Research Paper

Journal of Vascular Research

J Vasc Res 2009;46:177–187
DOI: 10.1159/000156703

Received: January 13, 2008
Accepted after revision: March 18, 2008
Published online: September 24, 2008

Oligonucleotide Microarrays Reveal Regulated Genes Related to Inward Arterial Remodeling Induced by Urokinase Plasminogen Activator

Olga Plekhanova[a] Bradford C. Berk[c] Pavel Bashtrykov[a] Andrew I. Brooks[c]
Vsevolod Tkachuk[a, b] Yelena Parfyonova[a]

[a]Cardiology Research Center, Moscow, and [b]Lomonosov Moscow State University, Moscow, Russia;
[c]Aab Cardiovascular Research Institute, University Rochester Medical Center, Rochester, N.Y., USA

Key Words
Urokinase plasminogen activator · Inward arterial remodeling · Inflammation · Oxidative stress · Microarray

Abstract
Accumulating evidence suggests that urokinase plasminogen activator (uPA) is involved in vascular remodeling and lumen stenosis after angioplasty and stenting. We have shown previously that increased uPA expression greatly promotes neointima formation and inward arterial remodeling after balloon injury. To evaluate the role of inflammation in early mechanisms responsible for inward arterial remodeling induced by uPA and elucidate the mechanisms of remodeling, we characterized changes in the expression profiles of 8,799 genes in injured rat carotid arteries 1 and 4 days after recombinant uPA treatment compared to vehicle. We used a standard model of the balloon catheter injury of the rat carotid followed by periadventitial application to the injured vessel of either uPA dissolved in Pluronic gel, or plain gel. Vessels were harvested and analyzed by immunohistochemistry, morphometry, microarray gene expression profiling and quantitative RT-PCR. Periadventitial application of uPA significantly reduced lumen size and vessel area encompassed by the external elastic lamina at both 1 and 4 days after treatment. Inflammatory cells accumulated in the arterial adventitia at both 1 and 4 days after uPA treatment. On the 4th day, increases in the areas and arterial cell numbers of all arterial layers were found. Among 79 differentially expressed known genes 1 day after uPA application, 12 proinflammatory genes, including TNF-α and TACE, and 15 genes related to mitochondrial metabolism and oxidative stress regulation were identified. Four days after injury in uPA-treated arteries, 3 proinflammatory and 2 oxidation-related genes were differentially expressed. We conclude that uPA likely promotes inward arterial remodeling by regulating oxidative stress and inflammation after arterial injury.

Introduction

Inward vessel remodeling and intima-media thickening are the fundamental mechanisms responsible for lumen narrowing [1] in a number of pathological conditions including restenosis after transluminal coronary angioplasty and intraluminal stenting [2, 3]. We and others have demonstrated previously that urokinase plasminogen activator (uPA) is a potent stimulator of neointima growth and unfavorable arterial remodeling [4–7]. Despite multiple studies focused on the problem of arterial remodeling, the exact mechanisms of uPA effects in the vessel wall are not fully understood.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

Accessible online at:
www.karger.com/jvr

Prof. Yelena Parfyonova
Molecular Endocrinology Laboratory
Institute of Experimental Cardiology
Cardiology Research Center, Moscow 121552 (Russia)
Tel. +7 495 414 67 13, Fax +7 495 414 67 12 (19), E-Mail yeparfyon@cardio.ru

Vascular inflammation induced by proinflammatory cytokines is one of the key mechanisms in the development of neointimal hyperplasia, restenosis and atherosclerosis [8, 9]. In particular, leukocytes contribute to the generation of reactive oxygen species (ROS) and augment oxidative stress and restenosis [10]. Moreover, monocytes/macrophages represent the main source of cytokines playing the key roles in the progression of vascular pathologies. In our previous studies in the rat model of balloon catheter injury of the carotid artery we found that uPA augmented inflammatory cell accumulation in the arterial adventitia [7]. These data are consistent with findings in uPA knockout mice that uPA mediated infiltration of leukocytes during arterial remodeling and neointima formation after injury [4].

We hypothesized that the inflammatory mediators could be involved in the effects of urokinase in the injured arterial wall. To evaluate the role of inflammation in early mechanisms responsible for inward arterial remodeling induced by uPA, we characterized changes in gene expression profiles in injured rat carotid arteries after the local application of recombinant uPA (r-uPA).

Materials and Methods

Animals and Experimental Design

Male Wistar-Kyoto rats (4–5 months old) were obtained from a colony maintained under standard colony conditions including 12-hour light/12-hour dark cycle, 22 ± 2°C temperature and free access to food and water. Their left common carotid artery was subjected to balloon catheter injury using surgical procedures approved by the Cardiology Research Center's Animal Experimentation Committee.

Following balloon catheter injury, 20 nmol/kg of r-uPA dissolved in Pluronic gel or 500 μl of plain Pluronic gel F-127 (BASF) were applied to the adventitial side of left common carotid arteries [11]. One and four days later, the animals were killed and total RNA was isolated from left and right carotids of both ballooned and sham-operated as well as nonoperated animals (9 rats per group). Changes in gene expression profiles were analyzed using U34A microarray hybridization (Affymetrix, Santa Clara, Calif., USA) and Quantitative RT- PCR (Applied Biosystems).

