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UROKINASE REQUIRES NAD(P)H OXIDASE TO TRANSACTIVATE THE EPIDERMAL GROWTH FACTOR RECEPTOR

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Abstract

Background—Cell migration is an integral component of intimal hyperplasia development and proteases are pivotal components in the process. Cell migration in response to urokinase is mediated through the aminoterminal domain (ATF) of the protein. This study examines the role of NAD(P)H oxidase during EGFR transactivation by ATF in human vascular smooth muscle cells (VSMC).

Methods—Human VSMCs were cultured in vitro. Linear wound and Boyden microchemotaxis assays of migration in response to ATF were performed in the presence and absence of NAD(P)H oxidase inhibitors (DPI and apocynin) and siRNA to Nox1. Additional assays were performed to examine the upstream pathways that lead to NAD(P)H oxidase activity. Assays were also performed for EGFR activation.

Results—ATF produced concentration-dependent VSMC migration, which was inhibited by increasing concentrations of DPI and apocynin. ATF was shown to induce time-dependent EGFR phosphorylation, which peaked at 4-fold greater than control. This response was inhibited by DPI and apocynin in a concentration-dependent manner. ATF induced a concentration-dependent increase in intracellular oxygen free radical species, which was mitigated by the presence of DPI and apocynin. Inhibition of $G\beta\gamma$ by βARK_{CT} reduced both NAD(P)H oxidase activity and EGFR activation. Inhibition of rac, which allows the NAD(P)H complex to assemble on the membrane, and inhibition of src, which induces assembly of the complex, both reduced ATF-dependent NAD(P)H oxidase activity and EGFR phosphorylation. siRNA to Nox1 prevented ATF-mediated EGFR activation and cell migration.

Conclusion—ATF requires NAD(P)H oxidase activity through a $G\beta\gamma$, rac and src-mediated pathway to facilitate transactivation of EGFR and VSMC migration.

Keywords

uPA; growth factor domain; NAD(P)H Oxidase; migration; cell signaling; human coronary smooth muscle cell

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INTRODUCTION

The migration of vascular smooth muscle cells (VSMC) involves the complex regulation of proteases, integrins and extracellular molecules leading to the sequence of attachment, detachment and contraction events which allow a cell to move through the extracellular matrix. Vascular smooth muscle cell migration in vivo can be induced by alterations in flow, inflammation, atherosclerotic processes and in response to angioplasty and stenting. Urokinase plasminogen activator (uPA) is the serine protease that is the primary serine protease (plasminogen activator) in these tissue-remodeling processes (1) and increased serum uPA is associated with development of restenosis after coronary angioplasty (2). uPA induces cell migration as a result of the binding of its aminoterminal domain (ATF) with the urokinase receptor (uPAR). We have shown that ATF can strongly induce G protein-mediated, plasmin-independent, VSMC migration in vitro (3), which is both PI3K-ERK1/2 and PI3K-akt dependent (4). We have also demonstrated that ATF induces an A Disintegrin and Metalloproteinase Domain-mediated epidermal growth factor receptor (EGFR) transactivation, which is important in ATF-mediated VSMC migration (5). In general terms, EGFR activation by G protein-coupled receptors (GPCR) occurs through a series of intracellular and extracellular signaling. Oxygen free radical production through NAD(P)H oxidase activation is considered an important early event in the intracellular activation pathway. These NAD(P)H oxidases (Nox1–4) represent a class of hetero-oligomeric enzymes that generate oxygen free radicals in VSMC. The intracellular pathway by which ATF mediates EGFR activation is not well described. This study examines the role of NAD(P)H oxidase during EGFR transactivation by ATF in human vascular smooth muscle cells.

METHODS

Experimental Design

Human coronary arterial VSMCs were cultured in vitro (passage 3–6; Clontech, Mountain View, CA). Linear wound and Boyden microchemotaxis assays of migration in response to ATF were performed in the presence and absence of NAD(P)H oxidase inhibitors (diphenyleneiodonium (DPI) and apocynin) and siRNA to Nox1. Additional assays were performed to examine the upstream pathways mediated by G $\beta\gamma$, rac and src that lead to NAD(P)H oxidase activity using β ARK_{CT}, NSC23766 and PP2 inhibitors, respectively. Supplementary experiments were performed with dominant negative src (DNsrc) and dominant negative rac (DNrac). Assays were also performed for EGFR activation in response to ATF in the presence and absence of inhibitors to NAD(P)H oxidase, G $\beta\gamma$, rac and src.

