Stimulation of the Mitogen-activated Protein Kinase Cascade and Tyrosine Phosphorylation of the Epidermal Growth Factor Receptor by Hepatopoietin*

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Hepatopoietin (HPO) is a novel human hepatotrophic growth factor, which specifically stimulates proliferation of cultured primary hepatocytes in vitro and liver regeneration after liver partial hepatectomy in vivo. Recently, the identification of the mitogenic effect of HPO on hepatoma cell lines and the existence of HPO-specific receptors indicate that HPO acts via its specific cell surface receptor. However, the molecular mechanism of HPO action is not fully elucidated. In this report, we examined the signal transduction events induced by HPO in hepatoma cell line (HepG2). Our results demonstrated that HPO induces phosphorylation of mitogen-activated protein kinase kinase (MAPK) in a rapid and transient manner. HPO stimulates tyrosine phosphorylation of epidermal growth factor receptor (EGFR). Furthermore, we observed that both MAPK activation and the mitogenic effect of HPO on HepG2 cells were completely blocked by AG1478, a specific inhibitor of EGFR tyrosine kinase activity. However, the effects of HPO were not antagonized by an EGFR-blocking antibody, mAb528, which blocks the interaction between epidermal growth factor receptor and EGFR, indicating that stimulation of tyrosine phosphorylation of EGFR by HPO was not mediated by epidermal growth factor receptor. In contrast, genistein, a general tyrosine kinase inhibitor, significantly attenuated the tyrosine phosphorylation of EGFR in response to HPO. In conclusion, our results suggest that tyrosine phosphorylation of EGFR may play a critical role in MAPK activation and mitogenic stimulation by HPO.

Hepatopoietin (HPO) is a novel human hepatotrophic growth factor, an orthologue of rat augmenter of liver regeneration or hepatic stimulator substance (1). In 1975, LaBrecque and Pesh (2) first reported that in the livers of weanling rats or partially hepatectomized rats, there existed a polypeptide, named hepatic stimulator substance, that could specifically stimulate DNA synthesis of hepatic cells. The existence of hepatic stimulator substance-related activities has been reported in other species including mice, cows, dogs, pigs, and humans (3). Hagiya et al. (4) cloned the cDNA of rat augmentor of liver regenerator, which is the same as rat hepatic stimulator substance. Subsequently, Giorda et al. (5) and Yang et al. (6) cloned the cDNA of human augmentor of liver regenerator or HPO by screening the cDNA library of human fetal liver. HPO encodes a novel protein with no sequence similarity to any known growth regulator. Interestingly, HPO is highly related to the yeast ERV (essential for respiration and viability) gene products. However, the functional relevance of HPO and ERV is currently unclear (7). Yang et al. (8, 9) demonstrated that the recombinant human HPO stimulated proliferation of hepatocytes as well as hepatoma cells in vitro. HPO also promotes regeneration and recovery of damaged hepatocytes and rescues acute hepatic failure in vivo (8, 9). Thus, these observations support the contention that HPO is a hepatotrophic growth factor.

Unlike other typical growth factors, HPO was discovered in the cytosol of liver parenchymal cells and was produced in an autocrine way (rather than in a paracrine way from mesenchymal cells or in an endocrine way from other glands) during liver regeneration or organogenesis (10). In addition, the effects of HPO are known to be liver-specific. HPO specifically stimulates proliferation of cultured primary hepatocytes in vitro and enhances liver regeneration after liver partial hepatectomy in vivo. HPO displays no significant effect on the proliferation of non-hepatocytes or tumor cell lines derived from tissues other than liver (10). These unique characteristics distinguish HPO from various well known hepatic stimulators, such as insulin, epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and transformation growth factor (TGF-α), which can stimulate proliferation of a wide variety of cell types (11). Because of the very specific effect of HPO, the mechanism of HPO action might be intriguing and different from other known growth factors.

