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Urokinase Plasminogen Activator Stimulates Vascular Smooth Muscle Cell Proliferation Via Redox-Dependent Pathways

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Objective—We showed previously that increased urokinase plasminogen activator (uPA) expression contributes to vascular smooth muscle cell (VSMC) proliferation and neointima formation after injury. Proliferation of cultured rat aortic VSMCs induced by uPA was inhibited by the antioxidant ebselen. Because increases in VSMC reactive oxygen species (ROS) contribute to VSMC proliferation, we hypothesized that uPA increases ROS generation by regulating expression or activity of cellular oxidases.

Methods and Results—uPA stimulated ROS production to levels equivalent to 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 and VSMC proliferation. Transfection of VSMCs with uPA cDNA to increase endogenous uPA expression enhanced ROS production dramatically, suggesting that autocrine uPA production may be an important mechanism for uPA-mediated VSMC events.

Conclusion—These data show that uPA is an autocrine VSMC growth factor that increases ROS generated by both Nox1 and Nox4 oxidases. **(*Arterioscler Thromb Vasc Biol*. 2006;26:801-807.)**

Key Words: urokinase ■ superoxide ■ VSMC proliferation ■ arterial remodeling

Plasminogen activators, their inhibitors, and receptors are the main components of the fibrinolytic (or plasminogen/plasmin) system that, together with the blood coagulation system, determine the balance between the formation and dissolution of blood clots.[1] In addition, much evidence suggests that the fibrinolytic system also participates in vascular remodeling processes such as atherosclerosis and postangioplasty restenosis.[2–6] The predominant processes that contribute to vessel remodeling in cardiovascular diseases are proliferation and migration of vascular smooth muscle cells (VSMCs), extracellular matrix deposition, adhesion of inflammatory cells, invasion into the vessel wall, and proliferation. These processes are regulated by growth factors and the components of the fibrinolytic or plasminogen system.[7,8]

The plasminogen system is composed of an inactive proenzyme, plasminogen, that is converted to active plasmin by 2 physiological plasminogen activators: tissue-type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA).[1,9] VSMCs use proteinases to degrade the extracellular matrix that encages them, releasing them to migrate into the wound.[10] Plasmin may trigger this process because it can directly degrade fibrin and matrix and also activate other matrix-degrading proteinases, including the metalloproteinases. Plasmin has been presumed to play a role in tissue remodeling via proteolysis of extracellular matrix components and activation of growth factors. Whereas tPA is primarily involved in clot dissolution, uPA, which binds to a membrane-anchored receptor (uPAR), has been implicated in pericellular proteolysis during cell migration and tissue remodeling.[6,11]

We[5,6] and others[12,13] have shown that after injury to the artery expression of tPA, uPA, and uPAR by VSMCs, endothelial cells and inflammatory cells are significantly induced, suggesting that a hyperfibrinolytic response may be important in the migration or proliferation of these cells. Studies with uPA and tPA knockout mice have shown that deficiency of uPA decreased neointima formation, whereas deficiency of tPA did not affect this process, indicating a

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more important role for uPA in the response to arterial injury. Additional studies provide direct evidence that uPA-mediated plasmin proteolysis promotes vascular wound healing and associated neointima formation in mice in part via stimulation of migration.[3] However, uPA has other functions, including changes in cell adhesion,[14] chemotaxis,[15] and cell proliferation. uPA-induced cell migration is associated with a rapid and transient activation of intracellular serine and tyrosine phosphorylation,[15,16] activation of Src kinases,[17] focal adhesion kinase,[18] mitogen-activated protein kinase,[18] and Jak kinases.[19]

Recently, we demonstrated that uPA applied periadventitially potentiated vessel wall thickening, reduced vessel lumen size, and caused inward vessel remodeling after balloon injury of rat carotid artery.[5,6,20] In contrast to uPA, application of recombinant tPA reduced neointima area, prevented lumen narrowing, and promoted outward remodeling.[20] These data suggest that the ability to stimulate neointima formation and cause negative vessel remodeling is specific for uPA among plasminogen activators. This suggests that mechanisms unique to uPA are involved in uPA-dependent vessel wall remodeling.

Reactive oxygen species (ROS) are important signaling molecules that regulate vascular tone and VSMC proliferation.[8] We previously showed increased O_2^- production in injured vessels and demonstrated the beneficial effects of inhibiting ROS on vascular remodeling.[21] Previous studies found that uPA augmented neutrophil generation of O_2^-, suggesting that uPA may regulate NAD(P)H oxidases.[22] Therefore, we hypothesized that the effects of uPA to promote vessel wall thickening and inward remodeling may be attributable to uPA-mediated increases in ROS production. Here we show that uPA stimulates O_2^- production by cultured VSMCs by increasing the activity and expression of both Nox1 and Nox4 oxidases. We demonstrated an autocrine role for uPA in O_2^- production, suggesting that locally generated uPA may contribute to vascular remodeling after injury. Finally, we showed that generation of ROS is essential for uPA-mediated VSMC growth.

Methods

VSMC Preparations, Growth Assays, and Transfection

Aortic VSMCs were isolated from 200- to 250-g male Harlan (Charles River, Cambridge, Mass) Sprague-Dawley rats and maintained in 10% FCS/DMEM as described.[23] Passages 5 to 10 VSMCs at 80% to 90% confluence in tissue culture dishes or glass slides were growth-arrested by incubation in 0.1% FCS/DMEM for 48 hours before treatment. Recombinant uPA (American Diagnostica) in DMEM supplemented with 0.1% FCS was added to the cells as indicated. For flow cytometric analysis of cell growth, VSMCs were incubated with 10 μmol/L 5-bromo-2′-deoxyuridine for 2 hours at 37°C in a humidified atmosphere with 5% CO_2. After being washed with 10 mL of 1×PBS, the cells were fixed with 70% ethanol overnight at 4°C. Fixed cells were rinsed with PBS and incubated with 0.2 mg/mL pepsin/2 N HCl/1×PBS for 20 minutes at 37°C. After 2 washes with 1×PBS/0.5% FBS/0.5% Tween 20, cells were treated with 1×PBS/2% FBS for 20 minutes at room temperature. Cells were incubated with 1 μg of anti-BrdU/fluorescein isothiocyanate (Boehringer Mannheim) for 45 minutes and washed in 1×PBS/0.5% FBS/0.5% Tween 20. They were then incubated in 1 mL of PBS containing 1 mg/mL of RNase A (Sigma) for ≥30 minutes at room temperature after resuspending in 500 μL propidium iodide (20 μg/mL in 1×PBS). Propidium iodide fluorescence (ie, DNA content) and fluorescein isothiocyanate fluorescence (BrdU incorporation) were determined by flow cytometry on a FACScalibur (BD Biosciences). A minimum of 20 000 cells per sample were analyzed. Data were collected and analyzed using CellQuest software.

