Binding of Urokinase to Low Density Lipoprotein-related Receptor (LRP) Regulates Vascular Smooth Muscle Cell Contraction*

Received for publication, July 17, 2002

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Urokinase plasminogen activator (uPA) is a multifunctional protein that has been implicated in several physiological and pathological processes involving cell adhesion and migration in addition to fibrinolysis. In a previous study we found that two-chain urokinase plasminogen activator (tcuPA) stimulates phenylephrine-induced vasoconstriction of isolated rat aortic rings. In the present paper we report that uPA−/− mice have a significantly lower mean arterial blood pressure than do wild type mice and that aortic rings from uPA−/− mice show an attenuated contractile response to phenylephrine. In contrast, the blood pressure of urokinase receptor knockout (uPAR−/−) mice and the response of their isolated aortic rings to phenylephrine were normal, indicating that the effect of uPA on vascular contraction is independent of uPAR. Addition of mouse and human uPA almost completely reverses both the impaired vascular contractility and the lower arterial blood pressure in vivo. The in vitro and in vivo effects of infused uPA on aortic contractility and the restoration of normal blood pressure in uPA−/− mice were prevented by antibody to low-density lipoprotein receptor-related protein/α2-macroglobulin receptor (LRP). A modified form of uPA that lacks the kringle failed to restore the blood pressure in uPA−/− mice, notwithstanding having a longer half-life in the circulation. Ligands that regulate the interaction of uPA with LRP, such as PAI-1 or the PAI-1-derived peptide (EEIIMD), abolished the vasoactivity of tcuPA and human uPA, also raising a question as to the nature of vasoactivity in vivo. These studies identify a novel signal transducing cellular receptor pathway involved in the regulation of vascular contractility.

Urokinase plasminogen activator (uPA)‡ is a multifunctional protein that has been implicated in several physiological and pathological processes, including fibrinolysis. Transgenic mice with a targeted disruption in the uPA gene (uPA−/−) are prone to form thrombi when exposed to endotoxin (1) or hypoxia (2, 3) or when the uPA gene is disrupted in otherwise healthy tPA−/− mice (1, 4). We recently reported that clearance of pulmonary microemboli in uPA−/− mice is delayed, despite the presence of an intact tPA system (5).

uPA has also been implicated in other pathophysiological processes, such as pulmonary inflammation and repair, in which the relationship to fibrinolysis is less clear. For example, uPA−/− mice are more susceptible to lethal pulmonary infection (6) and to the development of pulmonary fibrosis (7), endpoints that might reflect contribution of uPA in cell adhesion (8) and migration to these phenotypes. However, the mechanism by which uPA is involved in these processes has not been established.

In a previous study we found that uPA enhances phenylephrine and endothelin-induced vasoconstriction of aortic rings isolated from rats (9). The possibility that uPA contributes to the regulation of vascular tone may help to explain some of the phenotypic changes described in uPA−/− mice and perhaps provide a broader understanding of the role played by uPA in certain physiological and pathological processes, such as inflammation (6) and metastasis. It was, therefore, of interest to examine the contribution of endogenous uPA to the regulation of vasoactivity in vivo.

These experiments, having been performed using rat aortic rings and human uPA, also raised a question as to the nature of the vascular receptor that mediates the contractile properties of uPA (9). Because rat uPAR does not bind human uPA (10), the results suggest that uPAR is not directly responsible for the observed vasoactivity, but they leave the identity of this other “uPA receptor” unresolved. Also uncertain is the effect of PAI-1, which binds to tcuPA with high affinity and neutralizes its proteolytic activity (11). The results of several studies indicate that PAI-1 is elevated in the plasma of patients with hypertension, and reduction of blood pressure by certain classes of anti-hypertensive agents is associated with a decrease in PAI-1 concentration (12–15). Whether this is a cause or result of therapy is unclear.

To address these questions, we turned to uPA−/− mice. In the present paper we report that uPA−/− mice have lower blood pressure than wild type (WT) animals and that the contractility of their blood vessels is attenuated in vitro and in vivo. We also report that the stimulatory effect of tcuPA on vascular smooth muscle cell activation is mediated by LRP and through a process that inhibited by PAI-1 but independent of its proteolytic activity.
**MATERIALS AND METHODS**

**Materials**—Recombinant full-length uPA and a uPA variant lacking the kringle were prepared in S2 cells and purified as in Ref. 9. Phenylephrine (PE) was purchased from Sigma. uPA−/− and uPAR−/− mice on a C57/black background and wild type C57/black mice were purchased from Jackson Laboratories. Four-month-old male mice were used in all studies. Recombinant 39-kDa receptor-associated protein (rRAP) and purified soluble LRP were generously provided by Dr. D. Strickland (American Red Cross, Rockville, MD). Anti-LRP antibodies were also provided by D. Strickland and by American Diagnostica Inc. (Greenwich, CT). The peptides (E111MD and RE111MD) were synthesized as previously described (16). tcuPA and its amino-terminal fragment (ATF) were prepared and purified as in Ref. 9.

