Hemangiopoietin Promotes Endothelial Cell Proliferation Through PI-3K/Akt Pathway

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Key Words

Hemangiopoietin • Proliferation • Endothelial Cell • Akt • Cyclin D1

Abstract

Hemangiopoietin (HAPO) is a novel human growth factor acting on the primitive cells of both hematopoietic and endothelial cell lineages. Our previous study has shown that HAPO exerts a proliferative effect on endothelial cells. However, the mechanism of this action remains unclear. Thus, we studied the signal transduction pathway whereby HAPO promotes cell proliferation in human umbilical vein endothelial cells (HUVECs). In this study, recombinant human HAPO (rhHAPO) stimulated the proliferation of HUVECs in a dose-dependent manner. The transient phosphorylation of Akt occurred after addition of rhHAPO to HUVECs. LY294002, a specific inhibitor of PI-3K, significantly inhibited Akt phosphorylation and completely abrogated HAPO-stimulated proliferation of HUVECs. rhHAPO enhanced the expression of cyclin D1, where as LY294002 inhibited the up-regulation of cyclin D1. Moreover, rhHAPO is able to selectively enhance

the mitogenic activity of VEGF for vascular endothelial cells. Overall, these findings demonstrate that HAPO induces endothelial cell proliferation through the PI-3K/Akt pathway.

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Introduction

Hemangiopoietin (HAPO) is a novel growth factor, originally identified and purified from the urine of patients with aplastic anemia. Previously, we have demonstrated that human recombinant HAPO (rhHAPO), which has been produced based on the N-terminal amino acid sequence of purified native HAPO, supports survival and proliferation of primitive cells of both hematopoietic and endothelial cell lineages, and efficiently protects mice from radiation damage [1-3]. Recently, we have reported that rhHAPO protects MO7e cells from apoptosis by inactivating caspase 3 and blocking the PARP degradation through the PI-3K/Akt pathway [4]. However, the underlying mechanisms by which HAPO stimulates cell proliferation remain unclear. Thus, we studied the signal

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transduction pathway through which rhHAPO promotes cell proliferation in human umbilical vein endothelial cells (HUVECs).

Many growth factors and cytokines have been shown to regulate proliferation of endothelial cells via diverse signaling pathways. In endothelial cells, the majority of growth factor- or cytokine-induced responses are mediated by the activation of the PI-3K/Akt signaling cascade [5, 6]. Akt is believed to play a crucial role in apoptosis, cell cycle, glucose metabolism, angiogenesis, and tumor progression [7, 8]. Indeed, accumulating evidence suggest that Akt signaling activated by a variety of stimuli can regulate cell cycle progression through modulating numerous targets including cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors [9, 10].

In the present study, we investigated the mitogenic activity of HAPO for endothelial cells. We demonstrated that HAPO stimulated endothelial cell proliferation through the PI-3K/Akt pathway.

Materials and Methods

Materials

Recombinant human HAPO (rhHAPO) was produced in the GMP laboratory of our institute as previously described [1]. Anti-phospho-Akt (Ser473), anti-Akt polyclonal antibodies, anti-p21 Waf1/Cip1, anti-p27 Kip1, and anti-Cyclin D1 monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-GAPDH monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The PI-3K inhibitor LY294002 and MEK1 inhibitor PD98059 were also purchased from Cell Signaling Technology Inc. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Isolation and Culture of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase type I (Sigma) treatment and used in passages 3-5. The cells were cultured in M200 medium (Cascade Biologics, Portland, OR) supplemented with 1% low serum growth supplement (Cascade Biologics), 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a humidified 5% CO₂ atmosphere until they reached 80-90% confluence. HUVECs were identified by DiI-Ac-LDL uptake experiment and immunofluorescent staining for CD31. HUVECs from three different donors were pooled for each experiment. All experiments were performed on cells synchronized by incubation in serum-free M200 medium for 12 h.