Wound Assay

The wound assay was performed with VSMC as previously described (3, 6). Trials with each reagent or inhibitor were performed in six separate dishes, and the results were averaged. Cells were then allowed to migrate over 24 hours at 37°C in Dulbecco's modified Eagle's medium (DMEM) with or without ATF (10 nM). In a second series of experiments, migration in response to ATF was examined in the presence and absence of the inhibitors.

Boyden Chamber

Chemotaxis was measured using a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) and polycarbonate filters (Neuro Probe, Inc., 10 μ m pore size, 25 $\times$ 80 mm, PVP free) with VSMC as previously described (3, 6). ATF (10 nM) was added to the lower wells. For trials with the inhibitors, the inhibitor was added to 2 mL of the cell

suspension 1 hour prior to addition of cells to the upper wells. Trials included eight or twelve wells per reagent or inhibitor per trial, and were repeated no fewer than three times.

Western Blotting

Cells were allowed to grow to 80% confluence and starved for 48 hours. Cells were then stimulated with ATF alone and in the presence of pharmacological and molecular inhibitors and harvested at time points from 0 to 30 minutes. Western blotting was performed as previously described (7). Total protein was determined using antibodies against each intact kinase.

siRNA Transfection

Pre-designed HPLC-purified siRNA for gene knockdown for Nox1 was procured commercially. VSMC of 50% confluence in 60-mm plates were starved overnight in 4 mL Opti-MEM reduced serum medium (Gibco). siRNA was transfected using Lipofectamine 2000 from Invitrogen, Inc. (Carlsbad, CA) following the product protocol. Briefly, 22 μ L of Lipofectamine 2000 was first incubated in total volume of 250 μ L of Opti-MEM for 5 minutes at room temperature. It was then added to 250 μ L of Opti-MEM containing 440 pmoles of siRNA. The solution was mixed gently and incubated for 20 minutes at room temperature, after which it was added to the starved plates. The medium was changed after 4–6 hours of incubation. The cells were used between 24–72 hours after transfection for cell assays. Scrambled siRNA served as a control. Using the methodologies described, we conducted concentration-dependent experiments with siRNA against Nox1 and demonstrated a concentration-dependent decrease in protein expression that was specific for the protein targeted without altering the expression of the other proteins.

Adenoviral Infection

Adenoviral vectors were constructed by Welgen, Inc. (Worcester, MA) using commercially available purified plasmids encoding (β ARK_{CT}, DNsrc and DNrac. VSMC were plated at 70% confluence in 100-mm dishes and allowed to grow overnight. Recombinant adenovirus was then added at the appropriate concentrations (β ARK_{CT}: 50 MOI; DNsrc: 30 MOI; DNrac: 20 MOI) in a reduced volume of media (1.5 – 2 mL). After 48 hours of incubation, the media was changed and the cells were grown for an additional 24 hours. The cells were then used for assays. Empty vector served as a control. Transfection was performed as previously described (8).

DHE staining

Oxygen free radical (OFR) generation was assayed using the oxygen free radical-sensitive fluorescent dye dihydroethidium (DHE, 10 μ mol/L, Sigma). ATF (10 nM) was added to growth-arrested VSMC in 24-well plates for variable times from 0 min to 60 min. DMEM and Angiotensin II were used as positive and negative controls. Additional cells were examined in the presence of DPI and Apocynin to verify the role of NAD(P)H oxidase activation by ATF. Plates were washed with PBS, and then incubated in the dark for 5 min in DMEM lacking phenol red and containing DHE (10 μ mol/L). After the incubation, dishes were transferred to a fluorescent microscope for photography. Intracellular reactive oxygen species (ROS) generation was quantitated spectrophotometrically by measurement of the red fluorescent compound ethidium, which is produced when DHE specifically reacts with intracellular O₂^{•-}.