A full-length cDNA for rat uPA (generously donated by Dr Robert Beabealashvilly, Russian Cardiology Research Center, Moscow, Russia) was directionally cloned into the *Xba*I site of pcDNA3 (Invitrogen Corporation). As a control plasmid, we used enhanced green fluorescence protein cDNA (pEGFPC2). Plasmid DNA (0.9 μg/35-mm dish) was transfected using Lipofectamine 2000 (Invitrogen).

To target NADPH oxidase cDNA, Nox1 siRNA (5′-agatctatttctactggat-3′), and Nox4 siRNA (5′-aacgaaggggttaaacacc-3′) were designed and synthesized by Integrated DNA Technologies, Inc. Control nontargeting siRNA pool (No. D-001206-13) was from Dharmacon Research, Inc. Small interfering RNAs (siRNAs) were transfected with Lipofectamine 2000 at 0.932 μg/35-mm dish. After transfection, cells were incubated in air–CO_2 incubator for 4 hours, and then the media was changed for 16 hour. Cells were serum deprived in 0.1% FBS for 48 hours before use.

Measurement of ROS Production

Hydroethidium, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and lucigenin were used to measure ROS in VSMCs as described.[23] Growth-arrested VSMCs were incubated with recombinant uPA for 30 minutes to 48 hours, medium was aspirated, and VSMCs were incubated in Hank's balanced salt solution (HBSS) containing 1.3 mmol/L $CaCl_2$ and 5.5 mmol/L glucose supplemented with hydroethidium (5 μmol/L for 10 minutes) or H_2DCFDA (5 μmol/L for 30 minutes) in a light-protected humidified chamber at 37°C. Cells were rinsed in HBSS and images obtained with a Olympus BX51 epifluorescence microscope equipped with ×40 water immersion lens. For hydroethidium, excitation was at 488 nm with emission at 610 nm, and for dichlorodihydrofluorescein, excitation was 485 nm and emission at 535 nm. Lucigenin chemiluminescence was measured by a scintillation counter in out-of-coincidence mode as described.[21] For electron spin resonance (ESR) measurements, VSMCs were transfected with 25 nmol/L siRNA, grown to confluence (48 hours after transfection), and serum starved in DMEM containing 0.1% FBS for 2 days. The cells were then stimulated with 100 nmol/L uPA for 30 minutes and collected in PBS. After centrifugation, cells were resuspended in modified Krebs/HEPES buffer containing 25 μmol/L deferoximine and 3.5 μmol/L EDTA. Approximately 1×10^6 cells were mixed with the O_2^--specific spin trap cyclic hydroxylamine CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine; 1 mmol/L) in the presence or absence of manganese-containing superoxide dismutase (100 U/mL) and immediately analyzed for O_2^- production in glass capillaries using ESR spectrophotometer.[24] The ESR (Miniscope 200; Magnettech) settings were: Bio-field, 3350 G; field sweep, 45 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4096 points resolution and receiver gain, 500; and kinetic time, 10 minutes. Only superoxide dismutase–inhibitable fraction of O_2^- signal, normalized by cell counting on Beckman Z series counter, was compared between control and treatment groups.

Reverse Transcriptase–Polymerase Chain Reaction

RT-PCR was performed using 0.5 μg of total RNA, oligo$(dT)_{18}$ and RevertAid M-MuLV (Fermentas). Specific primers: Nox1 (fp GGA-ACA-AGA-GTA-GGA-CGA-ATT-A, rp GTC-AAC-CAG-CAA-GAT-TCA-GCT-A), Nox4 (fp GAA-TGC-AGC-AAG-ATA-CCA-GAA-T, rp GGC-TTG-ATG-GAG-GCA-GTA-GTA-A). To estimate the amplification midpoint, previous cycling was made with 16 to 30 cycles (for β-actin) and 22 to 36 cycles (for Nox1 and Nox4). The final PCR were made with 22 cycles for β-actin and 26 cycles for Nox4.

Western Immunoblotting

VSMC lysates, SDS-PAGE, transfer to polyvinylidene fluoride membranes, and immunoblotting were performed as described.[23]

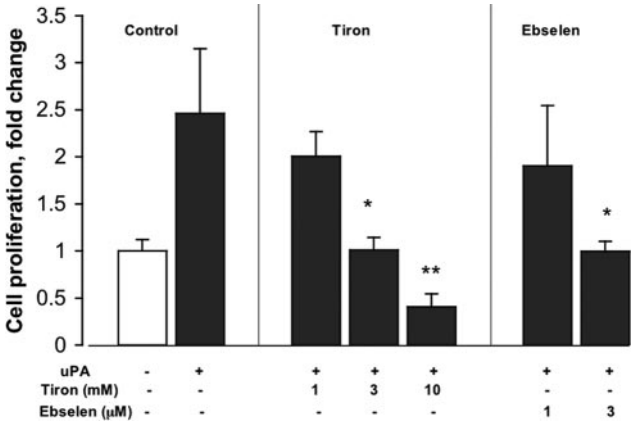

Figure 1. VSMC proliferation induced by uPA is redox sensitive. Growth-arrested VSMCs were treated for 48 hours with 100 nmol/L uPA, and cell proliferation measured by S-phase/G1-phase cell number ratio using flow cytometry as described in Methods. To inhibit ROS production, cells were treated with ebselen and Tiron as indicated. Results are fold change in cell number relative to uPA. Data are mean±SE of 3 independent observations. *$P<0.05$; **$P<0.01$ between cells stimulated by uPA in the absence and in presence of antioxidants.