Surgery and Tissue Collection

Briefly, after anesthetizing the rats with ketamine (100 mg/kg body weight), the common carotid arteries were injured with an inflated Fogarty 2 F balloon catheter, the external carotid arteries were ligated, then uPA or vehicle in Pluronic gel were applied around the common carotids as previously described [11]. One or four days after surgery, the animals were perfusion-fixed, the removal of endothelium was confirmed with the solution of Evans blue dye, carotids were harvested, embedded in paraffin [5], and cross-sections for morphometry (10 μm) and immunohistochemistry (5 μm) were made as described [7]. For microarray technique left and right common carotids were rapidly removed, rinsed with cold saline solution to remove blood, cleaned of extraneous material and frozen in liquid nitrogen.

Morphometry and Immunohistochemistry

For morphometry, cross-sections were stained with toluidine blue and analyzed using a Zeiss microscope coupled to a Prog-Ress-3008 camera (Kontron Elektronik) and a computer with a UTHSCSA ImageTool, version 3.0, image processing and analysis program (San Antonio, Tex., USA) as previously described [11].

Immunohistochemical staining was performed using either the anti-PCNA monoclonal antibody (4.8 μg/ml), the anti-ED-1 monoclonal antibody (3.3 μg/ml), the anti-α smooth muscle actin monoclonal antibody (3.6 μg/ml) or the mouse control nonimmune IgGs in concentrations coinciding with those of each immune IgG as previously reported [7].

Microarray Analysis

Total RNA was extracted from homogenized samples using the Qiagen RNAeasy Micro kit. RNA quality was assessed by gel electrophoresis and spectrophotometric analysis prior to cDNA synthesis. For each hybridization experiment, the probe was generated using material pooled from 3 animals; 3 hybridizations were performed per treatment group. The amplification and biotinylation of probes were done according to Affymetrix recommendations for microarray analysis [12]. Affymetrix rat genome U34A oligonucleotide microarrays, representing 8,799 known genes and expressed sequence tags (ESTs), were used (www.affymetrix.com) at the University of Rochester Microarray Core Facility. Hybridization, staining, washing, and scanning were performed as per the manufacturer's protocol. Streptavidin-phycoerythrin stain (SAPE, Molecular Probes) was used as the fluorescent conjugate to detect hybridized target sequences. The detection and quantitation of target hybridization were performed with a Gene Array Scanner (Hewlett Packard/Affymetrix). All arrays were assessed for 'array performance' prior to data analysis. Control transcripts were spiked into the hybridization mixture to control for hybridization efficiency and sensitivity. To evaluate inter-array variability, the Affymetrix software (Microarray Analysis Suite; MAS 5.0) was employed and the array data were normalized by a global scaling approach to a target intensity of 500 across all probe sets [12]. To reduce the errors due to cross-hybridizing probes and image contamination and lower the estimated false detection rate and the number of targets included in the further statistical analyses, we calculated model-based expression indexes (MBEI) for perfect match (PM)-only arrays using DNA-Chip Analyzer (dChip) software package [13]. The invariant set normalization method was applied prior to analysis. The percentage of probe sets called 'Present' in all arrays was >40%. The 'Array-outlier' percentage in all arrays did not exceed 5%, indicating the absence of dramatic image contamination or sample hybridization problems of our arrays. We also performed gene filtering to exclude genes that are 'Absent' in the majority of the samples, restricting the data analyses to targets called 'Present' in more than 20% of arrays. We confirmed that there was negligible inter-array variability (SD less than 2%) of the mean signal (with 5% of signals trimmed from both the high and low ends) across the 4,458 targets that passed the presence/absence filter. After dChip-filtering, we used significance analysis of microarrays (SAM) software as we previously described [14] to iden-