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (s.e.m.) and statistical differences between groups were analyzed using one-way ANOVA with post hoc Dunnett's

multiple comparisons correction where appropriate. A P-value of < 0.05 was considered significant.

RESULTS

Cell Migration

To examine the role of NAD(P)H oxidase in ATF-mediated cell migration, two migration assays were chosen: the linear wound assay that measures two-dimensional cell migration and the Boyden chamber assay that measures invasive three-dimensional migration. VSMC were pre-incubated with chemical (DPI and apocynin) and molecular (siRNA to Nox1) inhibitors of NAD(P)H oxidase and stimulated with ATF. ATF stimulated VSMC migration, which was inhibited by preincubation of DPI and apocynin in the wound assay and Boyden chambers (Figure 1). Application of siRNA to Nox1 reduced Nox1 expression by ~60%. In both the wound assay and the Boyden chamber, siRNA to Nox1 markedly reduced ATF-mediated VSMC migration (Figure 1). Scrambled siRNA had no effect.

OFR production

To confirm the generation of oxygen free radicals (OFR) by ATF and the role of uPAR and NAD(P)H oxidase in their generation, ATF was incubated with the cells and DHE staining and quantification performed in the presence and absence of chemical (DPI and apocynin) and molecular (siRNA to Nox1) inhibitors of NAD(P)H oxidase. ATF produced an increase in DHE staining, which was suppressed by preincubation with DPI and apocynin (Figure 2). siRNA to Nox1 markedly reduced ATF-mediated OFR generation while scrambled siRNA had no effect (Figure 2). Phospholipase can strip the cell surface of uPAR. To determine if ATF interactions with its receptor uPAR were involved, cells were pre-incubated with a phospholipase. Removal of the ATF receptor uPAR by pre-incubation with phospholipase negated the changes in DHE staining induced by ATF, confirming the need for uPAR for ATF to induce OFR generation. ATF-mediated cell migration is G protein-dependent. To examine the role of the key G-protein $G\beta\gamma$ on the generation of OFR in the VSMC, the $G\beta\gamma$ inhibitor $\beta\text{ARK}_{\text{CT}}$ was used. When the cells were transfected with $\beta\text{ARK}_{\text{CT}}$, ATF-induced OFR generation was markedly reduced. An empty vector did not produce the same effect (Figure 2). The intracellular kinase src allows the assembly of the NAD(P)H oxidase complex. Pre-incubation with PP2 (a src inhibitor) and DNsrc also reduced ATF-induced OFR generation; PP3 (an inactive analog for PP2) and an empty vector had no effect (Figure 2). The intracellular small GTPase, rac, allows the NAD(P)H complex to assemble on the membrane. Inhibition of rac by pre-incubation with NSC23766 (a rac inhibitor) and DNrac also reduced ATF-induced OFR generation (Figure 2).

EGFR Activation

ATF-mediated cell migration is dependent on EGFR and ATF has been shown to induce time-dependent EGFR phosphorylation, which peaked at 4-fold greater than control. This response was inhibited by DPI and apocynin (Figure 3A). Inhibition of $G\beta\gamma$, inhibition of rac and inhibition of src also reduced EGFR activation (Figures 3B, C, and D, respectively).

DISCUSSION

We have previously demonstrated that ATF requires EGFR for smooth muscle cell migration and that this response is mediated by $G\beta\gamma$ G-proteins to activate ADAM-9 and -10 (A Disintegrin And Metalloproteinase Domain) (5). The pathway whereby this ADAM activation occurs was not known. This study demonstrates that ATF requires NAD(P)H oxidase activity to induce this EGFR activation and that NAD(P)H oxidase activity is also required for smooth muscle cell migration. The activation of NAD(P)H oxidase by ATF