**Blood Pressure Recording**—Mean arterial blood pressure (MABP) was measured as in Ref. 9. Mice were anesthetized by intraperitoneal administration of 10 mg/Kg−1 ketamine and 50 mg/Kg xylazine. One cannula was placed in the left carotid artery to record the mean arterial blood pressure, and a second through which PE (0.3 μg per animal) was administered was placed in the right jugular vein. Blood pressure was monitored continuously through a transducer placed in the left carotid cannula using the CARDIOSYS computerized system (ExperimentiaE, Budapest, Hungary).

In some experiments, tcuPA, ATF, tcuPA/PAI−1, or tcuPA/E111MD complex were infused through the cannula placed in the right jugular using a multisyringe pump at a rate of 5 μl/min (maximum 60 min), and the MABP was determined beginning 10 min later. In other experiments, anti-LRP antibody or control IgG was administered by constant infusion for 5 min followed by co-infusion of antibody with tcuPA or ATF for the remainder of the experiment.

**Contraction Response**—Experiments were performed as described in Ref. 9. Mice were sacrificed by exsanguination. Thoracic aortae were removed with care to avoid damage to the endothelium, dissected free of fat and connective tissue, and cut into transverse rings 5 mm in length (17–20). In another set of experiments, the rings were gently rotated on a stainless steel rod to remove the endothelium (denuded aorta). To record isometric tension the rings were mounted in a 10-ml bath containing oxygenated (95% O2, 5% CO2) solution of Krebs-Henseleit (KHF) buffer (144 mM NaCl, 5.9 mM KCl, 1.6 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, and 11.1 mM glucose). Each aortic ring was then contracted by adding PE stepwise increments from 10−10 to 10−5 M. In other experiments, uPA was added 15 min before addition of PE. In each experiment, rings exposed to KHF buffer alone were analyzed in parallel. Isometric tension was measured with a force displacement transducer and recorded online using a computerized system (ExperimentiaE). The half-maximal effective concentration (EC50) was calculated by measuring the response of the aortic rings to increasing concentration of PE. In all studies, results are shown as the mean ± S.E. from three experiments.

The half-maximal effective concentration (EC50) was calculated from data that show the response of the aortic rings to increasing concentration of PE and includes the data from at least three repetitions of the same experiment. After drawing a figure portraying the outcome, two lines that intersect with the y and x axes were drawn to determine the PE concentration that induces 50% of the maximal effect.

**Half-life of uPA**—The life-time of uPA in the circulation was determined as described previously (5). Briefly, mice were injected intravenously with a single dose of 125I-labeled tcuPA or 125I-labeled ΔK-tcuPA (0.1 mg/kg). Blood was withdrawn by intracardiac injection at the indicated time points and counted for radioactivity.

**RESULTS**

We previously reported that tcuPA, its ATF, and kringle domain enhance PE-induced vasoconstriction of rat aortic rings (9). In the present paper, we examined the contribution of endogenous uPA to blood pressure regulation in mice. To do so, we first compared the MABP of uPA−/− mice with that of WT controls. The data presented in Table I show that the blood pressure of uPA−/− mice was significantly lower than that of WT animals. The MABP in WT mice was 73.17 ± 3.97 mm Hg (n = 6), whereas the uPA−/− mice had an MABP of 53.83 ± 3.54 mm Hg (n = 6; p = 0.0005 in the paired t test). The lower MABP in uPA−/− mice reflected both a lowered systolic and diastolic pressures. The systolic and diastolic pressures in the uPA−/− mice were 63.17 ± 2.88 and 44.51 ± 4.43 mm Hg, while those in the WT mice were 87.6 ± 3.93 and 58.81 ± 3.86 mm Hg, respectively.

Similar differences were observed when the contractions of isolated aortic rings from the two sets of mice were compared. Aortic rings from uPA−/− mice had a reduced response to phenylephrine, i.e. rings from WT mice had an EC50 of 7.94 nM PE, whereas uPA−/− had an EC50 of 251 nM (Fig. 1).

