17, 24). Recently, Azuara et al. (1) demonstrated that administration of NO with caspase inhibitor minimizes BT in SBT, suggesting that NO prevents increased BT, although it induces an apoptotic effect.

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SMALL BOWEL TRANSPLANTATION (SBT) is the effective curative treatment for patients with irreversible intestinal failure, such as short bowel syndrome. However, SBT causes many injuries in the graft due to ischemia-reperfusion, interruption of lymphatic drainage, and graft-vs.-host disease, whereas immuno-suppressive agents, such as tacrolimus, have reduced the early graft losses due to rejection. The loss of physical integrity of intestinal barrier and increased bacterial translocation (BT) in the graft leads to septic complications. Accumulated evidence indicates that increased production of nitric oxide (NO) is associated with BT, but the role of NO, which may have a detrimental or a beneficial effect, is still controversial.

Experiments with animal models and intestinal cells in vitro suggested that NO accelerates BT. It has been reported that BT during graft-vs.-host disease after SBT is reduced after inhibition of inducible NO synthase (iNOS) (19). Experiments with iNOS knockout mice suggested that endotoxin-induced gut injury, loss of barrier function, and BT are associated with increased NO production (6, 39). NO directly impaired intestinal barrier function and induced its permeability in enterocyte monolayer in vitro (11). Potoka et al. (29) proposed that NO can activate the cell apoptotic machinery through peroxynitrite generation, and this may promote BT.

In contrast, other reports have shown that NO may be an important protective molecule against gut-derived sepsis, burn injury, intestinal ulcerogeneity, intestinal lesions, and acute edematous pancreatitis (13, 23, 41). Exogenous administration of NO attenuates postsischemic lesion in organs, including intestine (37). Increased expression of iNOS through immunoregulatory cytokine IL-2 is implicated in the protective effect against intestinal injury after ischemia-reperfusion (27).

Epidermal growth factor (EGF), which is one of the trophic factors for intestinal adaptation after SBT, has been shown to prevent BT in a variety of experimental animal models (20, 22, 44), although there are contradictory results (10, 30). We have reported that EGF stimulates the intestinal structure and function in a rat model of isograft (16) and allograft (17, 24). Recently, Azuara et al. (1) demonstrated that administration of NO with caspase inhibitor minimizes BT in SBT, suggesting that NO prevents increased BT, although it induces an apoptotic effect.

We hypothesized that EGF stimulates the induction of iNOS gene expression in the graft after SBT, which is followed by increased production of NO, leading to the decrease of BT. In this study, we examined whether EGF can stimulate the production of NO under inflammatory conditions in rat-cultured intestinal epithelial cells (IEC).

MATERIALS AND METHODS

Materials. EGF and recombinant human IL-1β (2 × 10^7 U/mg protein) were generously provided by Otsuka Pharmaceutical (Toskushima, Japan). Tyrphostin A25 (TyrA25) and LY-294002 were purchased from Calbiochem (La Jolla, CA). [α-32P]ATP and [α-32P]dCTP were from DuPont-New England Nuclear (Tokyo, Japan).

Cultures. Normal rat IEC-6 (passages 10–15; Riken Cell Bank, Ibaragi, Japan) were grown at 37°C in DMEM ( Gibco, Grand Island, NY) containing glucose (4.5 g/l), 5% heat-inactivated fetal bovine serum, insulin (0.24 U/ml; Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml; Gibco). Cells were trypsinized (0.05% trypsin-0.02% EDTA), suspended in the completed medium, and cultured on plastic dishes (35
When the cell count reached 1.5 \times 10^5, the medium was replaced by fresh serum- and hormone-free medium, and cells were cultured overnight and then used for experiments.

**Measurement of NO production.** IEC-6 cells were washed with fresh serum- and hormone-free medium and then treated with EGF or/and IL-1β. Cells were placed on ice after the indicated times, and the accumulation of nitrite (NO\(_2\)), a stable metabolite of NO, in the medium was determined by the method of Griess (14). In experiments with inhibitors, LY-294002 and TyrA25 were added 30 min before medium was determined by the method of Griess (14).