Antibodies were goat polyclonal Nox4 (sc-21860) and Nox1 (sc-5821; Santa Cruz Biotechnology, Inc).

Statistics

Results are means±SE. Comparisons between multiple groups were performed using 1-way ANOVA and Student-Newman–Keuls test for multiple comparisons. Single comparisons were made using Student *t* test. $P<0.05$ was considered statistically significant.

Results

VSMC Proliferation in Response to uPA Is Inhibited by Antioxidants

To assay the effect of uPA on VSMC proliferation, cells were growth arrested and stimulated with 100 nmol/L uPA for 48 hours. Cell number increased significantly by 2.4±0.6-fold in response to uPA (Figure 1). To determine the requirement for ROS production, we used the glutathione peroxidase mimetic ebselen and the ROS scavenger Tiron to inhibit ROS generation. Both Tiron and ebselen inhibited uPA-stimulated VSMC growth in a concentration-dependent manner, with ≈100% inhibition at the highest concentrations.

Urokinase Stimulates a Biphasic Increase in ROS Production in VSMCs

To measure the effect of uPA on VSMC ROS production, we used 4 techniques to assay ROS. We also compared the effects of uPA with angiotensin II, which is known to stimulate NAD(P)H oxidases and ROS generation in VSMCs.[25] We first measured the time course and concentration dependence for uPA-stimulated ROS generation by lucigenin chemiluminescence. As shown in Figure 2A, 100 nmol/L uPA stimulated a biphasic increase in ROS, with peaks at 25 minutes and 240 minutes. The concentration response curve for ROS production at 25 minutes showed that the EC_{50} for uPA was ≈20 nmol/L with peak at 80 nmol/L (Figure 2B). The magnitude of uPA-induced ROS generation was similar to that observed with 200 nmol/L angiotensin II

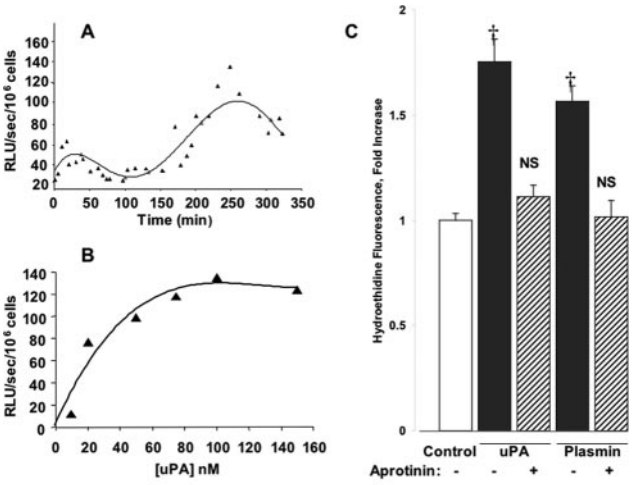

Figure 2. Time and dose dependence of uPA-stimulated $O_2^{\cdot-}$ production. A, Growth-arrested VSMCs were evaluated by lucigenin chemiluminescence after treatment with 100 nmol/L uPA. Results are means of peak $O_2^{\cdot-}$ levels from 4 independent experiments. B, Growth-arrested VSMCs were treated with the indicated uPA concentrations for 25 minutes. Results are mean of 4 experiments. The curve was fit with a polynomial equation using CricketGraph. C, Growth-arrested VSMCs were incubated for 30 minutes with 100 nmol/L uPA and 100 nmol/L plasmin with or without 7.6 μmol/L aprotinin. ROS production was measured by hydroethidium fluorescence. Data are mean±SE of ≥5 experiments. †$P<0.001$ vs control. NS indicates not significantly different from control.

(data not shown). The addition of the superoxide dismutase mimic (MnTPyP 100 μmol/L) into the mixtures decreased chemiluminescence to the baseline levels (data not shown).

We next measured uPA-stimulated fluorescence of 2 ROS-sensitive dyes: 2′,7′-dichlorofluorescein diacetate (DCFH-DA, specific for hydrogen peroxide) and hydroethidine (hydroethidine, specific for $O_2^{\cdot-}$). Confocal fluorescence images of VSMCs after incubation with hydroethidine indicated that ROS levels were increased in VSMCs after uPA (100 nmol/L) treatment (supplemental Figure IA and IB, available online at http://atvb.ahajournals.org). The maximal hydroethidine fluorescence (≈2-fold increase) was observed at 30 minutes of treatment with 100 nmol/L uPA (supplemental Figure IB). Hydroethidine fluorescence in response to uPA was equivalent to that observed with 1 μmol/L angiotensin II at 20 minutes (supplemental Figure IA and IB). The increase in ROS induced by uPA, as measured by DCFH-DA fluorescence, was similar, with a 1.8-fold increase at 30 minutes (supplemental Figure IC and ID).

Finally, we used ESR to assay the increase in ROS generation. uPA (100 nmol/L for 30 minutes) stimulated a 2.75±0.3-fold increase in ROS ($P=0.01$; see Figure 6). Thus by 4 techniques, uPA stimulated a significant increase in VSMC ROS production.

uPA Requires Proteolytic Activity for ROS Production

To determine the nature of uPA-mediated ROS production, we assayed the effect of the protease inhibitor aprotinin. As shown in Figure 2C, aprotinin completely blocked uPA-mediated ROS production measured by hydroethidine. Con-