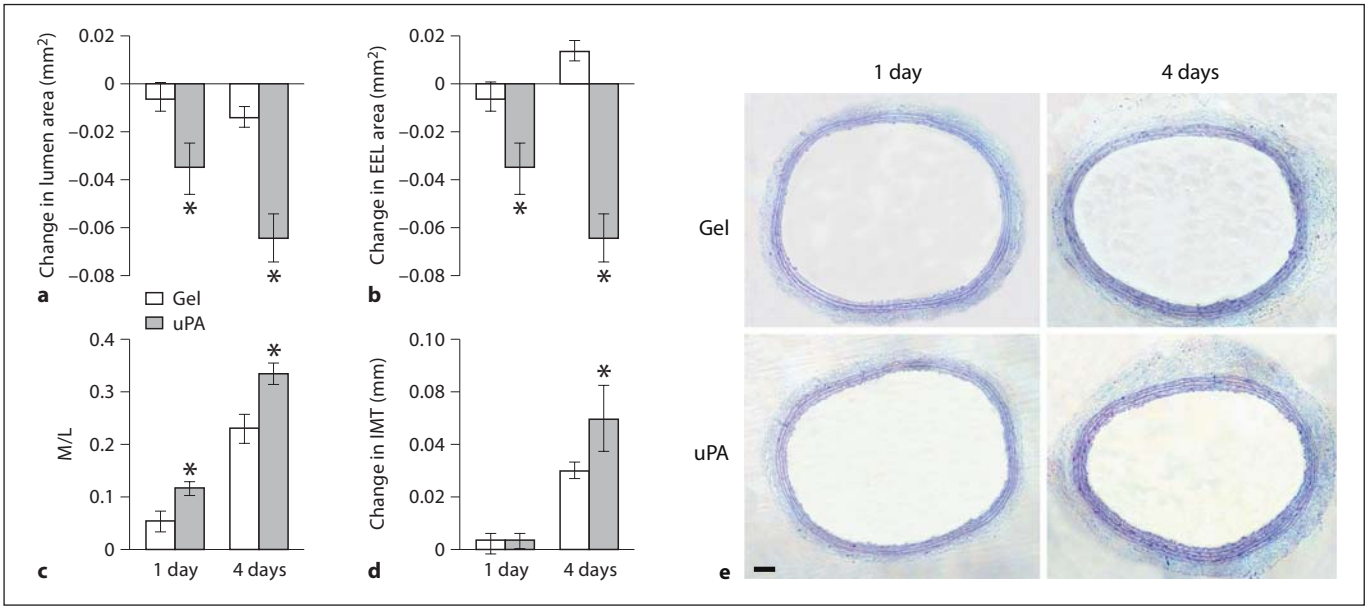

Fig. 1. Bar graphs summarizing the effects of perivascularly applied r-uPA 1 and 4 days after balloon injury on lumen area (**a**), EEL cross-sectional area (**b**), M/L (**c**) and IMT (**d**) 1 and 4 days after injury to carotid arteries. 'Gel' represents control vessels treated only with Pluronic gel/saline. For lumen and EEL areas, all injured vessels were compared with their corresponding right uninjured carotid artery in the same animals and the differences calculated. Results are the means ± SEM of 6 or 7 animals in each group. * $p < 0.05$ from control. **e** Histological figures depicting the effects of perivascular administration of r-uPA on the vessel structure 1 and 4 days after balloon catheter injury of the carotid artery. 'Gel' represents control vessels which only received Pluronic gel/saline. Sections are stained with toluidine blue; magnification ×63. Scale bar 500 μm.

tify genes with statistically significant changes in expression. We also performed statistical comparison analysis using parametric ANOVA or Welch's approximate t test for non-equal variances (GeneSpring Software; Silicon Genetics, Redwood City, Calif., USA). To obtain highly confident and robust data, we considered significant only genes confirmed by both analyses (SAM and Welsh's t test) [15].

Software

The statistical algorithms of Microarray Suite 5.0 were used with the default parameters to generate signals. DNA-Chip Analyzer software package was kindly provided by Drs. Cheng Li and Wing Hung Wong, Computational Biology Lab, Department of Biostatistics, Harvard School of Public Health (www.dchip.org). Data generated by Microarray Suite and DNA-Chip Analyzer were exported to Microsoft Excel for further analysis. SAM runs within Excel. SAM was kindly provided by the Department of Statistics, Stanford University (http://www-stat.stanford.edu/~tibs/SAM/). GeneSpring 5.0 software was obtained from Silicon Genetics (http://www.silicongenetics.com/).

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) analyses were performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems) [16]. Double-stranded cDNA template preparation and purification were performed with the Ambion MessageAmp aRNA kit. The qPCR primers and Master Mix from RT2 Real-Time Gene Expression Assay kits (SuperArray) were obtained for 6 rat genes: β-actin (V01217), manganese-containing SOD-2 (AA926129), TNF-α-converting enzyme (TACE) (AJ012603), TNF-α (L00981), MMP-2 (U65656), cAMP phosphodiesterase 4B (PDE4B) (M25350). Each reaction contained 2 μl of SYBR Green PCR Master Mix (Applied Biosystems) and 2 μl diluted cDNA (1:100). The PCR consisted of an initial enzyme activation step at 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Statistics

All results are reported as mean ± SEM. Statistical tests were performed with Jandel SigmaStat. Comparisons were made by the t test or ANOVA for repeated measurements, as appropriate. A value of $p < 0.05$ was considered statistically significant.

Results

Urokinase and Vessel Remodeling

To evaluate the effects of uPA on arterial remodeling after injury, we evaluated the areas, encompassed by the external elastic lamina (EEL), lumen areas, media/lumen ratio (M/L), and intima-media thickness (IMT), important parameters reflecting vessel remodeling after bal-

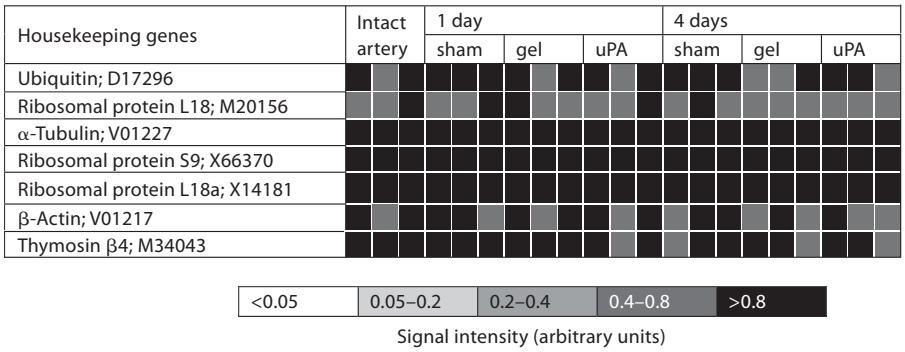

Fig. 2. Transcription profiles of highly expressed housekeeping genes in rat carotid arteries. Each column represents a gene expression pattern for one replicate. For each gene, the signal intensity level normalized to the average signals of 7 housekeeping genes is represented by a gray value according to the signal intensity scale at the bottom.