MTT colorimetric assay

HUVECs were seeded in triplicate in 96-well plates (3 × 10³ cells/well) and grown under normal conditions for 24 h. After starvation for 12 h, fresh medium plus 0.5% FBS with HAPO at different concentrations were added to each well, followed by indicated incubation at 37 °C and 5% CO₂. For the experiments conducted in the presence of pharmacological inhibitors of selected signaling pathways, the cells were treated with different inhibitors or vehicle for 1 h before the stimulation with HAPO. The viable cell numbers were estimated by MTT assay. Briefly, 20 µl of MTT (5 mg/ml) was added to each well, and these plates were maintained in culture for 4 h. The medium was discarded, and the product was solubilized with DMSO. Absorbance was measured at 570 nm using a spectrophotometer (ELISA reader, Dynatech MR 5000).

BrdU Incorporation Assay

DNA synthesis in HUVECs was measured using the cell proliferation BrdU ELISA kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. BrdU was added during the last 4 h of incubation. After fixation, the mouse monoclonal anti-BrdU-POD antibody was added. The immune complexes were revealed by the subsequent substrate reaction, and quantified by measuring the absorbance at 405 nm with a reference wavelength at 490 nm in a microliter plate reader.

Western blot analysis

After treatment, HUVECs were scraped off the plates and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40, 0.25% deoxycholic acid, 0.1% SDS, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄) with protease inhibitor cocktail (Sigma). Protein concentration of the lysates was quantified by the Bradford protein assay (Pierce Rockford, IL). Equal amounts of protein (50 µg) were electrophoresed in 10% or 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 2 h with 5% skim milk in TBS, and incubated with primary antibodies in TBST at 4 °C overnight. After washing, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Pierce) diluted 1: 20 000 in TBST. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Pierce). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 55 °C. After washing, membranes were blocked and reprobed with antibodies against the non-phosphorylated proteins. To quantify and compare levels of proteins, the intensity of each band was measured by densitometry (NIH Image J, version 1.38).

Statistical Analysis

All data are presented as mean ± standard deviation (SD) of at least 3 independent experiments. ANOVA with a subsequent Bonferroni/Dunnnett test was used to determine the significance of differences in multiple comparisons. A probability value <0.05 was considered statistically significant.

Fig. 1. Effects of HAPO on HUVEC proliferation analyzed by MTT and BrdU incorporation assays. (A, B) To determine the dose-dependent effect, serum-starved HUVECs were treated with the indicated concentration of rhHAPO for 72 h. Results are expressed as percent change versus control cells 72 h after treatment with rhHAPO, and each bar represents the mean $\pm$ SD of triplicate determinations. (C, D) For the time course experiment, serum-starved HUVECs were treated with rhHAPO (500 ng/ml) for 24, 48, and 72 h. The results are presented as the mean $\pm$ SD of three independent experiments. * P <0.05 versus untreated cells.

Results

Effects of HAPO on HUVEC proliferation

To determine the direct effects of HAPO on HUVEC proliferation, both MTT assay and BrdU incorporation assay were used. The growth experiments were performed in the presence of 0.5% FBS to allow optimal growth stimulation of synchronized cells. We demonstrated that rhHAPO induced HUVEC proliferation in a dose-dependent manner, with the maximum effect observed at a concentration of 500 ng/ml (Figure 1A, B). Then, we examined the time-dependent effect of HAPO on HUVEC proliferation. Serum-starved HUVECs were treated with 500 ng/ml of rhHAPO for various time points. As shown in Figure 1C, D, incubation with HAPO for 72 h significantly increased proliferation of HUVECs by approximately 28% (MTT assay) and 42% (BrdU assay). These results are consistent with those shown in Figure 1A, B.