Recently, we characterized the mitogenic effect of HPO on hepatoma cell lines and demonstrated the existence of HPO receptors on the membranes of these cells (12). Furthermore, we showed that HPO acts as an autocrine growth factor to maintain the autonomous growth of hepatoma cell lines in vitro. These findings suggest that HPO binds a specific receptor...
on the cell membrane and triggers the signal transduction pathway leading to cell proliferation. Considering the importance of the mitogen-activated protein kinase (MAPK) pathway in cell growth, we investigated activation of the MAPK pathway by HPO.

EGF is also an important growth factor in liver regeneration (13) and hepatoma progression in vitro (14). The autocrine loop of TGF-α/EGFR plays an important role in supporting autonomous growth of hepatoma cells in vitro (15). In addition to activation by its ligands, such as EGF and TGF-α, EGFR could act as a downstream mediator in various signaling pathways via a novel ligand-independent pathway. In some tumor cell lines, EGFR is essential for a wide variety of mitogens to stimulate the MAPK pathway and cell proliferation (16). In this study, we demonstrated that EGFR might play an important role for the mitogenic signaling triggered by HPO in hepatoma cell lines.

MATERIALS AND METHODS

Cell Culture—HepG2 (a hepatocellular carcinoma cell line) and GLC-82 (a lung cancer cell line) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (Hyclone).

Preparation of Recombinant Human Hepatopoietin—HPO open reading frame (375 base pairs) (6) was subcloned into expression vector pBV220 to create vector pBV220-HPO. The vector containing HPO cDNA was transformed into Escherichia coli strain JM109. A single colony of JM109 containing pBV220-HPO to LB (5 ml) was seeded and cultured overnight at 37 °C with vigorous shaking. The culture was diluted 2:10 into fresh LB and incubated for 3 h at 30 °C; then expression of HPO protein was induced by heating (42 °C, 5 h).

The bacteria cells were collected by centrifugation (4000 rpm, 10 min) and lysated with a sonicator. The sonicated cells were spun at 10,000 × g for 5 min; then the supernatant was discarded, and the inclusion body pellet was washed with 20 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 4 M urea. The washed inclusion bodies were dissolved in solubilization buffer (8 mM urea, 1 mM EDTA in 20 mM Tris-HCl, pH 8.0) and then centrifuged at 10,000 × g for 20 min. The supernatant was diluted to the protein concentration of 10 mg/ml. 5 ml of supernatant of the sample was loaded on the Superdex 75 prep grade 26/60 column (Amersham Pharmacia Biotech) equilibrated with the solubilization buffer. The column was eluted at 2 ml/min. The purity of the proteins of peak II was analyzed by silver-stained SDS-polyacrylamide gel electrophoresis. The purified protein was refolded by dilution in refolding buffer (1 mM reduced glutathione, 0.5 mM oxidized glutathione in 20 mM Tris-HCl, pH 8.0). The refolded protein was dialyzed against 20 mM Tris-HCl, pH 8.0; then the solution was spun at 10,000 × g for 20 min, and the supernatant was collected.

Antibodies and Other Reagents—EGF, IGF-1, tyrophostin AG1478, and genistein were from Sigma. Antibodies against active MAP kinase and pan-extracellular signal-regulated kinase were from Promega Corporation. Peroxidase-conjugated anti-rabbit and anti-mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. EGFR, IGF-1 receptor, and HGF receptor antibodies and Protein A/G-agarose were from Santa Cruz Biotechnology. Antibody mAb528 was from NeoMarkers. Phospho-tyrosine monoclonal antibody P-Tyr-100 was from BioLabs. Enhanced Chemiluminescence (ECL) kit and Hybond-P membrane were from Amersham Pharmacia Biotech.