loon injury to the rat carotid artery. Periadventitial application of uPA progressively decreased both lumen and EEL areas 1 and 4 days after injury ($p < 0.05$; fig. 1a, b). uPA significantly increased the M/L ratio 1 and 4 days after injury ($p < 0.05$; fig. 1c) and IMT 4 days after injury ($p < 0.05$; fig. 1d). These parameters show that the vessel remodeling response induced by uPA leads to lumen narrowing (fig.1e), a decrease in EEL and an increase in IMT.

Differential Gene Expression Profiling Induced by uPA

To identify the downstream mechanisms underlying uPA-stimulated neointima formation and inward remodeling, in vivo gene expression profiling was performed. The expression of 8,799 genes was analyzed 1 and 4 days after arterial injury followed by treatment with either exogenous recombinant uPA or vehicle (Plain gel/saline). To confirm successful hybridization, we analyzed the expression of 7 housekeeping genes including ribosomal proteins L18, L18a and S9, α-tubulin, thymosin β4, ubiquitin, β-actin, which consistently gave comparably positive signals in all samples including intact artery, sham-operated, operated controls and experimental arteries (fig. 2). Analysis of signal intensities revealed that 1 day after the operation in arteries treated with uPA 150 transcripts (62 known genes, table 1A) differed significantly compared to control ($p \leq 0.05$) confirmed by two statistical analyses. Among these genes, 34 (54.8%) were found to be downregulated, and 28 (45.2%) upregulated after uPA treatment compared to control (plain gel/saline). At day 4, 65 mRNAs (31 known genes, table 1B) were significantly different in the uPA group (complete information is available from the authors upon request). Among these genes, 8 (25.8%) were downregulated and 23 (74.2%) were upregulated compared to control.

uPA-Induced Upregulation of Proinflammatory Gene Expression

To evaluate the role of inflammatory mediators in inward arterial remodeling induced by uPA early after arterial injury, we analyzed changes in gene expression profiles in injured arteries after uPA application. Clusters of genes participating in inflammatory responses were found to be activated in injured artery both 1 and 4 days after uPA treatment. Proinflammatory gene patterns may come from infiltrating inflammatory cells such as macrophages and neutrophils (for example CD45 and Nramp 2, table 1A) or lymphocytes (for example CCR10, table 1B) or may be induced in vascular smooth muscle cells (VSMC) (for example interleukin-1β-converting enzyme (ICE, caspase-1) and TACE, table 1A). Caspase-1 enzymatically activating the IL-1β and IL-18 precursors is the founding member of the caspase family of enzymes, which are involved in maturation of cytokines and in initiation and execution of apoptotic processes [17]. Since IL-1β and TNF-α are central mediators in the cytokine network, they may act as the potent activators of cardiovascular cells involved in the pathogenesis of cardiovascular diseases including atherosclerosis and restenosis. The selective upregulation of TACE in uPA-treated vessels compared with vehicle-treated arteries deserves special attention since TNF-α is one of the most potent proinflammatory cytokines secreted by monocytes/macrophages. Moreover, a number of studies demonstrated the role of TNF-α signaling for proliferative arterial diseases and restenosis [18, 19].

uPA and Differential Expression of Oxidative-Stress-Related Genes

Of greatest interest, among genes differentially expressed 1 day after injury and uPA treatment were 13 genes related to mitochondrial metabolism and oxida-

Table 1. A Transcription profiles of genes differentially expressed in rat carotid artery 1 day after balloon injury treated with uPA compared to control vessels, which only received Pluronic gel/saline