HAPO activates Akt in HUVECs

We have previously demonstrated that HAPO activates PI-3K/Akt pathway to protect MO7e cells from apoptosis [4]. Therefore, we examined whether HAPO can stimulate Akt signaling in HUVECs. Activation of Akt was analyzed by Western blot using specific anti-phospho-Akt antibody. As shown in Figure 2A, rhHAPO induced the phosphorylation of Akt in a time-dependent manner. The phosphorylation peaked at 30 min after stimulation with 500ng/ml rhHAPO and declined thereafter. Then, we checked the effect of a specific PI-3K inhibitor, LY294002, on HAPO-induced Akt activation. As expected, pretreatment with LY294002 abolished HAPO-induced Akt activation, whereas the specific MEK inhibitor, PD98059, failed to abrogate Akt phosphorylation (Figure 2B). Moreover, we also investigated whether

HAPO could activate other signaling cascades such as ERK1/2 and STAT3/5 pathways. However, HAPO led to neither ERK1/2 activation nor STAT3/5 activation (data not shown), although these pathways have been reported to be involved in endothelial cell proliferation induced by numerous growth factors and cytokines stimulation [11, 12].

PI-3K/Akt pathway is involved in HAPO-induced HUVEC proliferation

To examine whether Akt activation is involved in HAPO-induced cell proliferation, HUVECs were treated with LY294002 and PD98059 for 1 h before the stimulation with HAPO. Cell proliferation was still evaluated by MTT assay and BrdU incorporation assay. Both methods showed that pretreatment of the cells with LY294002

Fig. 2. Akt activation in HUVECs stimulated with HAPO (A) and inhibition with LY294002 (B). Blots at top of panels are typical examples of Western blot of Akt and phosphorylated Akt in HUVECs treated with rhHAPO (500 ng/ml). Graphs at the bottom of the panels indicate the fold increase in phosphorylated Akt compared with pretreated cells. For the time course experiment, HUVECs were treated with rhHAPO (500 ng/ml) for 5, 15, 30, and 60 minutes, and then analyzed by Western blot for phosphorylated Akt. The blot was stripped and reprobed with antibody against the non-phosphorylated Akt to demonstrate protein loading. For inhibitory experiment, serum-starved HUVECs were pretreated with LY294002 or PD98059 at indicated concentration for 1 hour before HAPO treatment. * $P < 0.05$ versus untreated cells, ** $P < 0.05$ versus rhHAPO-treated cells.

Fig. 3. Effects of PI-3K inhibitor, LY294002 and MEK inhibitor, PD98059 on HAPO-induced HUVEC proliferation assessed by MTT assay (A) and BrdU incorporation assay (B). Serum-starved HUVECs were pretreated with LY294002 or PD98059 at different concentrations for 1 hour before rhHAPO treatment (500 ng/ml), and the cell proliferation was determined after 72 hours. Values are expressed as percentage of untreated cells, and each bar represents the mean $\pm$ SD of triplicate determinations. * $P < 0.05$ versus untreated cells, ** $P < 0.05$ versus rhHAPO-treated cells.

completely inhibited HAPO-induced HUVEC proliferation (Figure 3). In contrast, PD98059 had only marginal effect on HUVEC proliferation induced by HAPO, indicating that ERK signaling seems not to participate in HAPO-mediated HUVEC proliferation.

HAPO up-regulates the expression of cyclin D1 through PI-3K/Akt pathway

To better understand the molecular mechanisms involved in HAPO-induced HUVEC proliferation, we investigated the expression levels of cell-cycle regulatory proteins. In order to verify that the HAPO-stimulated

Fig. 4. Effects of HAPO on cell-cycle regulatory proteins in HUVECs. Blots on the top of the panel are typical examples of Western blot of cell-cycle regulatory proteins in HUVECs treated with rhHAPO (500 ng/ml). The graph at the bottom of the panel indicates the fold increase in cyclin D1 induced by HAPO in Western blots. Serum-starved cells were cultured in medium (M200 plus 0.5% FBS) with HAPO (500 ng/ml) for 24 h and 48 h, or pretreated with LY294002 (50 μM) for 1 h and then cultured with or without HAPO for 48 h. The results represent one of three separate experiments. * $P < 0.05$ versus untreated cells, ** $P < 0.05$ versus rhHAPO treatment (48 h).