Although aortic rings from uPA−/− mice showed an impaired contractile response to PE, their maximal response did not differ from that of WT animals at high concentrations of agonist (Fig. 1), indicating that these mice do not lack vascular receptors and that the downstream intracellular signal transduction mechanisms are intact. In support of this conclusion, injection of PE (0.3 μg) increased MABP in the WT and uPA−/− animals to the same level (127 ± 3 mm Hg in the WT compared with 128 ± 4 mm Hg in uPA−/− mice). Moreover, the impaired contractility of aortic rings from uPA−/− mice was almost completely reversed by the addition of 1 nM mouse or human tcuPA (Fig. 2). These results indicate that it is the deficiency of uPA itself and not a secondary defect in the vasculature of these mice that underlies the difference in phenotype.

Having observed that uPA is involved in the regulation of the vasoactivity in vivo, our next goal was to identify the responsible cellular receptor. The finding that human and mouse uPA had a similar effect on the tension in mouse aortic rings (Fig. 2) suggests strongly that uPAR is not directly involved in the uPA-mediated vasoactivity. However, to examine the involvement of uPAR directly, we measured mean aortic blood pressure in uPAR−/− mice. The MABP of uPAR−/− mice (71 ± 4.29 mm Hg, n = 6) and WT mice were virtually identical. Second, the response of isolated aortic rings from uPAR−/− mice to PE was similar to that of WT mice, with an EC50 of 8.45 nM.

It is known that tcuPA binds to LRP (21, 22) and that binding of ligands, such as α2-macroglobulin, to LRP triggers mobilization of Ca2+ in certain cell types (23). Therefore, we evaluated LRP as a candidate receptor involved in uPA-mediated vasoconstriction.

To examine the potential involvement of LRP in the tcuPA-induced vasoactivity, we used two different approaches. First, we studied the effect of the receptor-associated protein (RAP) on tcuPA-mediated contractility. RAP is a 39-kDa protein that co-purifies with LRP and inhibits the binding of tcuPA (24). rRAP (20 nM) prevented uPA from restoring normal contractility to aortic rings from uPA−/− mice. Second, anti-LRP antibodies prevented (20 nM) tcuPA-enhanced contraction, whereas a blocking antibody to the LDL receptor had no effect (Fig. 3). Neither rRAP nor anti-LRP affected PE-induced vasoconstriction in the absence of uPA (Fig. 3).

LRP has been shown to mediate the clearance of uPA from in vivo studies. The effect of LRP on the clearance of uPA has been studied in vivo, and it was found that the clearance of uPA is increased in the presence of LRP. This suggests that LRP may play a role in the regulation of uPA levels in vivo. The exact mechanism by which LRP affects uPA clearance is not fully understood, but it is likely that LRP binds to uPA and promotes its degradation or sequestration.

**TABLE I**

| MABP (mmHg) |
| --- |
| Wild type |
| Knock-out |
| 75 | 50 |
| 77 | 49 |
| 66 | 55 |
| 72 | 56 |
| 73 | 58 |
| 76 | 55 |
Data shown as percent of maximum effect.

In this and in each figure, the mean ± S.D. of three experiments is shown. Data shown as percent of maximum effect.

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A

FIG. 1. Contraction of mouse aortic rings. Contraction of aortic rings from wild type (empty squares) and uPA−/− mice (filled squares) was induced by adding increasing concentrations of phenylephrine. In this and in each figure, the mean ± S.D. of three experiments is shown.

B

FIG. 2. Effect of uPA on the EC50 of aorta rings from knockout mice. The half-maximal effective concentration (EC50) was calculated from the response of aortic rings from WT, urokinase (uPA−/−) or urokinase receptor knock-out (uPAR−/−) mice to increasing concentrations of PE. The aortic rings from uPA−/− mice were examined in the absence (uPA−/−) or presence of 1 nM human uPA (uPA−/− (1 nM HuPA)) or 1 nM mouse uPA (uPA−/− (1 nM MuPA)).

FIG. 3. Effect of rRAP and anti-LRP antibodies on the vasoactivity of tcuPA. The EC50 of PE-induced vasoconstriction in aortic rings from WT or uPA−/− mice was measured. In the case of uPA−/− mice, the EC50 was determined in the absence (uPA−/−) or in the presence of 1 nM tcuPA (uPA−/− (1 nM tcuPA)) and 20 nM anti-LRP antibodies (uPA+Ant-LRP), 1 nM tcuPA and 20 nM anti-LDL receptor antibodies (uPA+ir Ab), 20 nM rRAP (RAP) or anti-LRP antibodies alone (Anti-LRP).

