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MECHANISMS OF KRINGLE FRAGMENT OF UROKINASE INDUCED VASCULAR SMOOTH MUSCLE CELL MIGRATION

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Abstract

Background—uPA is involved in vessel remodeling and mediates smooth muscle cell migration. Migration in response to uPA is dependent on both the growth factor binding domain at the aminoterminal end and the kringle (K) domain of the molecule. uPA is readily degraded *in vivo* into these constitutive domains. The aim of this study is to examine cell signaling during the migration of smooth muscle cell (SMC) in response to the kringle domain of urokinase.

Methods—Murine arterial SMCs were cultured *in vitro*. Migration assays were performed in the presence of K with and without the plasmin inhibitors (aprotinin and ϵ -aminocaproic acid), the G α i inhibitor Pertussis toxin, the MMP inhibitor (GM6001), the PI3-K inhibitors, Wortmannin and LY294002, and the MAPK inhibitors PD98089 (MEK1 inhibitor) and SB203580 (p38^{MAPK} inhibitor). Western blotting was performed for ERK 1/2 and p38^{MAPK} phosphorylation after stimulation with K in the presence and absence of the inhibitors. Statistics were analyzed by one-way ANOVA (n=6).

Results—The kringle domain (K) induced a plasmin-independent, MMP dependent increase in cell migration (2-fold, $p<0.05$) compared to control. This migratory response to K was G α i mediated and dependent on both ERK1/2 and p38^{MAPK} activation. K-induced time-dependent increases in the phosphorylation of ERK1/2 (3-fold, $p<0.05$) and p38^{MAPK} (3-fold, $p<0.05$). Activation of p38^{MAPK} and ERK1/2 was completely inhibited by the PI3-K inhibitors. We explored a potential role for the epidermal growth factor receptor (EGFR). K induced EGFR phosphorylation and the presence of AG1478, the EGFR inhibitor, inhibited both cell migration and akt activation in response to K.

Conclusion—Kringle domain of uPA induces smooth muscle cell migration through a G-protein coupled PI3-K dependent process involving both ERK1/2 and p38^{MAPK} and is mediated in part through EGFR. Defining the differences in response of key molecular domains of uPA is vital in order to understand its role in vessel remodeling.

INTRODUCTION

The migration of vascular smooth muscle cells (VSMC) occurs during vessel remodeling in response to injury and alterations in flow. Such migration involves the complex regulation of proteases, integrins, and extracellular molecules within the microenvironment of the vessel

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wall. Urokinase plasminogen activator (uPA) is a serine protease that is the primary plasminogen activator in tissue remodeling processes (1,2). uPA can induce both cell proliferation and cell migration and these responses use different signaling pathways and involve the epidermal growth factor receptor (EGFR) (3). Within the micro-environment of the cell wall, uPA can also be cleaved into several biologically active fragments: aminoterminal fragment (ATF), kringle domain (K) and carboxyterminal fragment (CTF) by other proteases. Each appears to have unique characteristics (4-6). We have shown that ATF will induce plasmin-independent cell migration and that CTF will induce plasmin-dependent cell proliferation (4). We have also demonstrated that K domain will induce cell migration and not cell proliferation (4). Stepanova et al have suggested that K domain may be the most important fragment during uPA mediated migration (7,8). Kringle can mediate heparin binding and may interact with the E4 domain of the glycoprotein laminin and thus, induce cell migration separate from uPAR (9). The signaling processes that lead to migration in response to the kringle domain are not well understood. The aim of this study is to define the signaling pathways used by the kringle domain during smooth muscle cell migration.

MATERIALS AND METHODS

Experimental design

Murine arterial VSMCs were cultured *in vitro* as previously described (3). Linear wound and Boyden chamber assays of migration were performed in the presence of K with and without the plasmin inhibitors (aprotinin (100 units/ml) and ϵ -aminocaproic acid EACA, (100 μ M)), the MMP inhibitor (GM6001), the G-protein inhibitors, Pertussis Toxin (Gai; 100ng/ml) and GP-2A (Gaq; 10 μ M), the PI3-K inhibitors, Wortmannin (10nM) and LY294002 (10 μ M), the MAPK inhibitors PD98059 (MEK1 inhibitor; 10 μ M) and SB 203580 (p38^{MAPK} inhibitor; 10 μ M) and the EGFR inhibitor, AG1478 (AG; 100nM). The specificity of inhibitors and their concentrations have been discussed previously (10,11). Western blotting was performed for akt, MEK1/2 and ERK 1/2, RSK90, MKK3/6 and p38^{MAPK} and EGFR phosphorylation after stimulation with K in the presence and absence of the inhibitors.