**Western blot analysis.** Cells were placed on ice, washed twice with cold phosphate-buffered saline, and lysed in 100–200 μl of solubilizing buffer [in mM: 10 Tris-HCl, pH 7.4, containing 1 EDTA, 1 EGTA, 1 sodium orthovanadate, 1 PMSF, and 1% Triton X-100, 0.5% Nonidet P-40, plus 100 U/ml Trasylol (Bayer, Leverkusen, Germany)]. Cells were then passed through a 26-gauge needle 10 times and placed on ice for 30 min, followed by centrifugation at 16,000 g for 15 min. The supernatant (total cell lysate) was mixed with a one-third volume of 4× SDS-PAGE sample buffer (final concentrations: 125 mM Tris-HCl, pH 6.8, containing 5% glycerol, 2% SDS, and 1% 2-mercaptoethanol), boiled at 100°C for 3 min, subjected to SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Immunostaining was performed by using rabbit polyclonal antibody directed against mouse iNOS (Affinity BioReagents, Golden, CO), human inhibitory protein of NF-κB (IκBα), mouse IL-1 receptor 1 (IL-1R1) (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody, and an enhanced chemiluminescence detection agent (Amersham, Piscataway, NJ).

In the case of Akt, total cell lysate was precleared for 1 h with 25 μl of Staphylococcus aureus insoluble 10% protein A (Sigma) and centrifuged at 16,000 g for 5 min. The supernatant was mixed with a mouse monoclonal antibody against human Akt1 (AktSG3; Cell Signaling, Beverly, MA). After incubation overnight at 4°C, 25 μl of 50% G protein Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden) was added. The mixture was shaken for 1 h and centrifuged at 16,000 g for 5 min, after which the beads were washed four times with solubilizing buffer at room temperature. After a final wash, the beads were solubilized in SDS-PAGE sample buffer and analyzed by Western blotting using rabbit polyclonal antibodies against human Akt and phospho-(Ser\(^{473}\)) Akt (Cell Signaling) as the primary antibody.

**Northern blot analysis.** Total RNA was extracted from cultured cells using the acid guanidinium-phenol-chloroform method (5). Ten micrograms of total RNA were then fractionated by 1% agarose gel electrophoresis, transferred to nylon membranes (Nytran; Schleicher & Schuell, Dassel, Germany), and immobilized by baking at 80°C for 1 h before hybridization with DNA probes. A cDNA probe for rat iNOS (830 bp) (26) was kindly provided by Dr. Y. Nunokawa (Suntory Institute for Biochemical Research, Osaka, Japan). cDNA encoding rat IL-1R1 (15) was isolated by RT-PCR, and cDNA probe for rat iNOS (830 bp) (26) was kindly provided by Dr. Y. Nunokawa (Suntory Institute for Biochemical Research, Osaka, Japan). cDNA encoding rat iNOS (830 bp) was analyzed by Northern blotting. The filter was probed with labeled inserts of iNOS (middle) and GAPDH (bottom) cDNAs. Data are representative of 4 independent experiments.

**EMSA.** Nuclear extracts were prepared according to the method of Schreiber et al. (32) at 4°C, unless otherwise stated. Briefly, cultured cells (35-mm dish) were washed with Tris-HCl-buffered saline, har-

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**Fig. 1. Effect of epidermal growth factor (EGF) and IL-1β on the production of nitric oxide (NO) in intestinal epithelial cells (IEC).**

A: time course. Cells were treated with EGF (33 ng/ml) in the presence and absence of IL-1β (1 nM) for the indicated times. ×, control, without EGF and IL-1β; ○, EGF; □, IL-1β; ●, EGF and IL-1β. B and C: concentration dependency. Cells were treated with IL-1β (1 nM) in the presence of various concentrations of EGF (3.3–100 ng/ml) or with EGF (33 ng/ml) in the presence of various concentrations of IL-1β (0.1–10 nM) for 24 h. Levels of nitrate as NO production were measured in the culture medium (means ± SD, n = 3 dishes). *P < 0.05 or **P < 0.01 vs. control (without EGF and IL-1β).