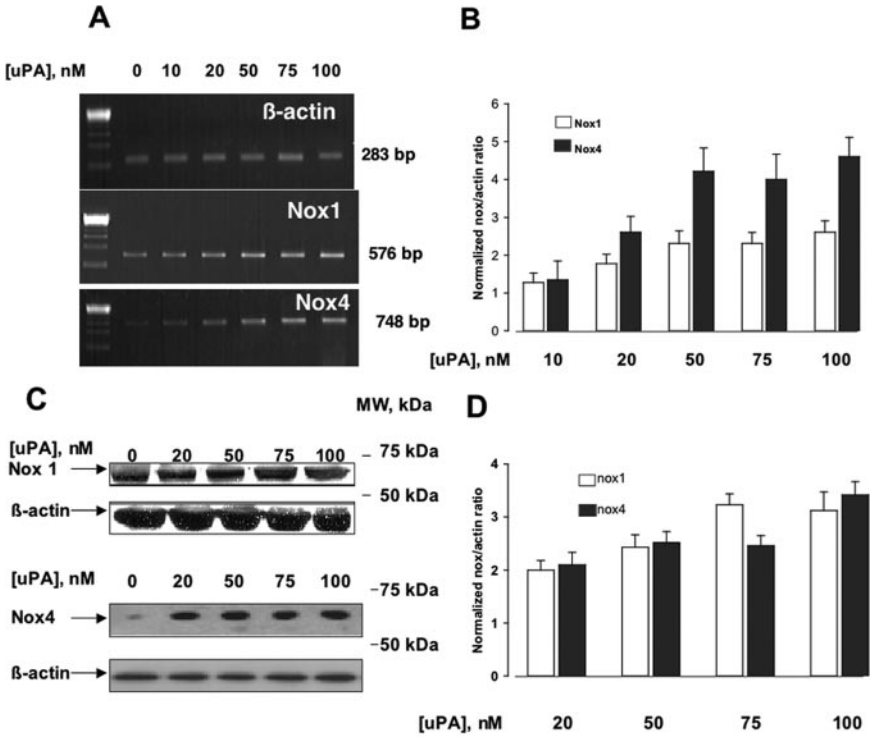

Figure 3. uPA increases expression of Nox1 and Nox4. A and B, VSMCs were serum deprived for 24 hours in DMEM with 0.1% FBS, followed by 3-hour treatment with uPA at the indicated concentrations. After incubation, total RNA was extracted and RT-PCR was performed. A, Representative Nox1, Nox4, and β-actin RT-PCR data. B, Quantitation of RT-PCR data by NIH Image. C and D, Growth-arrested VSMCs were incubated with uPA (0 to 100 nmol/L) for 4 hours. Expression levels of Nox1, Nox4, and β-actin were determined by Western blotting. Quantitation of Western blot results after analysis by NIH Image. For each experiment, the expression level of mRNA or proteins in 0 nmol/L uPA was arbitrarily normalized to 1.0 and the fold increase of Nox1 and Nox4 relative to β-actin determined. Results are mean$\pm$SE of 4 experiments.

sistent with these results, plasmin also stimulated an increase in ROS production that was blocked by aprotinin. Thus, proteolytic activity of uPA is required for ROS production.

Endogenous uPA Stimulates ROS Production

Because uPA is produced by VSMCs during the response to injury, we next investigated the effect of endogenous uPA on VSMC ROS production to characterize an autocrine pathway. To increase VSMC uPA production, we cotransfected VSMCs with pcDNA3-RuPA (encoding rat uPA) and pEGFPC2 vector (encoding green fluorescent protein [GFP]). After allowing gene expression for 24 hours, we measured ROS production in cells expressing GFP (likely cotransfected with RuPA) or in cells lacking GFP by hydroethidine fluorescence. As shown in supplemental Figure IIA and IIB, cells cotransfected with both vectors showed dramatically greater ROS production than neighboring nontransfected cells. To show that transfection and GFP had no effect on hydroethidine fluorescence, we transfected separate dishes with GFP alone. As shown in supplemental Figure IIC and IID, GFP alone had no effect on ROS generation. Thus, endogenous uPA potently increases VSMC ROS production.

uPA Stimulates VSMC Expression of NAD(P)H Oxidases

The biphasic time course for ROS generation in response to uPA (Figure 2) suggested that uPA might regulate NAD(P)H oxidase expression similar to angiotensin II. To assay the effect of uPA on expression of NAD(P)H oxidase components, we performed PCR and Western blot assays for expression of Nox1 and Nox4. In a concentration-dependent manner, uPA (10 to 100 nmol/L for 4 hours) increased Nox1 and Nox4 mRNA expression by 2.5-fold and 4.5-fold without significant change in actin (Figure 3A and 3B). The changes in mRNA expression were translated into similar increases in protein expression, which were $\approx$3-fold for both Nox1 and Nox4 at 100 nmol/L uPA (Figure 3C and 3D).

Inhibition of Nox1 and Nox4 Decreases uPA-Induced ROS Generation

To prove that NAD(P)H oxidase is the primary source of ROS production stimulated by uPA in VSMCs, we inhibited Nox1 and Nox4 function by both pharmacological and molecular approaches. To uncouple NAD(P)H oxidase, we exposed cells to 10 μmol/L diphenyliodinium (DPI) and

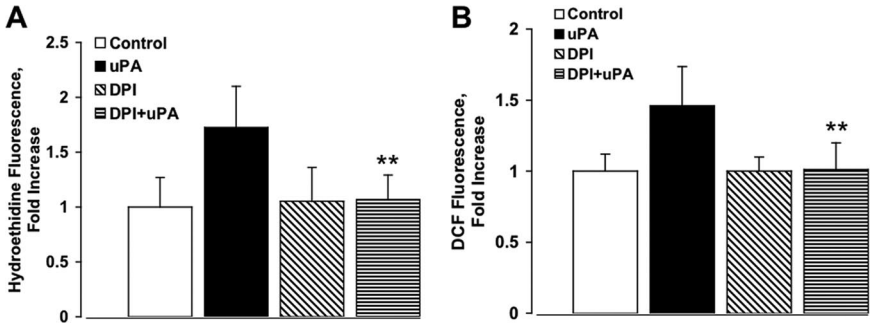

Figure 4. uPA-mediated ROS production in VSMCs is dependent on flavine-containing oxidases. Growth-arrested VSMCs were incubated with vehicle (1% DMSO) or 10 μmol/L DPI for 30 minutes before addition of 100 nmol/L uPA for 30 minutes. ROS production was assessed by fluorescence of hydroethidine (A) or DCFH-DA (B). Fluorescence images were quantified by NIH Image. Results are the mean of $\geq$10 experiments. **$P<0.01$ vs uPA alone.

