Pro-urokinase up-regulates the expression of urokinase-type plasminogen activator (u-PA) in human pulmonary arterial endothelial cells

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Abstract

The fibrinolytic function of endothelial cells plays an important role in the pathophysiology of pulmonary vascular diseases. In this study, the effects of pro-urokinase, a new thrombolytic drug that is currently being tested for the treatment of pulmonary embolism, on the expression of urokinase-type plasminogen activator (u-PA) and u-PA receptor (u-PAR) were assessed. The role of u-PAR was also investigated. Immunocytofluorescence and RT-PCR techniques were employed. In normal human pulmonary arterial endothelial cells (HPAECs), the expression levels of u-PA and u-PAR were very low. Incubation with pro-urokinase up-regulated u-PA expression at both the mRNA level and the protein level; however, the expression of u-PAR was not affected. The effect of pro-urokinase induction was totally inhibited by the release of u-PAR from the HPAECs' surface with PLC. This result suggests that the combination of u-PA with u-PAR may be a critical pathway for the induction of u-PA expression.

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KEYWORDS
Pro-urokinase; Urokinase-type plasminogen activator (u-PA); Human pulmonary arterial endothelial cells (HPAECs)

Introduction

Urokinase, a serine protease, dissolves blood clots by catalyzing the conversion of plasminogen to plasmin. It is an important drug in the treatment of thromboembolic disease. Abnormal fibrinolytic function has been reported in several lung diseases, such as pulmonary embolism, primary pulmonary hypertension [1] and acute pulmonary injury. Increased endothelial plasminogen activator production reduces thrombotic
events in vitro [2]: endothelial overexpression of recombinant plasminogen activators has anti-thrombotic benefits in both arterial-type and venous-type thrombosis [3,4].

Pro-urokinase is a relatively thrombus-specific, single-chain proenzyme that is converted to two-chain urokinase by fibrin-associated plasmin located at the thrombus site. It is currently the subject of several clinical trials for a variety of indications, including pulmonary embolism [5,6]. However, little is known about the direct effect that pro-urokinase has on human pulmonary arterial endothelial cells (HPAECs) under normal and pathological conditions.

The oral administration of pro-urokinase to animals or humans increases the endogenous u-PA level in the circulating blood [7–9]. The previously published data suggest that exogenous u-PA up-regulates endogenous u-PA production. However, the sources of u-PA production and its physiological significance are still unclear. It has been reported that exogenous u-PA induces u-PA expression in human umbilical vein endothelial cells (HUVECs), mononuclear cells, and lung epithelial cells. Also the u-PAR occupancy, a glycosyl phosphatidylinositol (GPI)-linked protein, is required for u-PA-induced u-PA expression in these cells [10,11]. Due to the close interaction between thromboembolism and pulmonary endothelial cells, we hypothesized that exogenous pro-urokinase could up-regulate the expression and secretion of u-PA in pulmonary endothelial cells, which may contribute to increased endogenous u-PA levels and facilitate rapid fibrinolysis in response to acute pulmonary embolism.

In this study, by using immunofluorescence and RT-PCR techniques, we explored the effect that pro-urokinase has on u-PA and u-PAR expression and its possible pathway in HPAECs.

Materials and methods

Materials

Human pulmonary arterial endothelial cells obtained from normal subjects, culture medium M200, and low serum growth supplement (LSGS) including 2% fetal bovine serum, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml human fibroblast growth factor, and 10 μg/ml heparin were obtained from Cascade Biologics (Portland, ME, USA), and fetal calf serum (FCS) was obtained from Gibco BRL laboratories (Grand Island, NY, USA). A monoclonal mouse antibody (IgG) to u-PA and a polyclonal goat anti-u-PAR antibody (IgG) were obtained from American Diagnostic (Greenwich, CT, USA). TRIzol for isolation of total RNA was obtained from Invitrogen (San Diego, CA, USA), and the RT-PCR kit was obtained from TaKaRa (Dalian, China). All primers were synthesized by Sangon Technology (Shanghai, China). Pro-urokinase was provided by the TianShili Pharmaceutical Group (Tianjin, China). GPI-specific phospholipase C (PLC) was obtained from Sigma (St. Louis, MO, USA).