cells are in a proliferative state, we examined the expression of cyclin D1, CDK inhibitors p21 Waf1/Cip1 and p27 Kip1, which participate in the progression through G1/S phase. As shown in Figure 4, rhHAPO markedly increased the expression of cyclin D1 in a time-course manner. However, the expression levels of p21 and p27 did not change during the 48 h of incubation. Notably, the PI-3K inhibitor, LY294002, potently inhibited the cyclin D1 induction by HAPO, whereas LY294002 alone did

Fig. 5. Effects of HAPO on bFGF- and VEGF-induced HUVEC proliferation. Serum-starved HUVECs were treated with combinations of different concentrations of rhHAPO and 10 ng/ml VEGF (A) or bFGF (B). After 72h, cell proliferation was determined by MTT assay. Values are expressed as percentage of control cells. The bars represent the mean $\pm$ SD of triplicate determinations. * $P < 0.05$ versus VEGF alone.

not suppress the expression of cyclin D1. These data suggest that HAPO-induced up-regulation of cyclin D1 requires PI-3K/Akt activation.

HAPO enhances VEGF-induced HUVEC proliferation

In this study, we have demonstrated that HAPO has modest effects on endothelial cell proliferation, as compared with bFGF and VEGF, two well-established potent angiogenic factors. Since many moderate endothelial cell mitogens can exert synergetic effects in angiogenesis [13, 14], we next examined whether HAPO could enhance bFGF- and VEGF-induced endothelial cell proliferation in vitro. As shown in Figure 5A, 10 ng/ml VEGF alone markedly stimulated the proliferation of endothelial cells by 1.8-fold. When VEGF and HAPO were combined together, 500 ng/ml rhHAPO enhanced 10 ng/ml VEGF-induced HUVEC proliferation by 1.45-fold. Notably, HAPO at a concentration of 50 ng/ml also enhanced VEGF-induced HUVEC proliferation by 1.35-fold, although the same dose of HAPO alone had no obvious effect on the HUVEC proliferation. However, rhHAPO did not significantly enhance bFGF-induced HUVEC proliferation (Figure 5B).

Discussion

In the present study, we have examined the effects of HAPO on endothelial cell proliferation and determined the signal transduction pathways involved. We have demonstrated here that HAPO is able to stimulate the proliferation of HUVECs in a dose-dependent manner, and to induce the phosphorylation of Akt in a time-dependent manner. Inhibition of the PI3-K/Akt pathway completely abrogated the proliferative effect of HAPO. In addition, HAPO induced the elevation of cyclin D1 expression level through PI-3K/Akt pathway. These findings indicate that HAPO exerts mitogenic activity on endothelial cells through the activation of Akt.

Akt, also known as protein kinase B (PKB), regulates essential cellular functions such as apoptosis, proliferation, differentiation, migration, and metabolism. Upon stimulation by growth factors and cytokines, Akt activation occurs immediately in endothelial cells. Subsequently, Akt modulates various downstream substrates to promote cell survival and cell cycle progression [6, 9]. To date, it has been known that Akt kinase activity is induced by a number of growth factors such as VEGF, angiopoietin and cytokines such as IL-4, IL12, and IL-13 in a PI-3K-dependent manner [15-18]. In the present study we found that HAPO could also activate the PI3-K/Akt pathway in endothelial cells, similar to the previous observation that HAPO activates Akt in megakaryocytic leukemia cells. Moreover, we demonstrated that the PI-3K/Akt pathway was involved in HAPO-induced HUVEC proliferation because the use of a specific inhibitor of this pathway, LY294002, dramatically reduced HUVEC proliferation after HAPO stimulation.