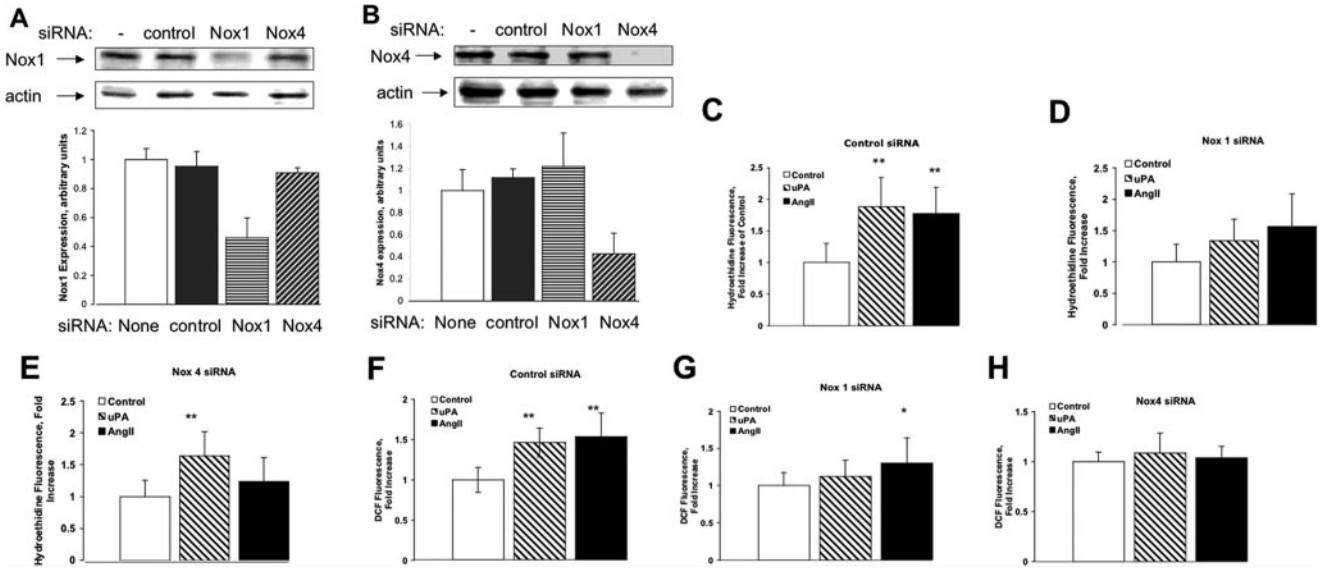

Figure 5. uPA- and angiotensin II–mediated ROS production are dependent on Nox1 and Nox4. A and B, VSMCs were transfected with control, Nox1, or Nox4 siRNA, followed by Nox1 and Nox4 evaluation by Western blot and quantitation by NIH Image. C through H, VSMCs were transfected with control siRNA (C and F), Nox1 siRNA (D and G), and Nox4 siRNA (E and H). The cells were then growth arrested for 24 hours before treatment with 100 nmol/L uPA for 30 minutes or 1 μmol/L angiotensin II for 20 minutes as indicated. ROS production was assessed by fluorescence of DCFH-DA (C through E) or by hydroethidium fluorescence (F through H). The images were quantified by NIH Image. Results are the mean±SE of ≥10 experiments. *$P<0.05$; **$P<0.01$ vs control.

measured ROS production by both hydroethidine (Figure 4A) and DCHF-DA fluorescence (Figure 4B). DPI had minimal effect on basal ROS generation. However, the increase in ROS induced by uPA was inhibited by nearly 100% with 10 μmol/L DPI (Figure 4A and 4B).

To provide further evidence that Nox1 and Nox4 mediate ROS production by uPA, we specifically decreased expression with siRNA. Transfection of each specific siRNA resulted in a 60% decrease of Nox1 or Nox4 expression in VSMCs without decreasing the other isoform (Figure 5A and 5B). VSMCs were transfected with control siRNA (Figure 5C and 5F), Nox1 siRNA (Figure 5D and 5G), or Nox4 siRNA (Figure 5E and 5H). The cells were then growth arrested for 24 hours before incubation with uPA for 30 minutes and angiotensin II for 20 minutes. As shown by both DCF and hydroethidium fluorescence, control siRNA had no effect on ROS generation. In contrast, both Nox1 and Nox4 siRNA significantly inhibited uPA-mediated ROS generation. The finding that siRNA for Nox 4 inhibited ROS generated in response to uPA detected by DCF but not by hydroethidium is likely attributable to the fact that DCF is less sensitive in our cells. Finally, we repeated the siRNA transfection and