Gene Identity	GenBank accession	Welch t test p/direction		Effects relevant to vascular remodeling
Adhesion and extracellular matrix				
Osteonectin (SPARC)	U75928	0.02	down	anti-adhesive, anti-proliferative, matrix remodeling
Prepro bone morphogenetic protein 3	D63860	0.001	down	matrix remodeling
Bone morphogenetic protein 3	S77492	0.01	down	matrix remodeling
Collagen alpha1 type I	Z78279	0.01	down	matrix remodeling
Procollagen C-proteinase enhancer protein	AF016503	0.018	down	cell growth and differentiation
Lumican	X84039	0.006	down	matrix remodeling
MT3-MMP-del	D63886	0.03	down	matrix remodeling
Embigin	AJ009698	0.01	up	cell/matrix interaction
Zn-peptidase aminopeptidase N	M25073	0.01	up	cell invasion
Migration and cytoskeleton				
β-Tropomyosin and fibroblast tropomyosin 1	L00382	0.03	down	cell motility
Tubulin	AB015946	0.007	down	migration, adhesion, proliferation
Vascular α-actin	X06801	0.03	down	SMC phenotype
Nerve growth factor	E03082	0.01	down	promigrative
Urokinase plasminogen activator	X63434	0.001	up	promigrative
Syndecan = heparan sulfate proteoglycan core protein	S61865	0.02	up	promigrative
GAS-7 protein	AJ131902	0.03	up	reorganization of microfilaments
Proliferation and apoptosis				
Defender against cell death-1 (DAD-1)	Y13336	0.03	down	anti-apoptotic
Platelet-derived growth factor A chain	D10106	0.016	down	mitogenic, pro-migrative
Heat shock protein 70	L16764	0.03	down	anti-apoptotic
PRG1 gene	X96437	0.01	up	mitogenic, pro-apoptotic
Double-stranded RNA-specific adenosine deaminase	U18942	0.03	up	proapoptotic
Inflammation				
Interleukin-15	U69272	0.003	down	proinflammatory
Protein S (activated protein C cofactor)	S78744	0.03	down	proinflammatory
Natural resistance-associated macrophage protein 2 (Nramp2)	AF008439	0.007	up	proinflammatory
Interleukin-1β-converting enzyme (ICE, caspase-1)	U14647	0.005	up	proinflammatory, pro-apoptotic
TNF-α-converting enzyme (TACE)	AJ012603	0.03	up	proinflammatory, pro-apoptotic
Leukocyte-common antigen (LCA, CD45 or T200)	M25823	0.04	up	proinflammatory
MHC class II-associated invariant chain	X14254	0.03	up	proinflammatory
MHC class I non-RT1.A alpha-1-chain	M31038	0.02	up	proinflammatory
MHC class I RT1.C-type protein	L40362	0.02	up	proinflammatory
MHC class I RT1.O type	L40364	0.04	up	proinflammatory
MHC class I antigen (RT1.EC3)	AF074609	0.03	up	proinflammatory
Transcription				
TIC transcription factor (HLH/PAS domain family)	AF015953	0.02	down	circadian transcription factor
Histone (H2A.Z)	M37584	0.02	down	transcription regulator
Retinoid X receptor γ (RXRγ)	AF016387	0.03	up	transcription factor
MSS1 protein	U13895	0.005	up	transcription regulator
Angiotensinogen gene-inducible enhancer-binding protein 1	M65251	0.03	up	gene expression regulator
Putative protein phosphatase 1 nuclear targeting subunit (PNUTS)	AF040954	0.02	up	nuclear function
Signaling				
Phospholipase C	J03806	0.007	down	mitogenic
Phospholipase C (β4)	L15556	0.02	down	mitogenic?
Receptor-linked protein tyrosine phosphatase	L19180	0.01	down	differentiation/inflammation?
Phospholipase C-β1	L14323	0.009	down	fibrosis
cAMP phosphodiesterase (PDE4)	M25350	0.035	down	mitogenic
Frizzled (Drosophila polarity gene homologue)	L02529	0.003	down	anti-proliferative
cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII)	U78517	0.04	down	unknown (small GTPase Rap1)
Protein phosphatase-2_A, A regulatory subunit	D14418	0.04	up	anti-apoptotic, proproliferative, pro-oxidant
MAP-kinase phosphatase (cpg21)	AF013144	0.007	up	anti-proliferative, promigrative
Lyn protein non-receptor kinase	L14951	0.002	up	mitogenic

Table 1. A (continued)

Gene Identity	GenBank accession	Welch t test p/direction		Effects relevant to vascular remodeling
Metabolism and others				
L-glutamine amidohydrolase	J05499	0.02	down	oxidative stress/inflammation
Aldehyde dehydrogenase	J03637	0.016	down	vasorelaxation
Lipoprotein lipase	L03294	0.04	down	unknown
P-glycoprotein-like ATP-binding cassette transporter	AF106563	0.03	down	unknown
Oxidosqualene cyclase	U31352	0.016	down	unknown
rPER2	AB016532	0.03	down	unknown
Drs (a gene down-regulated by v-src)	D78359	0.04	down	unknown
Submaxillary gland alpha-2u globulin	J00738	0.03	down	unknown
Homologue of Drosophila slit protein (MEGF5)	AB011531	0.003	down	unknown
BEST5 (hypothetical protein)	Y07704	0.01	up	unknown
Sulfated glycoprotein 1 (SGP-1)	M19936	0.03	up	unknown
Aquaporin	AB013112	0.003	up	unknown
S-adenosylmethionine synthetase	J05571	0.02	up	unknown
Vasopressin V1b receptor variant	AF064541	0.04	up	unknown

tive stress (table 2). Among these genes, 8 encoding mitochondrial proteins were downregulated, and 5 were upregulated (table 2). At day 4 after injury, the expression of 2 genes with putative relevance to oxidative stress was elevated in uPA-treated arteries (table 2). Thus, microarray gene analysis showed that uPA may cause unfavorable arterial remodeling at least in part through the upregulation of oxidative stress after arterial injury. These data are consistent with our previous findings that uPA can increase ROS production in VSMC and stimulate VSMC growth in a ROS-dependent manner [20].

uPA Effects on Gene Expression Profiling Relevant to Vascular Remodeling

Among genes differentially expressed after uPA treatment we also found several clusters of genes involved in mediating the key processes of vascular remodeling such as cell migration, proliferation and apoptosis, as well as adhesion and extracellular matrix (ECM) remodeling (table 1A, B). At the same time, the significant up-regulation of uPA mRNA (GenBank No. X63434) was observed ($p \leq 0.0005$) that may reflect the mechanism of uPA self-regulation as positive feedback, enhancing and elongating the uPA effects in the injured artery. The expression of matrix metalloproteinase-2 (MMP-2) was also up-regulated (table 1B), consistent with our previous findings that perivascular urokinase can increase the content and activity of MMP-2 in the injured artery [21]. Importantly, MMP-2 participates in vascular remodeling not only providing cell migration, but is also associated with the inflammatory response after injury [22].