Materials

Kringle was purchased from American Diagnostica, Inc. (Greenwich, CT). EACA and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated anti-rabbit IgG antibody (raised in goat) and Peroxidase-conjugated anti-mouse IgG antibody (raised in goat) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Phospho-ERK1/2 antibody was purchased from Promega, Inc. (Madison, WI). Total ERK 1/2 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Phospho-p38^{MAPK} antibody was purchased from Biosource (Camarillo, CA). Total p38^{MAPK}, phospho-MEK1/2 (ser217/221) and phospho-MKK3/6 (ser189/207) antibodies were purchased from Cell Signaling (Beverly, MA). Dulbecco's minimal essential medium (DMEM) and dulbecco's phosphate buffered saline (dPBS) were purchased from Cellgro.

Pertussis toxin catalyzes the ADP-ribosylation of the α subunit of G_i and G_o specifically, preventing coupling with receptors (12), and was used at a standard inhibitory concentration. Pertussis toxin does not effect G_{aq}, even when modified at its C-terminus to mimic G_i (13). Wortmannin specifically inhibits PI3-K at low nanomolar concentrations, and may affect myosin light chain kinase at the 10nM dose (14); however, it does not inhibit tyrosine kinases, protein kinase C, or PI 4-kinase (15). Wortmannin has been demonstrated to inhibit PI3-K activity and Akt phosphorylation at 10nM in VSMCs (16), and our own data on Akt phosphorylation confirmed its effectiveness at this dose (see Results). LY294002 is a competitive inhibitor of PI3-K, with an IC₅₀ of 1.4 μ M (17). LY294002 appears to be more specific than Wortmannin, with no effect on ERK, EGF receptor tyrosine kinase, PI4-K, protein

kinase C, protein kinase A, or S6 kinase (17). The 10 μ M dose was chosen as being slightly above the IC₅₀ to ensure complete PI3-K inhibition, and its effectiveness was confirmed with our own Akt phosphorylation data (see Results). PD98059 inhibits MEK1 (IC₅₀ 2-7 μ M) and MEK2 (IC₅₀ 50 μ M); it does not inhibit other MEKs or p70S6 kinase *in vitro* (18). PD98059 had no effect on p38^{MAPK} activation by K in our VSMCs at 25 μ M (data not shown), while it completely inhibited ERK 1/2 activation at this dose (13). SB203580 is a pyridinyl imidazole that specifically inhibits the activated α and β isoforms of p38^{MAPK} with an IC₅₀ 600nM (19). SB203580 demonstrates no inhibition of a wide range of other kinases, including ERK 1/2, JNK, ERK5, and several phosphatases in this dose range (20), although it has been shown to activate Raf-1 (without effect on MEK1/ERK) and to inhibit cytokine production (21). GM6001, a hydroxamic acid HONHCOCH₂CH(i-Bu)CO-L-Trp-NHMe, isomer 6A (GM 6001) completely inhibited all gelatinolytic activity on a zymogram (22,23)

Cell migration

VSMC migration in response to K (10nM) was performed in the presence and absence pharmacological inhibitors using both the linear wound assay and the Boyden microchemotaxis chamber assays were performed as previously described (13,24). PDGF (10nM) and uPA (10nM) were used as positive controls

Western Blotting

VSMC were allowed to grow to 80% confluence and starved for 48hours. Cells were then stimulated with K alone and in the presence of pharmacological inhibitors; cells were harvested at time points from 0 to 30 minutes. Western blotting for both activated and total kinases was performed as previously described (24).

Data and Statistical Analysis

All data are presented as the mean $\pm$ standard error of the mean (s.e.m.) for 6 experiments and statistical differences between groups were tested with a Kruskal-Wallis nonparametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant. Non-significant p-values were expressed as p=ns.

RESULTS

Cell migration

The kringle domain (K) induced a plasmin-independent, MMP-dependent increase in cell migration (2-fold, p<0.05) compared to control (Table 1). The presence of the G-protein inhibitors, PTx but not the GP-2A inhibited the migratory response (Table 1). This migratory response to K was both ERK1/2 and p38^{MAPK} dependent as demonstrated by the inhibitory effects of PD, a MEK1/2 inhibitor, and SB, a p38^{MAPK} inhibitor, on migration (Table 1). Inhibition of PI3-K also interrupted cell migration.