Protein Extraction, Immunoprecipitation, and Western Blotting—Cells were rinsed three times with ice-cold phosphate-buffered saline and lysed in 50 mM HEPES pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium-α-tena
date, 20 mM NaF, 10 mM sodium pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin for 20 min at 4 °C. The lysate was centrifuged at 12,000 rpm for 30 min. For detecting HGF receptor, IGF-1 receptor, and EGFR, the supernatant was collected and used in Western blot directly. For detecting the phospho-EGFR, the supernatant was incubated with anti-EGFR antibody for 2 h at 4 °C, followed by incubation with Protein A-Sepharose overnight at 4 °C. The immunoprecipitates were collected by centrifugation and washed by radioimmune precipitation buffer three times. The lysate or immunoprecipitate was fractionated via 10% SDS-polyacrylamide gel electrophoresis. The proteins were blotted onto Hybond-P membranes. The membranes were first blocked by incubation in TTBS containing 3% BSA overnight at 4 °C, then incubated sequentially with primary antibody and peroxidase-conjugated secondary

RESULTS

HPO Stimulates the MAPK Pathway in HepG2 Cells—Activation of the MAPK pathway is a key event in cell growth regulation. Both MEK and MAPK are activated by phosphorylation of conserved residues in the activation loop of the kinase domains. Activities of endogenous MEK and MAPK can be indirectly determined by Western blot with phosphospecific antibodies. We tested whether MAPK is stimulated in HepG2 cells by HPO. Fig. 1 indicates that HPO induces phosphorylation of MEK and MAPK in serum-starved HepG2 cells. The increase of phosphorylation appeared in a rapid and transient manner. MEK phosphorylation was at a maximum 5 min after HPO treatment and declined to the basal level at 30 min. Similarly, phosphorylation of MAPK reached the peak at 10 min and dropped to the basal level at 30 min after HPO stimulation. The kinetics of MEK and MAPK induced by HPO is very similar to those activated by other growth factors (17, 18). The above data demonstrate that HPO activates the MAPK signaling pathway in HepG2 cells.

HPO Induces Tyrosine Phosphorylation of EGFR—EGFR is activated not only by EGF but also by a wide variety of mitogens. We examined whether HPO stimulates tyrosine phosphorylation of EGFR in HepG2 cells. As shown in Fig. 2, HPO induces tyrosine phosphorylation of EGFR but has no effect on the tyrosine phosphorylation of HGF receptor (c-MET) nor on IGF-1 receptor. The kinetics of HPO action on the tyrosine phosphorylation of EGFR parallels that of the phosphorylation of MEK and MAPK, suggesting that phosphorylation of EGFR may play a role in the activation of MEK and MAPK in response to HPO. The level of phosphorylated EGFR induced by
HPO (50 ng/ml) and lysed. Immunoprecipitation was done with anti-HGF receptor antibodies. A, serum-starved cells were stimulated for 5 min with HPO (50 ng/ml), EGF (10 ng/ml), HGF (10 ng/ml), or IGF-1 (10 ng/ml) and lysed. Immunoprecipitation (IP) was done with anti-EGFR antibody. B, induction of EGFR tyrosine phosphorylation was analyzed upon stimulation of HepG2 cells for the indicated times with HPO (50 ng/ml). C and D, serum-starved cells were stimulated for 5 min, 10 min, and 20 min with HPO (50 ng/ml) and for 5 min with HGF (10 ng/ml) or IGF-1 (10 ng/ml) and lysed. Immunoprecipitatin was done with anti-HGF receptor antibody or anti-IGF-1 receptor antibody. PY, phosphorylated tyrosine.

The Tyrosine Kinase Activity of EGFR Is Required for HPO Signaling to the MAPK Pathway—To determine the significance of EGFR in HPO signaling, the effect of tyrphostin AG1478 was tested on MAPK activation by HPO. Tyrphostin AG1478 is a specific inhibitor of EGFR tyrosine kinase activity (19). Fig. 3 indicates that the MAPK activation triggered by both HPO and EGF were completely blocked by AG1478. In contrast, IGF-1-induced MAPK activation was not unaffected. Furthermore, the effect of HPO is more sensitive to the AG1478 inhibition than did EGF (data not shown). This could be due to the fact that HPO induced a much stronger activation of EGFR than did EGF (Fig. 2A). Our data show that EGFR is a prerequisite of the HPO signaling cascade leading to MAPK activation and that the role of EGFR in HPO signaling depends on its intrinsic AG1478-sensitive tyrosine kinase activity.