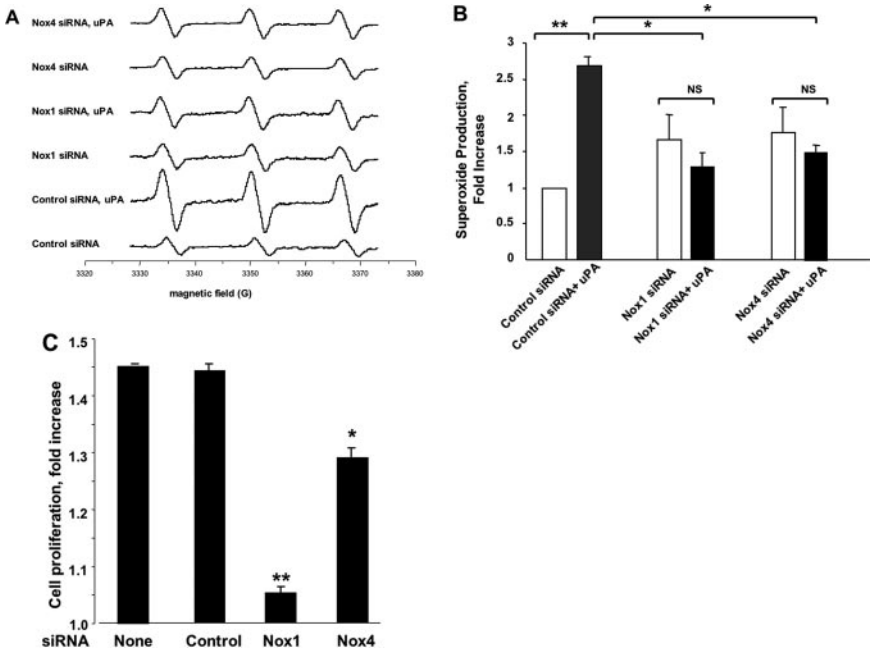

Figure 6. Nox1 and Nox4 are required for uPA stimulation of $O_2^{\cdot-}$ and cell proliferation. A and B, VSMCs transfected with control, Nox1, or Nox4 siRNA were stimulated with 100 nmol/L uPA for 30 minutes, and superoxide production was detected using ESR. A, Representative ESR spectra. B, Quantitation of data (n=5). C, Quantitation of cell cycle progression. VSMCs were stimulated with uPA as in Figure 1. Cell proliferation was measured as fold increase in (S+G2+M)/G1 stimulated by uPA for each condition. *$P<0.05$; **$P<0.01$.

measured ROS by ESR using the $O_2^{\cdot-}$-specific spin trap cyclic hydroxylamine CMH in the presence or absence of superoxide dismutase. uPA stimulated $O_2^{\cdot-}$ generation as shown by CMH oxidation (Figure 6A). Nox1 and Nox4 siRNA had no significant effect on basal $O_2^{\cdot-}$ generation. However, both siRNAs significantly decreased $O_2^{\cdot-}$ generation. Quantitation of 5 experiments showed that uPA stimulated a 2.7±0.2-fold increase (P=0.01) in $O_2^{\cdot-}$ (Figure 6B). The increases in $O_2^{\cdot-}$ generation by uPA in the presence of Nox1 and Nox4 siRNA were significantly decreased to 1.6±0.5- and 1.7±0.6-fold increases, respectively (P=0.05 versus control siRNA).

Finally, to determine the relative roles of Nox1 and Nox4 in uPA-mediated VSMC proliferation, we performed cell cycle analysis after transfection of Nox1 and Nox4 siRNA (Figure 6C). To assess cell proliferation, we measured the ratio of cells in S+G2+M relative to cells in G1 after 24-hour treatment with uPA or vehicle. In the absence of siRNA, uPA stimulated a 1.45±0.01-fold increase in cell proliferation. With control siRNA, the increase was 1.44±0.02-fold, whereas cell proliferation was significantly inhibited by both Nox1 (1.05±0.01) and Nox4 (1.29±0.02) siRNA.

Discussion

The major finding of the present study is that uPA increases ROS production in VSMCs by stimulating both the expression and activity of NAD(P)H oxidases. We defined important and equal roles for both Nox1 and Nox4 in mediating the uPA increase in ROS. We also found that uPA stimulated VSMC growth in an ROS-dependent manner, as shown by inhibition with ebselen and knockdown of Nox1 and Nox4. Finally, we found that endogenous generation of uPA was able to increase ROS production in VSMCs, suggesting an important role for uPA in VSMC autocrine ROS production and growth. In summary, these data support an important role for uPA as the plasminogen activator that regulates VSMC ROS production and growth after vascular injury.

The importance of uPA as a regulator of Nox1, Nox4, and ROS production in VSMCs has not been shown previously. Previous studies found that uPA greatly enhanced $O_2^{\cdot-}$ production by neutrophils.[22] The fact that uPA stimulates both activity and expression of NAD(P)H oxidases is not surprising because similar results have been reported for angiotensin II. Using cultured VSMCs, we observed that uPA increased ROS to the same level as angiotensin II, suggesting that in vivo uPA may be an important mediator of redox state in VSMCs. Like angiotensin II, uPA-stimulated growth of VSMCs was inhibited by antioxidants. It should be noted that there are likely other mechanisms by which uPA stimulates ROS production, as suggested by the finding that Nox1 and Nox4 siRNA decreased uPA-mediated ROS production by ≈50%. Similar data with angiotensin II and PDGF have been obtained.[26,27] For both agonists, a mitochondrial mechanism for ROS production appears likely. Future studies will be required to determine the effect of uPA on VSMC mitochondrial function.

Recently, Nox1 and Nox4 were found essential for $O_2^{\cdot-}$ production by VSMCs.[28] Among members of the Nox family, Nox1 confers mitogenic properties,[28] consistent with our findings that uPA-mediated VSMC cell cycle progression was more highly dependent on Nox1 than Nox4. The fact that Nox1 was more essential for cell proliferation, whereas Nox1 and Nox4 contributed equally to $O_2^{\cdot-}$ production, suggests that other roles for Nox1 may contribute to cell growth. The activity of NAD(P)H oxidases in cellular constituents of the arterial wall is modulated by cytokines known to influence vascular remodeling and neointima growth.[28] After balloon injury increased Nox1, Nox4 and p22phox stimulated ROS production, providing a signaling mechanism involved in vascular remodeling. Our findings that uPA induces $O_2^{\cdot-}$ production and upregulates Nox1 and Nox4 levels in cultured VSMCs are consistent with our previous data for uPA stimulation of VSMC proliferation,[11] neointima formation, and inward vessel remodeling.[5,6,20] Importantly, we found that endogenous uPA generated by uPA expression in cultured cells increased ROS production. These data suggest that autocrine growth mechanisms activated by uPA contribute to the vascular response to injury and suggests that inhibiting uPA function may have therapeutic benefits.

Acknowledgments

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Supplementary on-line text

Figure legends

Figure I. uPA increases ROS production in VSMC.

Growth arrested VSMC were incubated with 100 nM uPA, 1 μM angiotensin II (AngII) or 100 μM H_2O_2 for the indicated times. A-B. ROS production was assessed by hydroethidium fluorescence. C-D. Using the same protocol as in A, ROS production was assessed by fluorescence of DCFH-DA. For B and D, fluorescence images were quantified by NIH Image. Results are the mean of 5 experiments. ** = $p < 0.01$.