activity requires $G\beta\gamma$ G-proteins and is linked to rac and src activity. The pathway is illustrated in Fig 4. Receptor transactivation is the process whereby activation of a given receptor activates a heterologous receptor (9). Both G protein-coupled receptors and receptor-linked tyrosine kinases can induce rapid phosphorylation of EGFR, and suppression of this EGFR activation leads to reduced MAPK activation (9–11). The triple membrane passing signaling (TMPS) mechanism of GPCR-induced EGFR activation has been used as a model to explain receptor-linked tyrosine kinase transactivation by GPCR (9). In this model, there is a sequence of three transmembrane signaling events: G protein-coupled receptor activation followed by matrix metalloproteinase (MMP) activation, and subsequent activation of EGFR by HB-EGF, or other latent ligands of EGFR. Several different MMPs have been identified with HB-EGF release: ADAM-10, ADAM-12, ADAM-17 (TNF- α converting enzyme) and MMP3 (12). Activation of these protease pathways has been shown to involve one or more of the following signaling molecules: src, intracellular calcium, and protein kinase C (9). An alternative mechanism may be inactivation of protein tyrosine phosphatases due to the generation of oxygen free radicals by the NAD(P)H oxidase complex. This study strongly suggests that $G\beta\gamma$, rac and src activity are required for NAD(P)H oxidase activation and subsequent EGFR transactivation (Fig 4).

Recent work has shown that reactive oxygen species derived from NAD(P)H oxidases are important mediators of promigratory signaling pathways. These oxygen free radicals regulate the intracellular signals responsible for lamellipodia formation, actin cytoskeleton remodeling, focal adhesion turnover, and contraction of the cell body (13, 14). NAD(P)H oxidases (Nox) represent a class of hetero-oligomeric enzymes that generate oxygen free radicals, which contribute to the maintenance of vascular tone and regulate cell growth, proliferation, differentiation, apoptosis, cytoskeletal organization, and cell migration. Vascular NAD(P)H oxidases are involved in signal transduction and are a predominant source of oxygen free radicals in cardiovascular diseases (15). The enzyme in vascular smooth muscle cells is a multi-subunit complex consisting of membrane-bound (p22phox, Nox-1/Nox-4, gp91phox) (16, 17) and cytosolic components (p47phox, Rac-1) (16) and multiple studies suggest an important role of the p47phox subunit for ROS production in VSMC (18, 19). Agonist exposure leads to assembly and activation of the NAD(P)H oxidase complex, which is dependent on 1) the production of phosphatidic acid by phospholipase D; 2) protein kinase C (PKC)-dependent phosphorylation of p47phox; and 3) guanine nucleotide exchange of Rac-GDP to form Rac-GTP (20). Under pathological conditions, excessive Nox-dependent oxygen free radical formation induces dysregulation of the redox control systems and promotes oxidative injury of vascular cells (21). Inhibition of rac, which allows the NAD(P)H complex to assemble on the membrane, and inhibition of src, which induces assembly of the complex, have been shown to be important in angiotensin II-induced NAD(P)H oxidase activity in VSMC. (22). Our current work supports this role of both rac and src and extends the pathway to a second set of G protein-based receptors.

The generation of oxygen free radicals has been shown to be important in vessel wall remodeling. In humans, both ROS generation and p22(phox) expression significantly correlated with intravascular ultrasound-derived measures of remodeling(23). In a rat aorta balloon injury model, p47(phox) protein is increased after injury and expression of p47(phox) is localized to the neointima and media of injured arteries(13). In porcine balloon-injured arteries, NAD(P)H oxidase-dependent superoxide production increases significantly within 24 hours after balloon-induced injury (24). In a murine wire injury model, Nox1 plays a critical role in neointima formation by mediating VSMC migration, proliferation, and extracellular matrix production (25). Perivascular gene transfer of NAD(P)H oxidase inhibitor or the use of a cell-permeant peptide that inhibits NAD(P)H oxidase (gp91ds-tat) will both inhibit intimal hyperplasia development in the balloon-injured rat carotid (26, 27). Urokinase has been shown to stimulate the production of superoxide