ERK1/2 signaling

K-induced time-dependent increases in the phosphorylation of MEK1/2 (3-fold, p<0.05), ERK1/2 (3-fold, p<0.05) and RSK90 (4-fold, p<0.01) (Fig. 1A). We did not see sustained ERK1/2 activation (data not shown). Activation of both MEK1/2, the upstream kinase of ERK1/2 and ERK1/2 was completely inhibited by the presence of the MEK1/2 inhibitor, PD. In the presence of PD, the downstream kinase for ERK1/2, RSK90 at both phosphorylation sites was also inhibited (Fig. 1B). The presence of the G-protein inhibitors, PTx but not GP-2A inhibited K induced activation of the ERK1/2 pathway kinases (Fig. 1C). The ERK1/2 pathway was inhibited by both PI3-K inhibitors (Fig. 1D).

p38^{MAPK} signaling

K-induced time-dependent increases in the phosphorylation of MKK3/6 p38^{MAPK} and ATF2 (3-fold, $p < 0.05$) (Fig. 2A). Activation of MKK3/6, upstream kinase of p38^{MAPK}, was blocked by the G α i G-protein inhibitor but not the G α q G-protein inhibitor and unaffected by the p38^{MAPK} inhibitor, SB (Fig. 2B). Activation of p38^{MAPK} was blocked by the G α i G-protein inhibitor but not the G α q G-protein inhibitor and completely inhibited by the p38^{MAPK} inhibitor, SB (Fig. 2B). Activation of ATF-2, a downstream target of p38^{MAPK}, was completely inhibited by the p38^{MAPK} inhibitor, SB. Incubation with both PI3-K inhibitors prevented MKK3/6, p38^{MAPK} and ATF-2 activation (Fig. 2B).

akt signaling

K induced a time dependent increase in akt phosphorylation. This activation was inhibited by both PI3-K inhibitors (LY and Wn) (Fig. 3A). Incubation with G α i inhibitor blocked akt activation. Incubation with PD, the MEK1/2 inhibitor, or SB, the p38^{MAPK} inhibitor, had no effect on akt phosphorylation (Fig. 3B).

EGFR

K induced time dependent EGFR phosphorylation and the presence of AG1478 (Fig. 4A), the EGFR inhibitor, inhibited both cell migration and MAPK activation (ERK1/2 and p38^{MAPK}) in response to K (Table 1 and Fig. 4B). Activation of EGFR was inhibited by G α i inhibition but not by pre-incubation with PD and SB or both PI3-K inhibitors (Fig. 4B).

DISCUSSION

Plasminogen activation and arterial injury

Regulation of plasminogen activation has been shown to play a significant role in the development of intimal hyperplasia. Over-expression of uPA and deficiency of PAI-1 in transgenic mice have been shown to promote neo-intimal formation, while the absence of plasminogen prevents lesion formation (25-27). Unilateral partial ligation of the carotid system in the mouse induces a low flow state, which in turn leads to remodeling in the vessel and the development of a neointima without injury which can be correlated with a significant increase in the expression of u-PA (28,29). uPA consists of 3 domains, aminoterminal (ATF), kringle and carboxyterminal and the intact molecule can be broken down in vivo into these domains and several reports have suggested that Kringle domain of uPA may be the most important fragment during uPA mediated migration (7,8,30,31). Our study demonstrates that Kringle domain of uPA induces smooth muscle cell migration through a G α i G-protein coupled PI3-K dependent process involving both ERK1/2 and p38^{MAPK} and that this process is mediated through the EGFR. Kringle can mediate heparin binding and may interact with the E4 domain of the glycoprotein laminin and thus, induce cell migration (9). This activation profile is distinct from that observed with the ATF of uPA, which is G α i G-protein dependent, requires PI3K and is mediated through the activation of the FPRL-1 formic peptide G-protein coupled receptor to initiate MAPK-mediated VSMC migration (1-6,11).