The half-life of uPA (25). Therefore, we next examined the effect of anti-LRP antibodies on the regulation of blood pressure by uPA in vivo. Injecting human uPA to uPA−/− mice (0.1 mg/kg) restored the MABP to normal (Fig. 4A); higher doses of uPA had no additional effect (not shown). Preinjection of anti-LRP abolished the restoration of blood pressure by uPA (Fig. 4A) and increased its half-life in the circulation (Fig. 4B).

We previously reported (9) that the stimulatory effect of uPA on PE-induced vasoconstriction is mediated by its kringle. In agreement with this, a uPA variant in which the kringle was deleted (ΔΚ-uPA) was unable to restore blood pressure in uPA−/− mice (Fig. 4A). Moreover, the half-life of ΔΚ-uPA was prolonged comparably to that of WT uPA measured in the presence of anti-LRP (Fig. 4B).

Plasminogen activator inhibitor type 1 (PAI-1) binds tightly to tcuPA and enhances the affinity of the resultant complexes for LRP (26, 27). To examine the effect of PAI-1 on uPA-mediated contractility, aortic rings were contracted by adding increasing concentrations of PE in the presence of 1 nM tcuPA alone or together with equimolar concentrations of PAI-1. As expected, tcuPA (1 nM) augmented the PE-induced contraction of aortic rings from uPA−/− mice, decreasing the EC50 of PE from 254 to 9.43 nM (Fig. 5). In the presence of an equimolar concentration of PAI-1 the effect of tcuPA was abolished (EC50 248 nM) (Fig. 6).

tcuPA binds to PAI-1 through two distinct epitopes, one involving its catalytic site. The other site, EEIMD, comprises residues 350–355 in PAI, which interacts with residues 179–184 in uPA (28). We previously found that the isolated EEIMD peptide inhibits the binding of PAI-1 to tcuPA and regulates the binding of scuPA to cell-associated
and purified LRP (16). Therefore, we examined the effect of EEIIMD on the vasoconstrictive properties of tcuPA. EEIIMD (1 μM) abolished the capacity of tcuPA to restore PE-induced vasoconstriction of aortic rings from uPA−/− mice to normal (Fig. 6). In contrast, the control peptide, REIIMD, which has almost no effect on PAI-1 binding to uPA (16), did not affect tcuPA-mediated vasoactivity. Neither did PAI-1 nor EEIIMD alone alter the contraction of aortic rings. The inhibitory effect of PAI-1 and the PAI-1-derived peptide on tcuPA vasoactivity was also seen using aortic rings from WT mice (not shown). The vasoactive effect of the uPA ATF is similar to that of tcuPA (9), but ATF lacks proteolytic activity and the binding epitopes for PAI-1 and EEIIMD peptide. PAI-1 had no effect on ATF-induced vasoactivity (Fig. 7). The same result was obtained with 1 μM EEIIMD (not shown), excluding an effect of PAI-1 or EEIIMD on other components of the system.

PAI-1 also modulated the procontractile effect of tcuPA in vivo. tcuPA (0.1 mg/kg) no longer was able to restore the blood pressure of uPA−/− mice in the presence of an equimolar concentration (~40 nm) of PAI-1 (Fig. 8); the same outcome was observed after infusion of EEIIMD. In contrast, ATF (0.1 mg/kg), which had the same procontractile effect as full-length tcuPA, was refractory to PAI-1 and to EEIIMD (Fig. 8).

To examine the role of the endothelium and specifically that of eNOS synthase in uPA-mediated vasoconstriction, we first studied the effect of tcuPA on denuded rat aortic rings. We used rat aortic rings because it is technically difficult to denude aortic rings from mice completely and atraumatically. As previously reported by us (9), tcuPA stimulated the PE-induced constriction of isolated rat aortic rings but had no vasoactive effect on denuded rings (Fig. 9). Second, L-NAME inhibited uPA-mediated contraction of rat (Fig. 9) and mouse (not shown) aortic rings.