Accumulating evidence is defining a critical role of Akt in cell proliferation, although Akt has been implicated as an anti-apoptotic mediator in numerous cell death paradigms. Actually, Akt may regulate cell cycle progression through multiple mechanisms. One means by which PI-3K/Akt promotes cell proliferation is to up-regulate the expression of cyclin D1. Cyclin D proteins are controlled at the levels of transcription, translation, protein stability and translocation [9, 10]. Cyclin D proteins are important and essential regulators of cell cycle progression. Unlike other cyclins that are periodically induced during the cell cycle progression, the levels of cyclin D are regulated by the extracellular mitogenic environment. Thus, cyclin D proteins are believed to serve as "links" between the extracellular environment and the core cell cycle apparatus [19]. Many growth factors have been reported to increase the expression of cyclin D1,

and multiple signal transduction pathways, including the PI-3K/Akt pathway are involved [20, 21]. Therefore, the effect of HAPO on the expression of cyclin D1 was determined in this study. We observed that treatment of HUVECs with HAPO led to a marked increase of cyclin D1 expression. Notably, the PI-3K chemical inhibitor, LY294002, significantly inhibited the induction of cyclin D1 in HUVECs, suggesting that the PI-3K/Akt pathway is implicated in HAPO-induced cell proliferation, possibly through the induction of cyclin D1. However, the exact level at which cyclin D1 is regulated, and the potential components that Akt phosphorylates to induce cyclin D expression remain unclear, which will require further investigation.

Another important mechanism by which PI-3K/Akt signaling induces cell proliferation is the regulation of CDK inhibitor p21 Waf1/Cip1 and the related family member p27 Kip1. p21 has both positive and negative regulatory effects on cell proliferation, and its function during cell cycle progression appears to ensure appropriate CDK activation. The mechanisms of Akt-mediated regulation of p21 may be attributed to enhanced transcription, and reduced degradation. It has been shown that the PI-3K/Akt pathway is required for p21 induction because inactivation of this pathway by specific inhibitor or dominant-negative Akt expression decreased levels of p21, whereas expression of a constitutively active mutant Akt increased p21 expression [22]. On the other hand, Akt can phosphorylate Ser146 on p21, which inhibits p21 protein degradation via the proteasome machinery, and thus prolongs the half-life of p21 [23]. Interestingly, Akt can also phosphorylate Thr145 on p21, which causes the exclusion from the nucleus and prevents p21 from exerting regulatory effects, but this relocalization was not observed in endothelial cells [24, 25]. Therefore we investigated the effect of HAPO on p21 expression in HUVECs. However, the activation of PI-3K/Akt pathway induced by HAPO did not change the expression of p21.

In the current study, we investigated the effects of HAPO on VEGF- or bFGF-induced proliferation of endothelial cells. Interestingly, we found that HAPO selectively promoted VEGF-induced, but not bFGF-induced, HUVEC proliferation. There are two possible mechanisms that could explain our data. One possibility is that HAPO could selectively amplify the intracellular signaling transmitted by VEGFR, but not bFGFR, in endothelial cells. VEGF has been reported to induce HUVEC proliferation through different signaling molecules such as Src, ERK1/2, p38 MAP kinase, PI3-kinase, Akt, and p70 S6 kinase. [26-28]. In contrast, the

ERK1/2 pathway mainly contributes to the proliferative response of endothelial cells to bFGF, although other pathways could be involved in bFGF-triggered endothelial cell proliferation [29, 30]. Thus, it seems likely that HAPO enhances the mitogenic activity of VEGF through activating Akt pathway. Another possibility is that HAPO is able to regulate the surface expression of VEGFR, but not bFGFR in HUVECs. Further studies will be required to elucidate the mechanism by which HAPO selectively promotes VEGF-induced proliferation of endothelial cells.

In summary, we have demonstrated that HAPO, a novel growth factor, exerts a direct effect on HUVEC proliferation. Akt signaling has been shown to be involved in HAPO-induced cell proliferation. These observations

provide new insights into HAPO biology and elucidate the molecular mechanisms by which HAPO stimulates proliferation in endothelial cells.

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