Figure II. ROS production is increased in VSMC transfected with uPA.

A-B. VSMC were transfected with both pcDNA3-RuPA (encoding rat uPA) and pEGFPC2 vector (encoding GFP). C-D. VSMC were transfected with PEGFPC2 vector only. Cells were growth arrested for 24 hr after transfection, and ROS measured by hydroethidium fluorescence (red images, left panels) and GFP fluorescence (green images, right panels). Results are typical of 5 experiments.

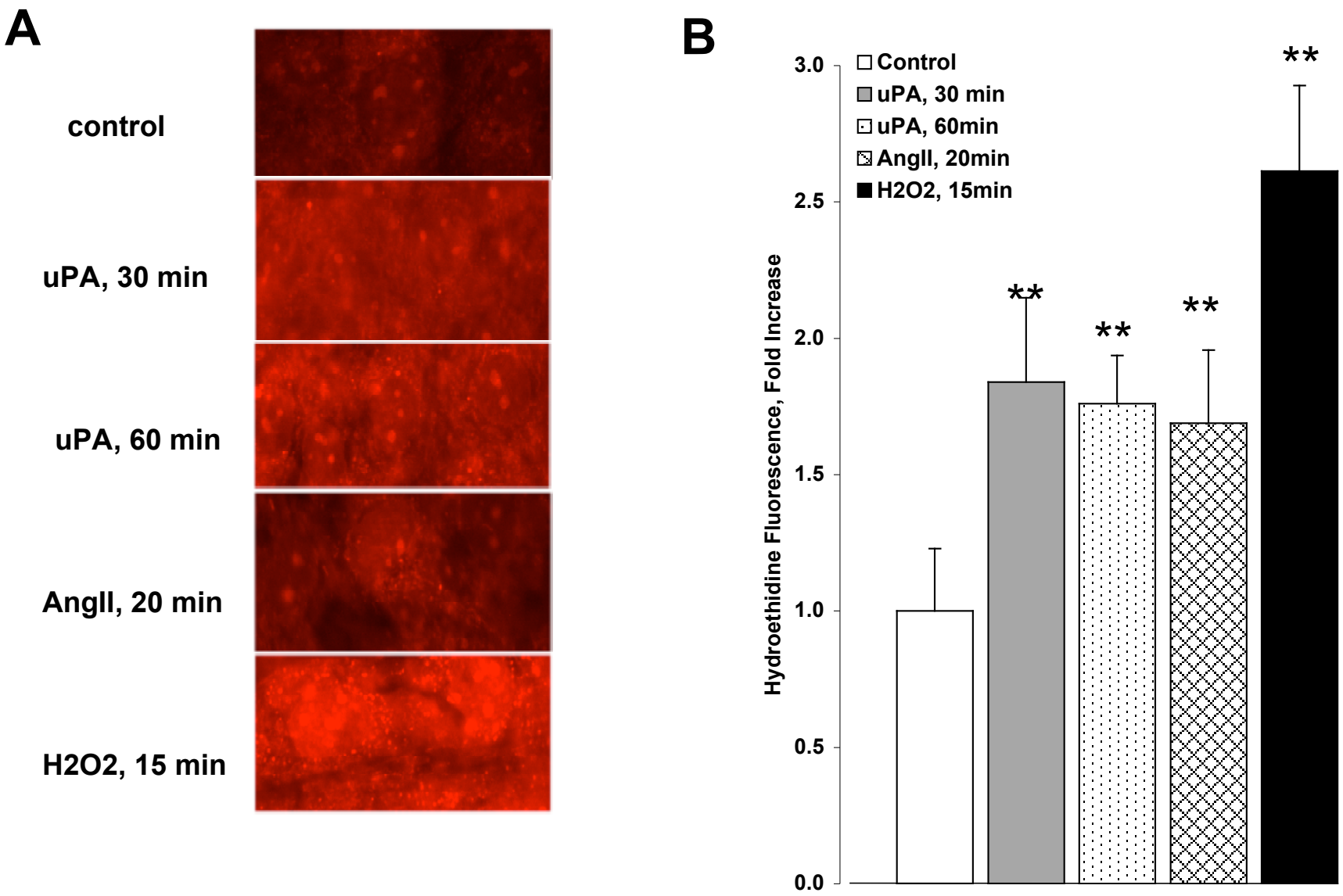

Figure I

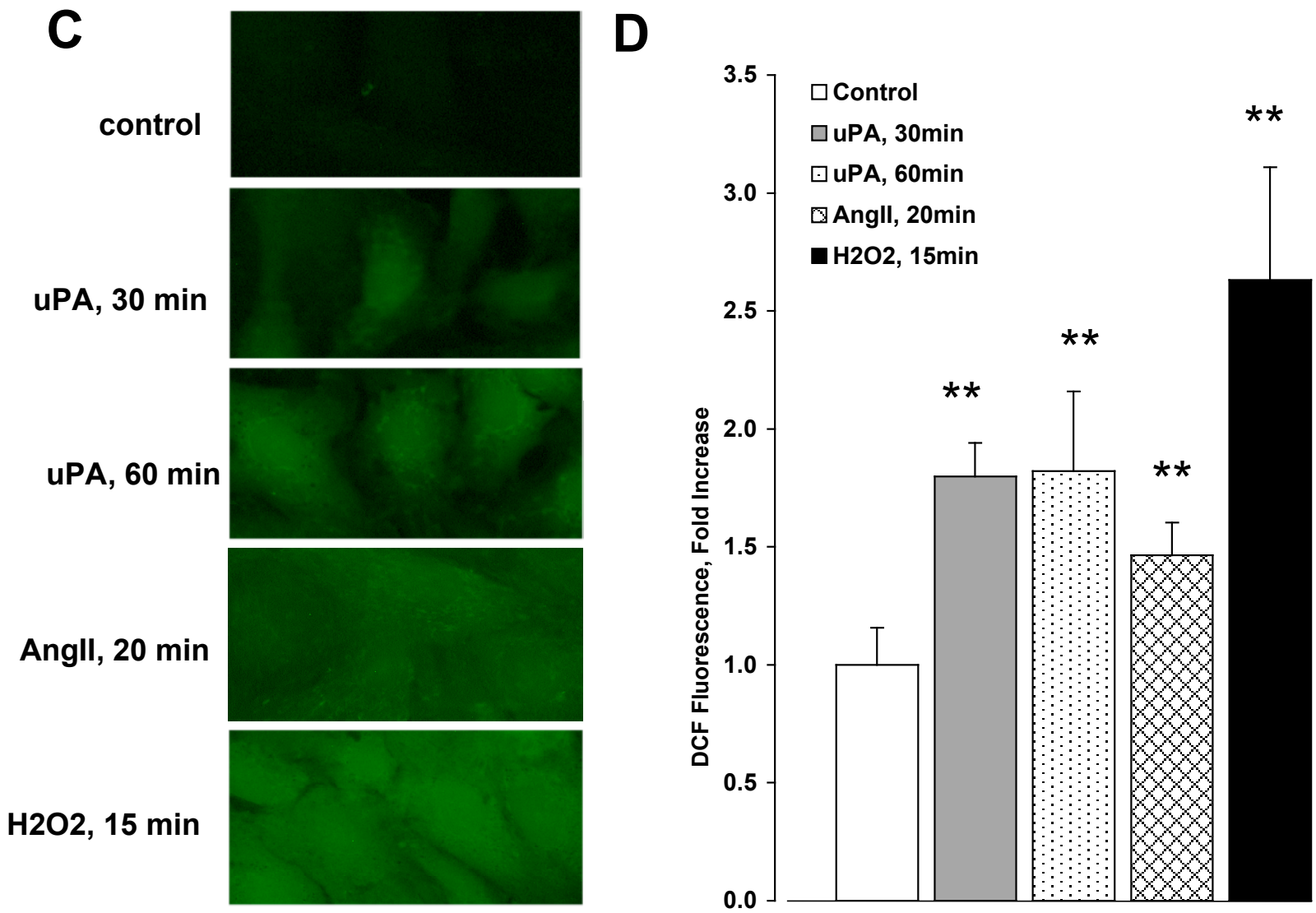

Figure I

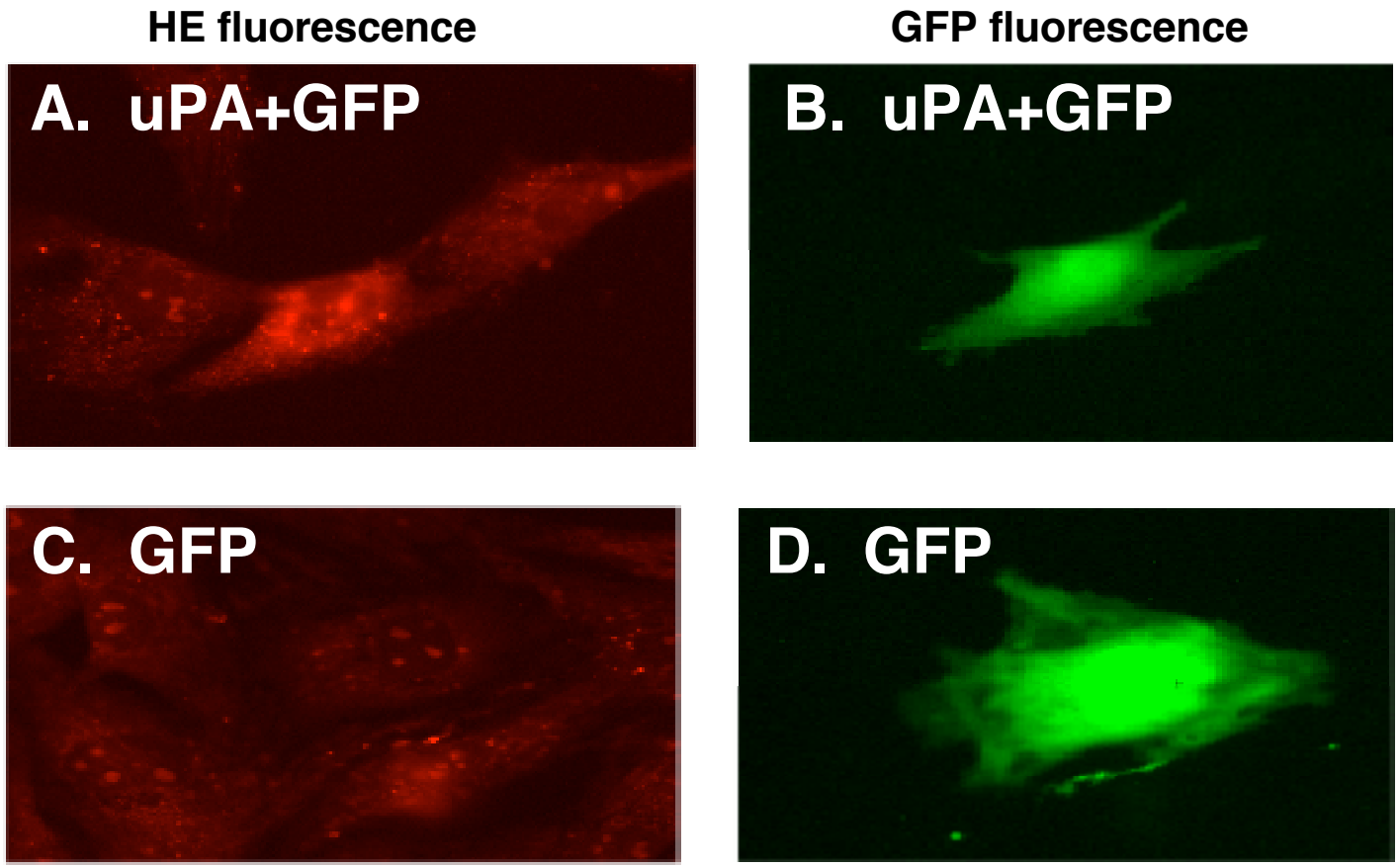

Figure II