BIOPHYSICS AND BIOCHEMISTRY

Urokinase Induces ROS Production in Vascular Smooth Muscle Cells

O. S. Plekhanova, M. Yu. Men'shikov, P. P. Bashtrykov, B. S. Berk*, V. A. Tkachuk, and E. V. Parfenova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 9, pp. 273-276, September, 2006
Original article submitted April 10, 2005

Urokinase stimulates the production of superoxide radical in cultured aortal smooth muscle cells simultaneously with activation of the expression of NAD(F)H-oxidases nox1, nox4, and phox22. Antioxidant ebselen abolishes the stimulating effect of urokinase on smooth muscle cell proliferation. The data showed that urokinase can potentiate oxidative stress in the arterial wall and can play an important role in the development of adverse arterial remodeling.

Key Words: *urokinase; superoxide radical; oxidative stress; smooth muscle cell proliferation; arterial remodeling*

Proliferation of vascular cells (smooth muscle cells (SMC), leukocytes, and fibroblasts) leading to thickening of the vascular wall and constriction of the arterial lumen is the cornerstone in arterial remodeling during various diseases including arterial hypertension, atherosclerosis, and restenosis after balloon angioplasty [1,5,12]. Urokinase plasminogen activator (urokinase) plays a key role in arterial remodeling after balloon angioplasty [1,8]. We found that urokinase stimulates the growth of neointima, media, neoadventitia, and the constrictive arterial remodeling after experimental balloon angioplasty [1,2,11]. An important role in the pathogenesis of cardiovascular diseases is played by oxidative stress resulting from damage to the vascular wall and promoting its adverse remodeling [3,4]. The formation of superoxide radical is one of the leading processes regulating vascular tone and SMC proliferation in the vascular wall [7]. After experimental balloon angioplasty, the production of superoxide radical and SMC proliferation in the damaged artery dramatically increased [10]. Urokinase promotes the release of superoxide radical from neutrophils [9]. It remains unclear whether urokinase stimulates the production of superoxide radical in SMC that could promote the development of oxidative stress and adverse arterial remodeling. Our aim was to examine the effect of urokinase on the formation of superoxide radical in aortal SMC.

MATERIALS AND METHODS

SMC were isolated from the aorta of Sprague-Dawley male rats weighing 200-250 g and cultured by the routine method [6] in DMEM (Gibco) containing 100 U penicillin, 100 μg/ml streptomycin, and 10% FCS. Passage 5-9 cells were grown to 80-90% confluence and incubated for 48 h in the medium with 0.1% FCS, thereafter 5-200 nM recombinant urokinase (American Diagnostica) in phosphate-buffered saline (PBS) was added and the cells were

Russian Cardiology Research-and-Production Complex, Ministry of Health, Moscow; *Cardiovascular Research Center, University of Rochester Medical Center, USA. ***Addresss for correspondence:*** yeparfyon@cardio.ru. E. V. Parfenova

incubated for various periods (from 15 min to 48 h); the corresponding volumes of PBS were added to control cells.

In situ production of superoxide radical (O_2^-) was evaluated using ethidium bromide fluorescence (excitation and emission wavelengths 488 and 610 nm, respectively). After deprivation and incubation with urokinase, the cells were washed with PBS and incubated with hydroethidium (5 μmol/liter) at 37°C in a humid chamber for 15 min [10]. Fluorescence was observed under a MRC-1024 confocal microscope (Bio-Rad; ×40) with a 585 nm filter. The control samples were analyzed simultaneously.

Lucigenin chemiluminescence was used as an independent method for measuring of O_2^- concentration in SMC. After deprivation, the cells were detached from the plastic with trypsin/EDTA. The cell suspension was placed into the test tubes containing lucigenin (5 μmol/liter) dissolved in Hank's solution and 5-200 nM recombinant urokinase was added. Chemiluminescence was recorded every 15 min over 4 h using an LS 7000 chemiluminometer (Beckman Instruments) [10]. The background chemiluminescence (cell-free samples) was subtracted from the experimental data and luminescence intensity was standardized per 1×10^6 SMC.