Validation of Array Data by PCR

In a separate experiment we validated our microarray data using the same samples of aRNA from the carotid arteries. For validation of hybridization signals by qPCR, we selected several genes of interest: TACE, TNF-α, MMP-2, manganese-containing SOD-2, cAMP phosphodiesterase 4B (PDE4B) and β-actin (table 3). Microarray analysis and qPCR showed similar results, indicating the confidence of microarray data.

Urokinase and Cell Numbers in Injured Arteries

Monocyte/Macrophage Infiltration

Since the expression of proinflammatory genes may originate from infiltrating macrophages, we evaluated the accumulation of monocytes/macrophages in arterial layers 1 and 4 days after balloon injury and uPA treatment. On the 1st day after uPA application, the frequency of ED1-positive cells in the media did not differ significantly between groups ($p > 0.05$; table 4), but was increased in the adventitia ($p < 0.05$; table 4). Four days after the operation, the proportion of ED1-positive cells was significantly increased only in the adventitia of uPA-treated arteries [7]. There were similar trends in the neointima and media, but changes were not statistically significant.

Table 1. B Transcription profiles of genes differentially expressed in rat carotid artery 4 days after balloon injury treated with uPA compared to control vessels, which only received

Gene Identity	GenBank accession	Welch t test p/direction		Effects relevant to vascular remodeling
Adhesion and extracellular matrix				
Osteonectin (SPARC)	U75928	0.04	up	anti-adhesive, anti-proliferative, matrix remodeling
Plakoglobin	U58858	0.03	up	proadhesive
Migration and cytoskeleton				
α-Actinin-2 associated LIM protein	AF002281	0.04	up	muscle contraction
Gelatinase A (MMP-2)	U65656	0.02	up	promigrative
Plectin	X59601	0.04	up	promigrative
Inflammation				
CCR10-related receptor	U92803	0.04	down	leukocyte chemotaxis
Interferon-γ-inducing factor (IGIF; IL-18) isoform α-precursor	U77777	0.03	down	proinflammatory
Similar to cyclophilin c	C07012	0.04	up	proinflammatory
Proliferation and apoptosis				
ICE-like cysteine protease (Lice)	U49930	0.01	down	proapoptotic
Tissue factor pathway inhibitor	D10926	0.02	down	proapoptotic/anti-thrombotic
Transforming growth factor β-3	U03491	0.01	up	cell growth and differentiation
Transcription				
VL30 element	M91234	0.002	down	transcription regulator
Proteasomal ATPase (MSS1)	D50694	0.04	up	transcription regulator
Signaling				
Phospholipase C-1	M20636	0.03	down	vasoconstriction/mitogenic
Sky	D37880	0.02	up	mitogenic/anti-apoptotic
Phosphatidylinositol synthase	D82928	0.047	up	mitogenic
Adenylyl cyclase type V (GTPase activating protein)	M96159	0.02	up	unknown
Guanine nucleotide-releasing protein (mss4) (GTFase regulation)	L10336	0.02	up	unknown
Prenylated rab acceptor 1 (PRA1) (binds Rab GTPases)	AF025506	0.005	up	unknown
eIF-2B β subunit (GEF)	U31880	0.02	up	unknown
p34 protein (activated by Rho family GTPases)	D13623	0.02	up	proproliferative
Annexin VI (GTP-binding protein)	X86086	0.035	up	proadhesive?
Metabolism and others				
Serine proteinase rPC7 precursor	U36580	0.04	down	migration/inflammation?
PKF-M (phosphofructokinase-M)	D21869	0.04	down	unknown
Alpha-soluble NSF attachment protein	X89968	0.04	up	unknown
Tat-binding protein homolog Sata	U77918	0.03	up	unknown
Glycogenin	U96130	0.01	up	unknown
Pyridoxine 5-phosphate oxidase	U91561	0.08	up	unknown
β-Chain clathrin associated protein complex AP-1	M77245	0.02	up	unknown
RB109 (brain specific protein)	D26154	0.03	up	unknown
Liver-specific transport protein	L27651	0.04	up	unknown

Proliferation Indexes and Total Cell Numbers

To further elucidate the processes induced by uPA-regulated genes, we compared the proportion of PCNA-positive VSMC in arterial layers of uPA- and vehicle-treated arteries. On the 1st day after uPA treatment, there was a small but not significant increase in the percentage of PCNA-positive cells in the adventitia without changes in the proportion of PCNA-positive cells in the media ($p > 0.05$; table 4). Four days after injury, uPA significantly increased the frequency of PCNA-expressing cells in the neointima [7].