radicals in cultured aortal smooth muscle cells simultaneously with activation of the expression of NAD(P)H-oxidases Nox1, Nox4, and Phox22 (28). uPA stimulated ROS production to levels equivalent to those induced by angiotensin II as measured by electron spin resonance and fluorescent redox indicators (dichlorofluorescein diacetate, lucigenin, and hydroethidine). The increase in ROS was biphasic, with the first peak at 30 minutes and the second peak at 4 hours. uPA increased expression of the NAD(P)H oxidases Nox1 and Nox4 as measured by RT-PCR and Western blot analysis. Knockdown of Nox1 and Nox4 expression with small interfering RNA showed that both isoforms (Nox1>Nox4) contributed significantly to uPA-stimulated ROS production (29). There is currently no data on the role of NAD(P)H oxidase activity and uPA-mediated migration. Kalmes et al have recently shown that thrombin, another coagulation factor, will also mediate EGFR-dependent VSMC migration and induce EGFR activation through a transactivation pathway (30). Our current data with ATF supports a similar NAD(P)H oxidase-mediated pathway. Thrombin has been shown to increase the expression of p47(phox) and Rac2 and stimulate their translocation to the cell membrane. Accompanied by these changes, thrombin increases O₂⁻ and H₂O₂ generation and NADH/NAD(P)H consumption (13). In other cell types, cell migration and invasion can be attenuated by diphenyleneiodonium and transfection of Nox1 siRNAs. Both DPI or Nox1 siRNAs blocked up-regulation of MMP-9 (31). We have shown that the subunit Nox1 is important in ATF-mediated cell migration and NAD(P)H oxidase activity.

Conclusion

The aminoterminal fragment of urokinase induced NAD(P)H oxidase activity through a Gβγ, rac and src-mediated pathway in order to facilitate transactivation of EGFR and VSMC migration. Targeting one or more of these pathways will reduce intimal hyperplasia and help control restenosis after endovascular intervention.

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B

Figure 1. Cell Migration

ATF produced VSMC migration, which was inhibited by preincubation of DPI and apocynin in the wound assay (A) and Boyden chambers (B). siRNA to Nox1 markedly reduced ATF-mediated VSMC migration. Scrambled siRNA had no effect. All values are the mean $\pm$ s.e.m. percent of the control for five experiments (* $p<0.05$, ** $p<0.01$). HPF, high-power field.

A

B

Figure 2. OFR production

ATF produced an increase in OFR generation, which was suppressed by pre-incubation of DPI and apocynin. siRNA to Nox1 markedly reduced ATF-mediated OFR generation while scrambled siRNA had no effect. When the cells were transfected with β ARK_{CT}, ATF induced OFR generation was markedly reduced. An empty vector did not produce the same effect. Preincubation with PP2 (a src inhibitor) and DNsrc also reduced ATF-induced OFR generation; PP3 (an inactive analog for PP2) and an empty vector had no effect. Inhibition of Rac with pre-incubation with NSC23766 and DNrac also reduced ATF induced OFR generation. All values are the mean $\pm$ s.e.m. percent of the control for five experiments (* $p < 0.05$, ** $p < 0.01$).

A

B

D

Figure 3. EGFR Activation

ATF induced EGFR phosphorylation, which was inhibited by DPI and apocynin (A). Inhibition of $G\beta\gamma$ by βARK_{CT} (B), inhibition of rac (C) and src (D) also reduced EGFR activation. All values are the mean $\pm$ s.e.m. percent of the control for five experiments (* $p < 0.05$, ** $p < 0.01$).

Cell Migration

Figure 4. Mechanism of EGFR activation by ATF

ATF binds uPAR, which then dissociates, and the soluble fragment of uPAR binds the formic receptor FPRL-1, which in turn activates Gβγ. Gβγ interacts with the GTPase, rac, and with src to facilitate the assembly and membrane localization of the components of NAD(P)H oxidase, including Nox1. Generation of oxygen free radicals (OFR) leads to activation of EGFR. Phospholipase activity will remove uPAR from the cell surface. βARK_{CT} will block Gβγ. NSC23766 and dominant negative rac (DNrac) block rac activity while PP2 and dominant negative src (DNsrc) block src. Diphenyleneiodonium (DPI), apocynin (Apo) and siRNA to Nox1 block NAD(P)H oxidase.