DISCUSSION

Our data show that uPA participates in the endogenous regulation of blood pressure in vivo. This conclusion is based on the following observations. First, uPA−/− mice have reduced systolic and diastolic arterial blood pressures. Second, aortic rings isolated from uPA−/− mice have impaired contractile responses to low doses of phenylephrine. Third, the lower blood
pressure in uPA−/− mice and the attenuated response of isolated aortic rings from these mice are restored to normal by addition of uPA exogenously. Fourth, ligands that bind to uPA, such as PAI-1 or the PAI-1-derived peptide, neutralize its vasoactivity.

The observations that aortic rings from uPAR−/− mice respond normally to PE and that these mice have a normal blood pressure indicates that the effect of uPA is independent of this receptor. The difference in blood pressure observed in uPA−/− and uPAR−/− mice is reminiscent of other differences in their phenotypes (4) and supports the existence of additional uPA receptors.

The finding that anti-LRP antibodies and rRAP abolish the effect of uPA in vitro and in vivo suggests that the effect of uPA on vascular contractility is mediated through LRP. This conclusion is supported by the observation that ligands that affect the interaction of tcuPA with LRP (PAI-1 and EEIIMD) attenuate the vasoactive effect of uPA. Our findings support the hypothesis of Baaskei et al. (23) that LRP can act as a signal-transducing receptor to mobilize release of intracellular Ca2+, which is necessary for contraction of vascular smooth muscle cells.

The ability of PAI-1 to neutralize the provasocontractile effects of uPA is relevant to understanding the behavior of LRP as well as smooth muscle cell contraction. PAI-1 binds to LRP (26, 27), but does not affect PE-induced vasocostriction unless complexed with tcuPA. Indeed, PAI-1 and its derived peptide (EEIIMD) inhibit the vasocontractile effect of uPA although both augment binding of urokinase to LRP (16, 26, 27). These findings indicate that not all LRP ligands transduce signals leading to vasocostriction. Additional study is needed to understand how LRP differentially handles the diverse ligands that it encounters. Several groups have reported that PAI-1 levels are elevated in hypertensive patients and that reduction of blood pressure by certain, but not all, forms of medical treatment are associated with a decrease in PAI-1 (12–15). Whether changes in PAI-1 are a cause or consequence of changes in vascular tone is unknown. Nevertheless, our studies raise the possibility that vascular tone and other critical behaviors of vascular smooth muscle cells are regulated in part through LRP and can be modified by PAI-1-derived peptides.

The mechanism by which uPA internalized by LRP signals intracellularly requires additional study. It is widely accepted that LRP is an endocytic receptor that delivers ligands to lysosomes. Recently, LRP has also been shown to initiate intracellular signaling. The human immunodeficiency virus-Tat protein binds to LRP and is internalized into endosomes and later translocated to the nucleus, by a process that is poorly understood, where it stimulates transcription (29). Furthermore, Pseudomonas exotoxin A, which mediates ADP-ribosylation of elongation factor 2 in the cytoplasm, enters the cell exclusively via LRP (30). The toxin is composed of a single-chain polypeptide that harbors a fusogenic domain that mediates its translocation into the cytoplasm (30). It is possible that a fraction of the unbound uPA may escape proteolysis and exit the lysosomes to reach another location where it can initiate signal transduction. Conversely, binding of uPA to PAI-1 may change its destination by accelerating its degradation, thereby impeding signal transduction. This hypothesis is in accord with our previous data that show that PAI-1 and PAI-1-derived peptide (EEIIMD) increases the degradation of uPA (16, 31).

It is noteworthy that the differences we found in blood pressure of anesthetized WT (and uPAR−/−) compared with uPA−/− mice were recorded in the absence of any exogenous vasoactive compounds. Injection of PE increased the MABP in WT and uPA−/− mice to the same level, and a similar effect was seen in isolated aortic rings. These findings suggest that under certain physiological and/or pathological conditions, such as pain or stress, the decreased blood pressure in uPA−/− may not be evident. This is consistent with the absence of an effect of uPA on aortic blood pressure measured through an open abdominal incision in a setting associated with significant hypotension (32).

Although we have shown that urokinase acts on LRP as a ligand to modulate blood vessel reactivity, the details of the downstream signaling pathway and the involvement for eNOS will also require additional study. Better insights into this process may provide information that will help to elucidate the relationship among the fibrinolytic and inflammatory systems, vasoactivity, and cancer invasiveness, in which urokinase and PAI-1 are known to play key roles (33). Furthermore, a better understanding of the mechanism by which uPA regulated vasoactivity may facilitate the design of thrombolytic agents with more selective fibrinolytic activity or antagonists that block vascular effects that contribute to unwanted inflammatory actions or tumor cell migration.

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