After deprivation and incubation with recombinant urokinase (20-100 nM), SMC were lysed for 4 h. The lysates were separated by electrophoresis in 12% polyacrylamide gel with sodium dodecyl sulfate, the proteins were transferred to PVDF membrane, and immunoblotting with monoclonal antibodies against nox1, nox4, and p22phox oxidases (Santa Cruz Biotechnology) was carried out. The

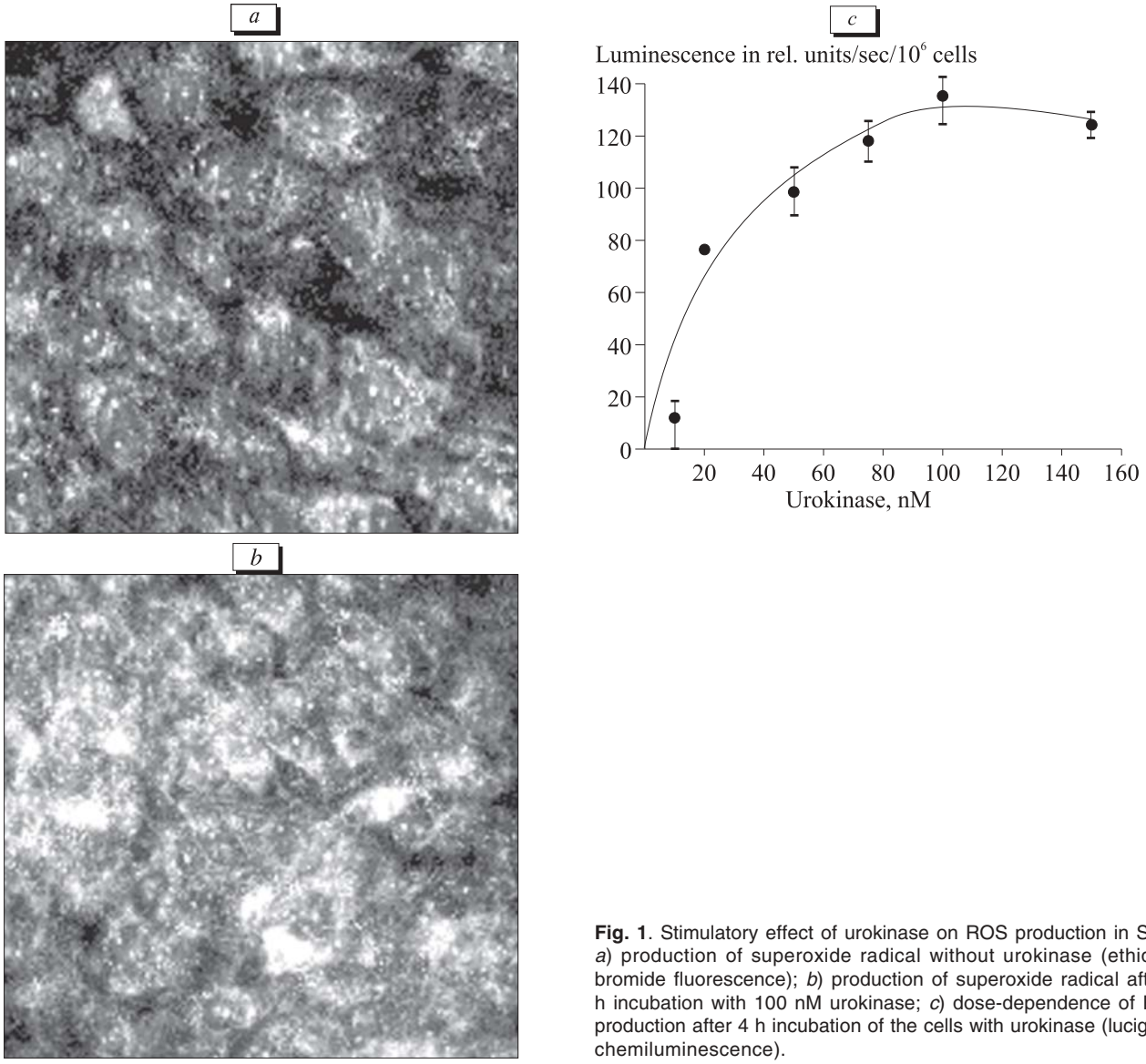

Fig. 1. Stimulatory effect of urokinase on ROS production in SMC. *a*) production of superoxide radical without urokinase (ethidium bromide fluorescence); *b*) production of superoxide radical after 4 h incubation with 100 nM urokinase; *c*) dose-dependence of ROS production after 4 h incubation of the cells with urokinase (lucigenin chemiluminescence).