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**Fig. 2. Effect of EGF and IL-1β on the induction of inducible NO synthase (iNOS) expression in IEC.** Cells were treated with EGF (33 ng/ml) in the presence and absence of IL-1β (1 nM) for the indicated times. Total cell lysate (75 μg of protein) was subjected to SDS-PAGE with 7.5% gel, followed by immunoblotting with anti-iNOS antibody (top). Total RNA (10 μg) was analyzed by Northern blotting. The filter was probed with labeled inserts of iNOS (middle) and GAPDH (bottom) cDNAs. Data are representative of 4 independent experiments.
vested with the same buffer, and centrifuged at 1,840 g for 1 min. Cells from two dishes were suspended in 400 μl of lysis buffer (in mM: 10 HEPES-KOH, pH 7.9, containing 10 KCl, 0.1 EDTA, 0.1 EGTA, 0.5 PMSF, and 1 dithiothreitol, plus 500 U/ml Trasylol) in a centrifuge tube and allowed to stand for 15 min, and 25 μl of 10% Nonidet P-40 was added to the lysis buffer. The tube was then vigorously vortexed for 1 min at room temperature and centrifuged at 15,000 g for 1 min. After removal of the supernatant, the nuclear pellet was suspended in 75 μl of nuclear extraction buffer (in mM: 20 HEPES-KOH, pH 7.9, containing 1 EDTA, 1 EGTA, 1 PMSF, and 1 dithiothreitol, plus 0.4 M NaCl and 500 U/ml Trasylol). The tube was incubated for 20 min with continuous mixing and then was then centrifuged at 15,000 g for 5 min. Aliquots of the supernatant were frozen in liquid nitrogen and stored at –80°C until use.

Binding reactions were performed by incubating the nuclear extract (4 μg of protein) with reaction buffer [20 mM HEPES-KOH, pH 7.9, 1 mM EDTA, 60 mM KCl, 10% glycerol, 1 μg of poly(dl-dc)] for 30 min at 4°C in a total volume of 15 μl, followed by incubation for 20 min at room temperature with the probe (~40,000 counts/min). Products were subjected to electrophoresis at 100 V on 4.8% polyacrylamide gel in high-ionic-strength buffer (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5), and the dried gels were analyzed by autoradiography. The NF-κB consensus oligonucleotide sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) from the mouse immunoglobulin-κ light chain was purchased from Promega (Madison, WI) and was labeled with [γ-32P]ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford (4) with a dye binding assay kit (Bio-Rad).

Statistical analysis. Results shown in the figures are representative of three to four independent experiments yielding similar findings. Differences were analyzed by the Bonferroni/Dunn test, and P < 0.05 was considered to indicate statistical significance.
RESULTS

Effect of EGF and IL-1β on the production of NO in IEC. As shown in Fig. 1, B and C, proinflammatory cytokine IL-1β (1–10 nM) showed practically no effect on the production of NO in IEC-6, as reported previously (40). EGF (33–100 ng/ml), which is one of the important trophic factors influencing an adaptation of the graft after SBT, also had no effect on NO production at all. However, simultaneous addition of EGF (33 ng/ml) and IL-1β (1 nM) markedly stimulated the induction of NO production time-dependently, showing the maximal accumulation of NO at ~30 h (Fig. 1A). EGF increased the production of NO in the presence of IL-1β (1 nM) dose-dependently, with the maximal effect at 33 ng/ml (Fig. 1B), and vice versa; IL-1β increased the production of NO in the presence of EGF (33 ng/ml) dose-dependently, with the maximal effect at 1 nM (Fig. 1C).

Effect of EGF and IL-1β on the induction of iNOS gene expression in IEC-6 cells. Western blot analysis revealed that iNOS protein (130 kDa) increased time-dependently with the addition of EGF and IL-1β, showing the maximal level at 24 h (Fig. 2, top). Northern blot analysis revealed that levels of iNOS mRNA increased at 6 h and thereafter after the addition of EGF and IL-1β (Fig. 2, middle). These results suggest that EGF and IL-1β synergistically stimulate the induction of iNOS at the transcription level in IEC-6 cells, which is followed by the enhancement of NO production. In the following experiments, we examined the mechanisms involved in the induction of iNOS gene expression by EGF (33 ng/ml) and IL-1β (1 nM).