Cell culture

HPAECs were routinely maintained in culture at 37 °C in the M200 medium supplemented with LSGS. All endothelial cells were grown in T25 flasks (for molecular experiments) or collagen-coated sterile glass cover slips in 24-well tissue culture plates (for immunofluorescence experiments) and kept in a humidified 5% CO2 atmosphere. After growing to sub-confluence, the cells were switched to a starvation medium (M200) for 12 h. Then, the HPAECs were incubated with pro-urokinase as described below. After incubation, either total cellular RNA was isolated or the immunofluorescence experiment was carried out. Cells between passages 3 and 6 were used for the experiments.

RNA isolation and reverse transcriptase PCR

RT-PCR was used to quantify target mRNA, normalized to housekeeping gene GAPDH mRNA.

Cells cultured in T25 flasks were incubated with increasing doses of pro-urokinase (0, 1.5, 15, 150 IU/ml) for 8 h or with a fixed concentration of pro-urokinase (150 IU/ml) for 0, 4, 8, 12, or 24 h. Total

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Amplification length (bp)</th>
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<td>Sense</td>
<td>5′-ATCTGCCCGCCCGTGTATAAC-3′</td>
<td>286</td>
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<td></td>
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<td>5′-ATCTCCGCTCAAGTATCCGCGCT-3′</td>
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<td>Sense</td>
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<td></td>
<td>Anti-sense</td>
<td>5′-GCCCTCTCATGCACTGAT-3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>5′-AACGACACCTTCTAAGGT-3′</td>
<td>498</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>5′-TCCAGCAGTACTCAGGCT-3′</td>
<td></td>
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</table>
RNA was isolated from the HPAECs using TRIzol reagent and then reverse transcripted to cDNA. PCR was performed in a total reaction volume of 20 μl including cDNA 2 μl, 5 × PCR Buffer 4 μl, dH2O 12.75 μl, Ex Taq 0.25 μl, and primer 0.5 μl×2. The mixture was denatured for one cycle of 5 min at 95 °C, followed by 20–35 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and completed by a final extension step of 5 min at 72 °C. The PCR products were subjected to 1.5% agarose gel electrophoresis stained with ethidium bromide. Band intensities were quantified based on densitometric analysis using Quantity One software. The data were normalized using the ratio of the target cDNA concentration to the GAPDH concentration.

The primers used for PCR amplification are described in Table 1.

Cellular immunofluorescence of u-PA and the u-PAR antigen

HPAECs cultured in 24-well tissue plates were incubated with pro-urokinase (0 or 150 IU/ml) for 8 h, washed with PBS, and fixed with 4% paraformaldehyde for 30 min at 4 °C. After 0.1% Triton X-100 was added for 20 min, the cells were incubated overnight with 1:60 monoclonal primary antibodies, mouse anti-u-PA antibody, and goat anti-u-PAR antibody. Then, the

Figure 1  u-PA mRNA expression induced by pro-urokinase in HPAECs. HPAECs were incubated with 150 IU/ml pro-urokinase for different times (A, B); or with different doses of pro-urokinase (0–150 IU/ml) for 8 h (C, D). u-PA and GAPDH mRNA expressions were investigated at the mRNA level using RT-PCR. A, C: The upper band represents the fragment of 286 bp given by primers specific to u-PA mRNA, and the lower band represents the fragment of 498 bp given by primers specific to GAPDH mRNA. B, D: Scatter plots of densitometric data from 3 separate experiments are shown. *P<0.05 compared with control.