MAPK

Kringle activates MEK-ERK1/2-RSK90 in a G α i, PI3K dependent manner and this pathway is sensitive to the MEK inhibitor PD98059. The ERK pathway is one of the classical pathways described in cell signaling. It is accepted that ERK1/2 can be activated by ras-raf-MEK1/2 cascade. Additionally, there is data to suggest that PI3-K can also initiate ERK1/2 independent of ras activation. Multiple reports have shown that ERK1/2 activation is involved in both migration and proliferation. However, the difference between migration and proliferation appears to be the finding that sustained ERK1/2 activation is required for proliferation (32).

We did not see a prolonged activation of ERK1/2 consistent with a presumption that only short term activation is necessary for cell proliferation. Furthermore, our data suggests that K can interact with raf signaling and PI3K signaling to mediated ERK1/2 activation. In addition to ERK1/2 activation p38^{MAPK} activation was also observed. The p38^{MAPK} kinase pathway has been implicated in the control of both cellular proliferation and apoptosis (33,34) and is considered to be activated by cellular stress. Both G-protein coupled receptors and receptor tyrosine kinases will induce p38^{MAPK} activation. In vitro, LPA and PDGF-BB induce vascular smooth muscle cell proliferation and phosphorylation of both ERK1/2 and p38^{MAPK}. The presence of the p38^{MAPK} inhibitor, SB203580, has been shown to inhibit both cell proliferation and phosphorylation of p38^{MAPK} in a dose dependent manner (35). SB203580 does not effect ERK 1/2 activation and the presence of PD98059 (a MEK1 inhibitor) has no effect on the phosphorylation of p38^{MAPK} (35). Our data suggests that Kringle activates p38^{MAPK} and that p38^{MAPK} is involved in VSMC migration. p38^{MAPK} interacts with the cytoskeleton through Heat Shock Proteins (HSP) and there is evidence that HSPs are important in SMC contraction, a necessary mechanism for migration (36). We did not see a similar response with the intact molecule, uPA. When uPA was applied to the cells p38^{MAPK} inhibition did not affect VSMC migration. However there is evidence that alternative uPA-uPAR initiated signal transduction may occur depending on integrin clustering. In tumor cells, uPA-uPAR- α 5b1 complexes bind fibronectin (FN). Through a caveolin-independent mechanism, the active uPA-uPAR- α 5b1-FN fibril complex triggers activation of the ERK1/2 pathway while the presence of fibronectin fibrils along with cytoskeleton reorganization will suppresses p38^{MAPK} (37). Thus, it is possible that accumulation of different integrins within the uPA multi-protein complex or simply the activation of different integrins may alter p38^{MAPK} activation with the intact molecule. The intact molecule may induce a different clustering of integrins compared to fragments which may explain these divergent findings.

EGFR and uPA

Our previous data suggests that uPA induces EGFR transactivation and that EGFR is involved in both cell migration and proliferation (3). The current data suggests that EGFR is involved and that activation of EGFR by K is dependent on a G-protein mediated event. Receptor transactivation is the phenomenon whereby activation of a given receptor activates a heterologous receptor (38). This situation is commonly encountered in respect to G-protein coupled receptors (GPCR) and receptor linked tyrosine kinases (RTK). ET-1, LPA and thrombin induce rapid phosphorylation of the EGFR and suppression of this EGFR activation leads to reduced ERK1/2 activation. At present, the triple membrane passing signaling (TMPS) mechanism of GPCR-associated EGFR activation is a widely accepted model of RTK transactivation. In this model, there is a sequence of three transmembrane signaling events: GPCR activation followed by MMP activation and subsequent activation of the EGFR by the tethered ligand heparin binding-EGF (HB-EGF) or other latent ligands of the EGFR. K can bind to the glycoprotein laminin and interact with heparin binding, the former can be linked to integrin receptors and the latter to tethered ligand release. Integrin-mediated adhesion induces assembly of a macromolecular complex containing c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosine residues (39). Integrin related transactivation of EGFR which can be G-protein linked via integrin linked kinase (ILK) activation (40). While we have identified a role for EGFR in this model further work on integrin mediated signaling and focal adhesion complex activation in response to K will be required (41).

Conclusion

Kringle domain of uPA induces smooth muscle cell migration through a G α i, G-protein coupled PI3-K dependent process that requires EGFR and involves both ERK1/2 and p38^{MAPK} (Fig

- 5). Defining the differences in response of key molecular domains of uPA is vital in order to understand its role in vessel remodeling.