We also evaluated the effects of uPA on cell accumulation in arterial layers. One day after the operation, uPA increased adventitial cell numbers by nearly 20% compared to control arteries ($p < 0.05$; table 4) without affecting total cell numbers in the media ($p > 0.05$; table 4). On the 4th day, perivascular uPA significantly increased the numbers of cells in the neointima (155.9 ± 33.4 vs. 84.7 ± 8.6 in control, $p < 0.05$), media (234.3 ± 57.6 vs. 152.4 ± 12.9 in control, $p < 0.05$) and adventitia (303.7 ± 69.7 vs. 171.8 ± 13.8 in control arteries, $p < 0.05$).

Table 2. Transcription profiles of genes related to mitochondrial metabolism and oxidative stress regulation differentially expressed in rat carotid artery 1 day after balloon injury treated with uPA compared to control vessels, which only received Pluronic gel/saline

Gene identity	GenBank accession	Welch t test p/direction		Group
1 day after injury: uPA treatment vs. control				
NADH ubiquinone oxidoreductase Fe-S protein 6	L38437	0.02	down	Complex I
Mitochondrial uncoupling protein 1	A04674	0.02	down	Complex IV
Cytochrome c oxidase subunit VIa polypeptide 2	X12554	0.01	down	Complex IV
Cytochrome c oxidase subunit VIa	X72757	0.003	down	Complex IV
ATP synthase γ-subunit	L19927	0.01	down	Complex V
Metallothionein 2 and metallothionein 1	M11794	0.01	down	Anti-OxStress
Glutathione transferase subunit 8	X62660	0.04	down	Anti-OxStress
Inducible carbonyl reductase	D89069	0.001	down	Anti-OxStress
αB-crystallin-related protein	D29960	0.001	down	Anti-OxStress
Fumarylacetoacetate hydrolase	M77694	0.04	down	Anti-OxStress
Pyruvate dehydrogenase kinase	L22294	0.001	down	OxStress
Cyclooxygenase 1	U03388	0.03	down	OxStress
L-glutamine amidohydrolase	J05499	0.02	down	OxStress
Mitochondrial uncoupling protein-2	AB010743	0.004	up	Anti-OxStress
PP2A regulatory subunit	D14418	0.04	up	Anti-OxStress?
Chaperonin 60 and chaperonin 10	U68562	0.04	up	Anti-OxStress
Manganese-containing SOD-2	AA926129	0.02	up	Anti-OxStress
Testosterone 6-βa-hydroxylase (CYP3A1)	L24207	0.04	up	OxStress
4 days after injury: uPA treatment vs. control				
Alcohol dehydrogenase/PCR	M15327	0.003	up	OxStress
Aldose reductase	M60322	0.01	up	OxStress

Table 3. Gene expression in rat carotid arteries by Microarray and qPCR

Gene	GenBank accession	uPA 1 day vs. Gel 1 day		uPA 4 days vs. Gel 4 days	
		Microarray/ Welch t test p	qPCR fold change	Microarray change/Welch t test p	qPCR fold change
TACE	AJ012603	up/0.03	up/1.48	no change	up/1.28
TNF-α	L00981, E02468cds_s_at	N/A	no change	N/A	up/1.9
MMP-2	U65656	no change	no change	up/0.02	up/1.5
Manganese-containing SOD-2	AA926129	up/0.02	up/1.39	no change	no change
β-Actin	V01217	no change	no change	no change	no change
PDE4B	M25350	down/0.035	down/–1.61	no change	no change

For statistical analysis, we used Superarray online Analyzer software, which compared expression data from multiple membranes (we had 3 in each group). Expression of GAPDH (1) was similar on all membranes ($p > 0.05$). qPCR was repeated 3 times.

N/A: gene was excluded by filtering.

Discussion

In the present study, changes in gene expression profiles after balloon catheter carotid injury and local administration of recombinant uPA were analyzed. Four major results were obtained. First, exogenous uPA significantly reduced the size of the arterial lumen area both 1 and 4 days after the operation. Second, we observed increases in the percentage of monocytes/macrophages and total cell numbers in the adventitia, suggesting a proinflammatory effect of uPA. The increased inflammation in uPA-treated arteries may be at least in part mediated

Table 4. Total cell numbers and percentages of cells expressing PCNA or ED-1 in injured arteries 1 day after treatments

	1 day	
	Control	uPA
Neointima		
Total cells, n	N/A	N/A
PCNA, %		
ED-1, %		
Media		
Total cells, n	78.3 ± 9.2	84.5 ± 12.6
PCNA, %	1.5 ± 0.2	2.1 ± 1.6
ED-1, %	3.8 ± 2.3	7.7 ± 3.4
Adventitia		
Total cells, n	112.2 ± 10.7	137.5 ± 14.3*
PCNA, %	6.8 ± 2.3	10.1 ± 3.2
ED-1, %	2.2 ± 1.5	7.3 ± 2.9*

Data represent mean ± SEM of total cell numbers or the percentages of cells expressing PCNA or ED-1 in arterial layers of at least 7 vessels 1 day after balloon catheter injury of the carotid artery and perivascular administration of uPA. Control represents vessels which only received vehicle (Pluronic gel in saline).