Blockage of Tyrosine Kinase Activity of EGFR Inhibits the Mitogenic Effect of HPO—It was demonstrated that GLC-82 cell line (human lung cancer cells) does not express the specific receptor of HPO (12). To identify the potential role of the specific receptor of HPO in HPO signaling, GLC-82 cells were utilized to test whether the specific HPO receptor is required for the activation of EGFR and MAPK triggered by HPO. The results in Fig. 5 indicate that HPO had no effect on the phosphorylation of EGFR nor MAPK in GLC-82 cells, whereas EGF stimulated phosphorylation of both EGFR and MAPK. These data suggest that the specific receptor of HPO might be required for the activation of EGFR and MAPK during HPO signaling.

The EGFR-dependent MAPK Activation by HPO Is Independent of the Ligand Interaction with EGFR—It has been demonstrated that HPO has its own specific receptor that is different from the receptors of EGF, TGFA, and insulin on the HepG2 cells, because EGF, TGFA, and insulin have no competitive effect on 125I-HPO binding to the cell surface sites (12). In vitro binding experiments indicate no direct physical interaction of HPO with the HepG2 cells, because EGF, TGFα, and insulin have no competitive effect on 125I-HPO binding to the cell surface sites (12).
HPO with the receptors of EGF, TGF-α, and insulin. Therefore, the induction of EGFR tyrosine phosphorylation by HPO may be a ligand-independent event, i.e. the triggering of tyrosine phosphorylation of EGFR by HPO might be different from a direct ligand-receptor interaction. To test this hypothesis, the specific antibody mAb528 against EGFR, which has been widely utilized to block the extracellular ligand-interacting domain (20), was used. Our results show that mAb528 indeed had no effect on the tyrosine phosphorylation of EGFR triggered by HPO, although it completely blocked the tyrosine phosphorylation triggered by EGF (Fig. 6). The above results suggest that the tyrosine phosphorylation of EGFR triggered by HPO is independent of EGFR-ligand interaction. The effect of HPO on EGFR is not due to a direct binding of HPO to EGFR. The results in Figs. 5 and 6 indicate that HPO binds its own receptor and indirectly induces activation of a tyrosine kinase(s) and EGFR tyrosine phosphorylation.

Genistein, a Universal Inhibitor of Tyrosine Kinases, Significantly Attenuates the Effect of HPO on EGFR Phosphorylation—Genistein is a universal inhibitor of tyrosine kinases (19). To provide further evidence, we compared the effect of genistein on EGFR phosphorylation triggered by HPO and EGF. The results (Fig. 7) show that in the absence of genistein, tyrosine phosphorylation of EGFR was induced 64.0 ± 7.5- and 24.0 ± 4.3-fold by EGF and HPO, respectively. In comparison, genistein significantly attenuated the effect of HPO on EGFR phosphorylation (reduced approximately 20-fold) at a relatively low dose (5 μg/ml) and completely blocked the effect of HPO at 10 μg/ml. However, genistein had no significant effect on EGF phosphorylation induced by EGFR at the same doses (5 and 10 μg/ml) and displayed a mild effect at 15 μg/ml. These results indicate that HPO and EGF probably use different mechanisms to stimulate the tyrosine phosphorylation of EGFR. We propose that HPO may activate a tyrosine kinase(s), which is different from EGFR and more sensitive to genistein inhibition. Activation of this unidentified tyrosine kinase is likely to play an important role in HPO signaling because genistein completely blocked the HPO effect on the tyrosine phosphorylation of EGFR.