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A Kringle and ERK Pathway

c Concentration Response Curve for PD98059

D Concentration Response Curve For LY294002

E Concentration Response Curve For AG1478

Figure 1.

Kringle induced time dependent activation of MEK1/2, ERK1/2 and RSK90 (A). A representative Western blot is shown (B). MEK1/2, ERK1/2 and RSK90 activation was blocked by pertussis Toxin (PTx), PI3K inhibitors (Wn and LY), MEK1/2 inhibitor PD, and EGFR inhibitor (AG, AG1478) (F). Concentration response curves for PD, LY and AG are shown (C, D and E). Values are the mean $\pm$ SEM fold increase over DMEM control (ratio of activated/total kinase). * $p < 0.05$ compared to control at maximal activation ($n=6$).

A Kringle and p38^{MAPK} Pathway

c Concentration Response Curve for SB203580

D Concentration Response Curve for LY294002

E Concentration Response Curve for AG1478

Figure 2.

Kringle induced time dependent activation of MKK3/6, p38^{MAPK} and ATF-2 (A). A representative Western blot is shown (B). MKK3/6, p38^{MAPK} and ATF-2 activation was blocked by pertussis Toxin (PTx), PI3K inhibitors (Wn and LY), and EGFR inhibitor (AG1478) (C and D). The p38^{MAPK} inhibitor SB inhibited p38^{MAPK} and ATF-2 activation (B). The MEK1/2 inhibitor PD had no effect. A representative Western blot is shown. Values are the mean±SEM fold increase over DMEM control (ratio of activated/total kinase). *p<0.05 compared to control at maximal activation(n=6).

A

Kringle and akt

B 0 5 10 20 30 mins

Figure 3.

Kringle induced time dependent activation of akt (A). A representative Western blot is shown (B). Akt activation was blocked by pertussis Toxin (PTx), PI3K inhibitors (Wn and LY), and EGFR inhibitor (AG1478). Neither the MEK1/2 inhibitor PD nor the p38^{MAPK} inhibitor SB inhibited akt activation. Values are the mean±SEM fold increase over DMEM control (ratio of activated/total kinase). *p<0.05 compared to control at maximal activation (n=6).

c Concentration Response Curve for AG1478

Figure 4.

Kringle induced time dependent activation of EGFR (A). A representative Western blot is shown (B). EGFR activation was blocked by pertussis Toxin (PTx) and EGFR inhibitor (AG1478) (C and D). There was no inhibitory effect of the PI3K inhibitors (Wn and LY), the MEK1/2 inhibitor PD, the p38^{MAPK} inhibitor SB on akt activation. Values are the mean±SEM fold increase over DMEM control (ratio of activated/total kinase). *p<0.05 compared to control at maximal activation(n=6).

Figure 5.

Proposed Kringle interaction with MAPKs and the role of EGFR: This flow diagram illustrates the proposed pathway by which K activates MAPK and induces VSMC migration. K induces VSMC migration through ERK1/2 and p38^{MAPK} mediated pathways. Activation of MAPK by K is dependent on a Gαi protein that leads to EGFR phosphorylation and EGFR transactivation induces PI3K-dependent, MEK1-mediated ERK1/2 and MKK3/6 mediated p38^{MAPK} activation. Blockade of either pathway prevents VSMC migration.

Table 1

	KRINGLE AND CELL MIGRATION	
	Wound Assay % decrease in area after 24 hrs	Boyden Chamber Fold change compared to DMEM
PDGF (10nM)	-30±1	4.1±0.3
uPA (10nM)	-15±2	2.2±0.2
Kringle (K, 10nM)	-17±4	2.3±0.1
Protease inhibitors+K		
aprotinin	-16±3	2.1±0.3
ε-aminocaproic acid	-15±4	2.2±0.3
GM6001	-7±1 **	1.3±0.2*
G-protein inhibitor		
Gαi (Pertussis Toxin)	-6±5 **	1.2±.2*
Gαq (GP-2A)	-15±4	2.2±.3
PI3-K inhibitors		
Wortmannin	-7±2 **	1.1±.1*
LY294002	-3±3 **	1.1±.1*
MAPK inhibitors		
PD98059	-8±1 *	0.8±.1 **
SB203580	-3±1 *	0.9±.1 **
EGFR inhibitor (AG1478)	-6±4 **	1.2±0.1

Values are mean±SEM (n=6; p<0.05, *; p<0.01, **).