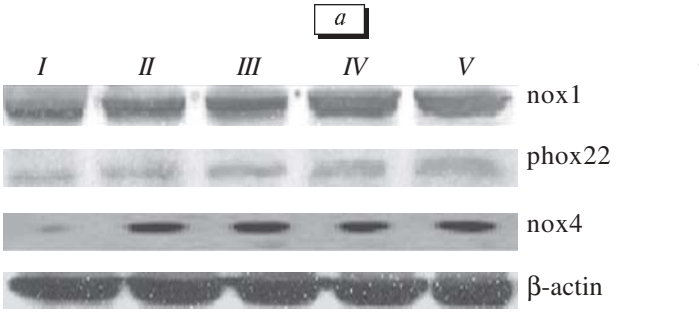

Fig. 2. Increase of the content of NAD(F)H oxidases nox1, nox4, and phox22 after 4 h incubation with urokinase. *a*) immunoblotting after incubation of cells in urokinase-free medium (*I*) and in the presence of urokinase (*II*, 20 nM; *III*, 50 nM; *IV*, 75 nM; *V*, 100 nM); *b*) dependence of expression of nox1 (*1*), nox4 (*2*), and phox22 (*3*) on urokinase concentration (averaged data from 4 experiments).

blots were visualized with ECL kit (Amersham). The intensity of stained bands was assessed with PCBAS 2.08 software.

Passage 5-7 SMC were cultured on glass. After deprivation, the cells were incubated for 24 h with 100 nM recombinant urokinase and/or with 40 μM ebselen (antioxidant, glutathione peroxidase mimic, Sigma). The cells were fixed with paraformaldehyde (3.7% in PBS), treated with Triton X-100 (1%), incubated with monoclonal antibodies against proliferating cells nuclear antigen (PCNA; 20 μg/ml, Dako), and then with second goat antibodies against mouse immunoglobulins supplied with fluorescent label (Alexa Fluor 488, Molecular Probes). The number of fluorescent PCNA-positive cells and the total cell number were counted under a Zeiss Axiovert 200M microscope. The proliferation index (I) was calculated by the formula $I=n_1\times100/n_2$, where n_1 is the number of antigen-positive cells, n_2 total number of the cells. The proliferation index in the control (in PBS without cells) was taken as 1.

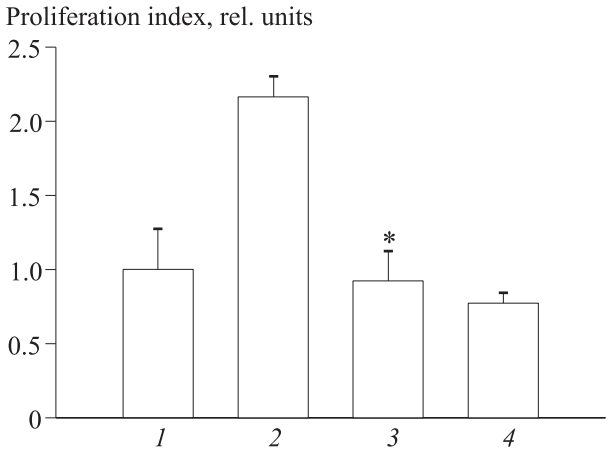

Fig. 3. Inhibitory effect of antioxidant ebselen on urokinase-stimulated proliferation of aortal SMC. Averaged data (n=4) are presented. *1*) control; *2*) 100 nM urokinase; *3*) urokinase (100 nM)+ebselen (40 μM); *4*) 40 μM ebselen. *p<0.01 compared to urokinase.

The data were analyzed statistically using Student's t test at $p\leq0.05$, Student—Newman—Keuls multiple range test (ANOVA), and a Jandel SigmaStat software.

RESULTS

Urokinase stimulated the production of superoxide radical in cultured vascular SMC. Ethidium bromide fluorescence showed that this process was most intensive after incubation with 75-100 nM urokinase for 4 h (Fig 1). Lucigenin chemiluminescence detected maximum production of superoxide radical in intact SMC also 4 h after introduction of 100 nM urokinase. The effect of urokinase was dose-dependent (Fig. 1). Addition of 100 μM MnTPyP (SOD mimic) abolished the effect of urokinase on the production of superoxide radical. It is known that urokinase promotes production [9] and release [13] of superoxide radical by neutrophils, although there are no data on the effect of urokinase on SMC. Our findings indicate the ability of urokinase to stimulate the production of superoxide radical in cultured vascular SMC.