Effect of EGF and IL-1β on the degradation of IkB and the activation of NF-κB. It is known that the transcription factor NF-κB is crucial for the induction of iNOS gene expression, because the promoter of mouse, rat, and human genes encoding iNOS contains a consensus sequence for the binding of NF-κB (7, 9, 43). NF-κB is attached to IkB in the cytoplasm of cells. Activation of NF-κB involves proteolytic degradation of IkB, translocation of NF-κB to the nucleus, and binding of NF-κB to the promoter κB site. Western blot analysis revealed that IL-1β and IL-1β plus EGF, but not EGF, decreased the levels of IkBα protein (Fig. 3A). Consistent with this observation, the EMSA demonstrated that that IL-1β and IL-1β plus EGF, but not EGF, increased the activation of NF-κB (nuclear translocation and DNA binding) (Fig. 3B).

Effects of TyrA25 (EGF receptor kinase inhibitor) and LY-294002 phosphatidylinositol 3-kinase inhibitor on NO production, iNOS induction, and NF-κB activation. Several reports indicate that phosphatidylinositol 3-kinase (PI3-kinase) plays an important role in the regulation of iNOS induction in

Fig. 7. A schematic model for the induction of iNOS gene expression in IEC. IL-1β stimulates the NF-κB activation through IkB kinase, and EGF stimulates the IL-1R1 induction through PI3-kinase/Akt. Accordingly, EGF and IL-1β activate 2 signals that are essential for the induction of iNOS and NO production. EGFR, EGF receptor; P, serine phosphorylated.

Fig. 6. Effect of EGF and IL-1β on the induction of type 1 IL-1 receptor (IL-1R1) in IEC. A: time course of IL-1R1 mRNA; cells were treated with EGF (33 ng/ml) in the presence and absence of IL-1β (1 nM) for the indicated times. B: concentration dependency of EGF and the effect of LY-294002. Cells were treated with EGF (1–33 ng/ml) and IL-1β (1 nM) in the presence and absence of LY-294002 (10 μM) for 3 h. Total RNA (10 μg) was analyzed by Northern blotting. The filter was probed with labeled inserts of IL-1R1 and GAPDH cDNAs. The bands corresponding to IL-1R1 mRNA were quantitated by densitometry (means ± SD, n = 3 experiments). C: induction of IL-1R1 protein and the effect of LY-294002 and TyrA25. Cells were treated with EGF (33 ng/ml) and IL-1β (1 nM) in the presence of LY-294002 (10 μM), TyrA25 (100 μM), or vehicle (0.1% of DMSO) for 8 h. Total cell lysate (100 μg of protein) was subjected to SDS-PAGE with a gradient 6–9% gel, followed by immunoblotting with anti-IL-1R1 antibody. Data are representative of 4 independent experiments.

Nitric oxide

G1191

AJP-Gastrointest Liver Physiol • VOL 287 • DECEMBER 2004 • www.ajpgi.org
glial cells (28), macrophages (8), and hepatocytes (42). TyrA25 and LY-294002 significantly blocked the increases of NO production stimulated by IL-1β plus EGF, with the maximal effects at 100 and 10 μM, respectively (Fig. 4A). TyrA25 and LY-294002 also abolished the induction of iNOS protein (Fig. 4B). However, both inhibitors had no effects on the degradation of IkBα and the activation of NF-κB induced by IL-1β or IL-1β plus EGF (data not shown). These results implied that the EGF action on the enhancement of NO production is PI3-kinase-dependent via its receptor, whereas IL-1β action on the activation of NF-κB is PI3-kinase-independent.

Effect of EGF and IL-1β on the activation of Akt (protein kinase B). We then examined whether EGF and IL-1β influence the activation (phosphorylation) of Akt, which is a downstream kinase of PI3-kinase. In support for the above observation, EGF and EGF plus IL-1β markedly increased the phosphorylation of Akt time-dependently, with the maximal effect at 30 min, whereas IL-1β alone showed only faint bands corresponding to phosphoAkt (Fig. 5, top). The phosphorylation of Akt by EGF or EGF plus IL-1β was blocked by LY-294002 without changes of Akt levels (Fig. 5, bottom).