Figure 2  Effect of pro-urokinase on u-PAR mRNA expression in HPAECs. HPAECs were incubated with different doses of pro-urokinase for 8 h. u-PAR and GAPDH mRNA expressions were investigated at the mRNA level using RT-PCR. A: The upper band represents the fragments of 140 bp given by primers specific to u-PAR mRNA, and the lower band represents the fragments of 498 bp given by primers specific to GAPDH mRNA. B: Scatter plot of densitometric data from 3 separate experiments is shown. *P<0.05 compared with control.
samples were incubated on ice in the absence of light for 30 min with either 1:200 FITC-labeled goat anti-mouse pAb or TICC-labeled rabbit anti-goat pAb in PBS. After being washed three times with ice-cold PBS, the cells were mounted. The images were captured at ×100 magnification using an Olympus fluorescence microscope. For quantitative analysis, the mean fluorescence intensity was determined using a digital analysis program, Image-pro Plus version 6.0, and normalized to the total area of cells in each image.

PLC treatment of HPAECs

The starved HPAECs were treated with PI-PLC (0.5 IU/ml) for 2 h at 37 °C, washed three times with M200, and incubated with pro-urokinase 150 IU/ml for 8 h. Then, the cells were harvested and processed for RT-PCR experiments.

Statistical analysis

The data are presented as scatter plots with mean of three independent experiments. One-way analysis of variance was used for the statistical analysis of the differences in the mRNA expression of each factor. The difference was considered to be statistically significant when the P value was less than 0.05.

Results

Effect of pro-urokinase on u-PA mRNA expression by HPAECs

A single band was detected (Fig. 1A and C), and fragment lengths fit the predicted products by the primers specific to u-PA and GAPDH, respectively. u-PA mRNA was detected in unstimulated HPAECs, and its expression level was significantly higher after incubation with pro-urokinase. The amount of u-PA mRNA relative to that of GAPDH increased after incubation with pro-urokinase (150 IU/ml) for 4 h, reached the maximum level after 8 h, and then returned to the normal level after at least 24 h (Fig. 1B). As shown in Fig. 1D, when HPAECs were treated for 8 h with increasing amounts of pro-urokinase (0–150 IU/ml), the u-PA mRNA expression levels increased as the pro-urokinase concentration increased.

Effect of pro-urokinase on u-PAR mRNA expression by HPAECs

The association of u-PA with u-PAR plays an important role in many of u-PA’s biological activities, and
our results demonstrated that pro-urokinase could up-regulate u-PA expression. Therefore, the expression of u-PAR induced by pro-urokinase was also assessed.

As shown in Fig. 2, u-PAR mRNA was detected in unstimulated HPAECs, and the expression level remained stable after incubation for 8 h with different doses of pro-urokinase (0–150 IU/ml).

**Effect of pro-urokinase on u-PA and u-PAR protein levels in HPAECs**

Having determined that pro-urokinase could up-regulate u-PA mRNA expression levels by HPAECs, changes in the u-PA protein levels were also assessed in HPAECs. The presence of cellular u-PA and u-PAR antigen was determined by immunological fluorescence. Both u-PA and u-PAR antigen were detected with no stimulation (Fig. 3A and C). After incubation for 8 h with pro-urokinase 150 IU/ml, the fluorescence intensity of u-PA became stronger (Fig. 3B), while the fluorescence intensity of u-PAR did not change (Fig. 3D).

**The role of u-PAR in the induction of u-PA**

u-PAR is a glycosyl phosphatidylinositol (GPI)-linked protein. Phosphatidylinositol-phospholipase C (PI-PLC) treatment can completely remove GPI-linked protein, including u-PAR, from the cell surface. After treatment with PLC, HPAEC growth remained normal, suggesting that the PLC effect was not toxic. However, enhancement of pro-urokinase-mediated u-PA expression was completely inhibited (Fig. 4). These findings confirm that the u-PA/u-PAR pathway is involved in the induction of u-PA expression.

**Discussion**

It has been reported that, at baseline, impressively high u-PA concentrations are present in human and bovine lung capillary endothelial cells in vitro [10,11]. Evidence of u-PA expression has also been found in human venous endothelial cells isolated from patients and healthy volunteers [12]. It was previously reported that exogenous u-PA induces u-PA expression in HUVECs [11]; however, there have been no published reports dealing with the expression of u-PA and the effect that pro-urokinase has on the fibrinolytic system of HPAECs.