In damaged arteries, the expression of NAD(F)H oxidases significantly increases and represents an important mechanism regulating the growth of neointima and arterial remodeling [15]. The production of superoxide radical in damaged arteries is predominantly controlled by NAD(F)H-oxidases nox1, nox4, and phox22 [14]. We evaluated the effect of urokinase on the expression of NAD(F)H-oxidases in cultured SMC. The content of these oxidases was

determined by immunoblotting 4 h after cell stimulation with 20-100 nM urokinase. This enzyme significantly stimulated expression of nox1, nox4, and phox22 (Fig. 2) in a dose-dependent manner: the content of these oxidases increased with increasing urokinase concentration. The maximum increase in oxidase expression was observed at urokinase concentrations of ≥75 nM. Therefore, our findings that urokinase regulates the level of NAD(F)H-oxidases in vascular SMC reveal a novel mechanism in the action of this enzyme, which can play a key role in the regulation of neointima growth and arterial remodeling.

Urokinase promotes proliferation of SMC [1, 11]; superoxide radicals also stimulate cell proliferation. For evaluation of the role of superoxide radical production in the regulation of SMC proliferation by urokinase, we assessed the effect of this enzyme on proliferation of cultured SMC in the presence of antioxidant ebselen. Proliferation was evaluated by immunofluorescence in the presence of antibodies against PCNA in 24 h after stimulation of the cells with 100 nM urokinase. The enzyme increased SMC proliferation index by more than 2-fold (Fig. 3). The antioxidant significantly inhibited the stimulatory effect of urokinase on SMC proliferation, which indicates the existence of presently unknown mechanism of urokinase-effected regulation of cell division.

Thus, our experiments showed that urokinase stimulates the production of superoxide radical in vascular SMC, which can up-regulate damage-induced oxidative stress in the arterial wall. This novel mechanism of urokinase action plays a key role in the regulation of SMC division, stimulation of neointima growth leading to narrowing of the arterial lumen, and development of adverse arterial remodeling.

This work was supported by the Russian Foundation for Basic Research, grant No. 06-04-49303, Supplementary Grant NHLBI HL 62826 (B.C.B.), and NATO grant LST.CLG.979209

REFERENCES

1. E. V. Parfenova, O. S. Plekhanova, and V. A. Tkachuk, *Biokhimiya,* **67**, No. 1, 119-134 (2002).
2. O. S. Plekhanova, M. A. Solomatina, C. P. Domogatskii, *et al*., *Fiziol. Zh.,* **87**, No. 5, 584-593 (2001).
3. J. Abe and B. C. Berk, *Trends Cardiovasc. Med*., **8**, 59-64 (1998).
4. R. W. Alexander, *Hypertension,* **25**, No. 2, 155-161 (1995).
5. S. M. Arribas, C. Hillier, and C. Gonzales, *Ibid.*, **30**, No. 6, 1455-1464 (1997).
6. A. S. Baas and B. C. Berk, *Circ. Res*., **77**, No. 1, 29-36 (1995).
7. B. C. Berk, *Physiol. Rev*., **81**, No. 3, 999-1030 (2001).
8. A. Bobik and V. Tkachuk, *Curr. Hypertens. Rep*., **5**, No. 6, 466-472 (2003).
9. D. Cao, I. F. Mizukamil, and B. X. Garni-Wagner, *et al*., *J. Immunol*., **154**, No. 4, 1817-1829 (1995).
10. G. L. Nunes, K. Robinzon, A. Kalynych, *et al., Circulation,* **96**, No. 10, 3593-3601 (1997).
11. O. Plekhanova, J. Parfyonova, R. Bibilashvily, *et al., Atherosclerosis,* **159**, No. 2, 297-306 (2001).
12. R. Schwartz*, Am. J. Cardiol*., **81**, No. 7A, 14E-17E (1998).
13. R. G. Sitrin, P. M. Pan, H. A. Harper, *et al., J. Immunol*., **165**, No. 6, 3341-3349 (2000).
14. K. Szocs, B. Lasseque, D. Sorescu, *et al., Arterioscler. Thromb. Vasc. Biol*., **22**, No. 1, 21-27 (2002).
15. N. L. Weintraub*, Ibid.*, **22**, No. 1, 4-5 (2002).