Effect of EGF and IL-1β on the induction of type 1 IL-1 receptor in IEC-6 cells. Recently, we found that IL-1β stimulates the upregulation of IL-1R1 through PI3-kinase/Akt in addition to the activation of NF-κB in hepatocytes, which is required to induce the expression of iNOS gene (42). In the present study, EGF and IL-1β induced the activations of Akt and NF-κB, respectively, in IEC-6 cells, leading to the induction of iNOS expression. Accordingly, we examined whether EGF and IL-1β upregulate the induction of IL-1R1 in IEC-6 cells. As shown in Fig. 6, EGF and EGF plus IL-1β, but not IL-1β, increased the induction of IL-1R1 mRNA time- and dose-dependently (Fig. 6, A and B), which was blocked by LY-294002. Levels of IL-1R1 protein were enhanced by EGF and EGF plus IL-1β and were also blocked by LY-294002 and TyrA25 (Fig. 6C).

DISCUSSION

In this study, we found that EGF induced the expression of the iNOS gene in the presence of the proinflammatory cytokine IL-1β in IEC, leading to increased production of NO (Figs. 1 and 2). It is known that IL-1β activates the transcription factor NF-κB, which is critical for the inducible expression of genes involved in inflammation. However, accumulated evidence suggested that the activation of NF-κB is not sufficient to induce the NF-κB-dependent transcription, and the existence of a second signaling was postulated (2, 3, 38). IL-1β alone has been reported to induce the expression of iNOS gene in human and rat hepatocytes (12, 18). Furthermore, we recently demonstrated (42) that IL-1β upregulated the induction of IL-1R1 through PI3-kinase/Akt and that its enhancement is essential for the induction of iNOS in concert with the activation of NF-κB in hepatocytes. The upregulation of IL-1R1 enhanced the phosphorylation of NF-κB subunit p65, resulting in the increased activation of NF-κB-dependent transcription as reported (21, 33).

In IEC-6 cells, IL-1β alone stimulated the pathway of IkB kinase involving the phosphorylation and degradation of IkB, followed by the activation of NF-κB (nuclear translocation and DNA binding), but had no effect on the induction of iNOS. In contrast, EGF had no effect on the activation of NF-κB. However, EGF but not IL-1β activated PI3-kinase/Akt pathway involving the enhancement of IL-1R1 induction (Figs. 5 and 6). Thus simultaneous addition of EGF and IL-1β activated the IkB kinase/ NF-κB and PI3-kinase/Akt/IL-1R1 pathways, resulting in the induction of iNOS. PI3-kinase inhibitor (LY-294002) blocked the Akt activation, IL-1R1 induction, and iNOS induction (Figs. 4–6), whereas it had no effect on the degradation of IkB and the activation of NF-κB induced by IL-1β, indicating that the upregulation of IL-1R1 by EGF through PI3-kinase/Akt is also crucial for iNOS induction in IEC-6 cells.

SBT is associated with high incidence of infectious complications and BT. NO is an important molecule with diverse roles in various organs, and the role of NO on intestinal injury including BT is still unclear. However, Sola et al. (34–37) reported that NO has a cytoprotective effect in intestinal ischemia-reperfusion injury, because endogenous accumulation after preconditioning and exogenous administration of NO can inhibit glycolytic enzyme and GAPDH, resulting in the decrease of lactate and the increase of fructose-1,6-bisphosphate. Inhibition of NO production with Nω-nitro-L-arginine methyl ester eliminated the protective effect by preconditioning (35, 37). Furthermore, exogenous administration of NO with the inhibitor of apoptosis maintains the graft of SBT in optimal conditions in terms of BT (1). These reports insisted that NO is cytoprotective in the graft after SBT. We hypothesized that an increased production of NO is involved in the adaptation effect of EGF after SBT. In fact, EGF stimulated the induction of iNOS and NO production in IEC under pathological conditions, such as in the presence of proinflammatory cytokine IL-1β.

In conclusion, our results demonstrate that EGF and IL-1β stimulate two essential signals for iNOS induction, the upregulation of IL-1R1 through PI3-kinase/Akt and the activation of NF-κB through IkB kinase, as shown in Fig. 7. Simultaneous addition of EGF and IL-1β can induce the expression of iNOS gene, resulting in increased production of NO.

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REFERENCES