The present study confirmed that low concentrations of u-PA and u-PAR are expressed in normal HPAECs. Pro-urokinase could up-regulate u-PA expression at both mRNA and protein levels in vitro, and could induce time-dependent promotion of u-PA mRNA in HPAECs without affecting the u-PAR expression level. This inductive effect of pro-urokinase could be totally inhibited by the release of u-PAR from HPAECs’ cell surface.

u-PAR is a GPI-linked protein that is present on the surface of a number of cells, including monocytes, endothelial cells, and many tumor cells. u-PA-mediated activation of plasminogen is closely regulated by its high-affinity receptor, u-PAR. Compared to u-PA in the fluid phase, the binding of u-PA to u-PAR on the cell surface leads to a greatly increased rate of u-PA activation by plasmin. Plasminogen activation by u-PA results in the acceleration of plasmin generation. In addition to its effect on facilitating plasmin formation, this pathway is also involved in many cellular functions, such as migration, adhesion, signaling, and mitogenesis. Most of these biological activities are dependent on the association of u-PA with u-PAR [13–16]. u-PAR plays a major regulatory role in the plasminogen–plasmin system [17]. In the present study, the results showed that pro-urokinase could not induce u-PA gene expression to increase after u-PAR was released from the cell surface by PI-PLC. The data indicate that u-PAR plays a critical role in the inductive effect of pro-urokinase.

Although u-PAR is devoid of a cytoplasmic domain, it has been shown to transduce a signal to the interior of the cell. The intracellular signal transduction pathway of u-PA bound to its cell receptor u-PAR has been investigated in many studies. Common signal transduction pathways, such as Src kinases [15], serine kinases [16], G proteins [16], and diacylglycerol [18], that are dependent on different cell types have been suggested for u-PA and u-PAR signaling. Many biological factors, such as cytokines [19], growth factors, hemoglobin, and steroid hormones, can also regulate the cellular expression of u-PA with different mechanisms and models of action [20]. These involve both transcriptional and posttranscriptional aspects. The posttranscriptional pathway was shown to influence u-PA mRNA levels in lung cancer-derived cell lines and in nonmalignant lung epithelial cells [21]. Cytokines involved in the setting of acute lung injury or in the tumor microenvironment stimulated u-PA expression by this mechanism [10]. Similar findings were also reported in HUVECs and U937 cells. The signal transduction pathway employed in the u-PA-induced u-PA expression of HPAECs needs to be explored in further experiments.

The maximum dose of pro-urokinase used in the present study (150 IU/ml) was based on the clinical dose; a higher dose may cause bleeding. In vivo, the
HPAECs may be exposed to this pro-urokinase concentration. At this concentration, pro-urokinase regulates the expression of u-PA mRNA in a time-dependent manner. However, the biological function of auto-up-regulation of u-PA is still unclear. u-PA has been confirmed to play a central role in venous thrombus resolution and vascular recanalization [22]. Retroviral gene transfer of cDNAs encoding t-PA or u-PA amplifies plasminogen activator production 10-fold and confers anti-thrombotic efficacy for both platelet-rich and fibrin-rich thrombus formation in vivo [3]. Gene deletion studies involving plasminogen activator-deficient mice provide complementary evidence that thrombo-occlusive events are associated with decreased levels of plasminogen activator in vivo [23]. The up-regulation of u-PA by pro-urokinase may contribute to the process of fibrinolysis [24] and the reduction of thrombus formation. This suggests a new pathway for pharmacological positive feedback of fibrinolysis.

Conclusions

In summary, the present findings indicate that pro-urokinase stimulates the expression of u-PA in cultured human pulmonary arterial endothelial cells. The u-PA pathway was also characterized. To the best of our knowledge, this is the first study to describe the ability of u-PA to regulate its own expression in pulmonary endothelial cells. This novel pathway may contribute to the regulation of u-PA-dependent fibrinolysis and other functions of endothelial cells involved in thrombosis and thromboembolism.

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References