**FIG. 6.** MAb528, the specific antibody against EGFR, had no effect on HPO function. Serum-starved cells were pretreated with mAb528 (500 ng/ml) for 1 h before stimulation for 5 min with HPO (50 ng/ml) or EGF (10 ng/ml). Phospho-EGFR (A) and phospho-MAPK (B) were detected as described above. PY, phosphorylated tyrosine.

**FIG. 7.** Genistein significantly attenuated the effect of HPO on EGFR. Serum-starved cells were pretreated with genistein of the indicated concentrations for 30 min or without genistein and then stimulated with HPO (50 ng/ml) or EGF (10 ng/ml). The cells pretreated without genistein, HPO, or EGF were used as controls. A, phosphorylated EGFR was detected as described above. B, the data were obtained by scanning Western blot bands. Bars, S.D. of triplicated samples. PY, phosphorylated tyrosine.

**DISCUSSION**

Although the role of HPO as a stimulator of hepatocyte proliferation in liver regeneration has been systematically investigated since the 1970s, the molecular mechanisms of HPO action are unclear. The MAPK pathway is well demonstrated to be an important growth-related pathway in liver regeneration, which was activated by some hepatic growth factors, including the three most important mitogens, EGF, TGF-α, and HGF (11). Those mitogens take effect in the course of the whole cell cycle, including an initial phase in which cells become primed to proliferation and a second phase in which competent hepatocytes progress through G1 and undergo proliferation (22). However, HPO appears to act at the late G1/S interface and stimulates DNA synthesis of those hepatocytes that have already entered the second phase (3). Intriguingly, our results here show that MAPK is also activated by HPO. However, in the view of divergent characteristics of HPO compared with those mitogens, it is reasonable to hypothesize that the HPO signaling leading to MAPK activation might be by a mechanism different from other hepatocyte mitogens.

More and more evidence shows that EGFR acts not only as a receptor for EGF-like ligands but also as a mediator of diverse
signaling systems (23, 24). A wide variety of mitogens, including some agonists of G protein-coupled receptors such as lysophosphatidic acid, thrombin (25), and agonists with unknown receptors (21), stimulate cell growth via active EGFR in a ligand-independent pathway. In this study, we demonstrate that HPO triggers MAPK activation and proliferation in HepG2 cells through induction of tyrosine phosphorylation of EGFR. Furthermore, our data suggest that activation of EGFR requires an HPO-specific receptor but not the direct binding of HPO or EGF to EGFR. The characteristics of EGFR activation by HPO are similar to those stimuli previously identified in an EGF-independent way. Our findings suggest that HPO binds its receptor and then triggers a tyrosine kinase(s) to activate EGFR or that HPO directly initiates the phosphorylation of EGFR. The difference of sensitivity to genistein of EGFR activation induced by HPO and EGF supports this hypothesis. Although HPO is capable of inducing phosphorylation of EGFR, HPO cannot cover the diverse function of EGF completely. For instance, unlike EGF, HPO cannot induce the phosphorylation of STAT3 (signal transducer and activator of transcription 3) in HepG2 cells (data not shown) and cannot stimulate growth of various tissues and cells other than liver and hepatocyte. This further indicates the difference of signal pathways between HPO and EGF.

This report provides the first biochemical data about the signal transduction pathway of HPO. We demonstrated that HPO stimulates the activation of EGFR and MAPK. Furthermore, our results show that tyrosine phosphorylation of EGFR is important for the effect of HPO on activation of MAPK and stimulation of cell proliferation. These data provide important information for further investigation about the mechanisms of HPO action and the relationship between HPO and other hepatocyte mitogens, especially EGF. Elucidation of the molecular mechanisms of HPO action will be valuable for our understanding of liver organogenesis, regeneration, and oncogenesis. Considering that the HPO receptor has not been cloned so far, the significance of activating EGFR in HPO signaling will shed some light on the way to approach the HPO receptor.

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