* $p < 0.05$.

by the group of proinflammatory genes upregulated by urokinase based on microarray analysis and qPCR including such potent cytokine as TNF-α. Third, changes in oxidation-related gene expression suggest that uPA promotes oxidative stress, which is consistent with our previous study showing increased Nox1 and Nox4 expression in response to uPA [20]. Taking into account that oxidative stress may regulate vascular tone and induce vasoconstriction (uPA itself possesses vasoactive properties [23]), we may hypothesize that the day 1 decrease in lumen and EEL areas after uPA could reflect changes in vascular tone. Fourth, the changes in gene expression were much greater at day 1 than day 4, possibly reflecting the disappearance or inactivation of uPA [11].

As we previously reported [7, 11], on the 4th day after injury exogenous uPA treatment induced inward arterial remodeling by stimulating several processes such as neointima formation, VSMC accumulation, intima-media thickness and decrease in EEL area. We also observed the accumulation of adventitial monocytes/macrophages. In uPA-treated arteries, microarray study revealed changes in the expression of 3 proinflammatory and 2 oxidation-related genes, indicating that these systems still play a role in uPA-induced changes 4 days after injury. We may suppose a trigger mechanism of action of uPA in the injured artery as soon as its effects on gene expression develop longer than exogenous uPA is detected there [11].

Our data on inflammatory cell accumulation after uPA treatment both 1 and 4 days after injury are consistent with previous studies in uPA knockout mice which showed that uPA mediates infiltration of leukocytes during arterial remodeling and neointima formation after injury [4]. Inflammation is the primary response to vessel wall injury during angioplasty and stenting. The important role for leukocyte accumulation is postulated for neointima formation and restenosis [9]. It was shown that inhibition of the inflammatory response in the adventitia limited the development of arterial constrictive remodeling by reducing adventitial constrictive fibrosis [24]. Moreover, macrophages express multiple proteases, including metalloproteinases and urokinase, that degrade the extracellular matrix and secrete numerous other effectors including ROS, TNF-α and interleukin-1 [25]. The present findings indicate that monocyte/macrophage accumulation in the adventitia is one of the earliest effects of uPA in the injured vessel wall, which may represent an important step in the effects of urokinase effects on inward arterial remodeling.

The microarray results suggest mechanisms of action of uPA leading to dramatic arterial remodeling. Of great interest are the significant increases in TNF-α and its converting enzyme (TACE) compared with control arteries. TNF-α has pleiotropic functions in cardiovascular diseases [26]. The periprocedural release of plaque-derived TNF-α was supposed to represent the amount and activity of the atherosclerotic process and to be a predictor for restenosis [27]. The upregulation of TNF-α is a potent mechanism for leukocyte recruitment to the injured region [28]. Moreover, TNF-α stimulates VSMC migration, one of the key processes of vascular stenotic lesion formation [29]. It was also shown that TNF-α may stimulate proliferation of VSMC [30] and enhance VSMC proliferation induced by monocytes [31], which is important for neointima growth and vessel remodeling. Finally, TNF-α was shown to be a key transcriptional inducer of several MMPs [32, 33], active participants of arterial response to intravascular injury promoting VSMC migration and replication [34, 35].

Our microarray study revealed changes in oxidation-related gene expression after r-uPA application compared with control arteries. The potential ability of uPA to regulate oxidative stress after arterial injury may represent one of the important mechanisms of inward remodeling of uPA-treated arteries. The oxidative stress induced by injury contributes to unfavorable vessel remodeling and

plays an important role in a number of cardiovascular pathologies [36, 37]. ROS are important signaling molecules regulating VSMC proliferation and tone [38]. We have previously shown increased superoxide production after balloon injury and demonstrated the beneficial effects of inhibiting ROS on vascular remodeling [39]. Previous studies showed that uPA contributes to superoxide production [40] and release [41] by neutrophils. Our microarray data are consistent with our recent findings that uPA increases ROS generation by VSMC [20]. Taken together, these data suggest that uPA has the potential ability to regulate oxidative stress in the injured artery that may promote inward arterial remodeling.

The cross-interaction between inflammation-related and oxidation-related gene patterns may occur in the injured artery, for example, TNF-α is able to cause oxidative stress in a variety of cell types [42] and to increase mitochondrial ROS [43]. In a recent study, a model for an interaction between ROS signaling and pathways activated by upregulation of the TACE/TNF-α system leading to vascular remodeling was proposed [44].

In conclusion, the major findings of the present study are that the early mechanisms leading to inward arterial remodeling induced by uPA in the balloon injury model involve the increased recruitment of leukocytes, early upregulation of the potent proinflammatory TACE/TNF-α system and changes in oxidation-related gene expression. Our results provide mechanistic insights into the role of uPA in remodeling, and provide a basis for combination therapy targeting both uPA and inflammatory mediators to prevent unfavorable arterial remodeling.

Acknowledgments

This study was supported by National Institutes of Health grant HL-62836 to B.C.B., by NHLBI funds for exchanges of scientists under the auspices of the US-Russia Joint Agreement in Cardiopulmonary Research, by NATO LST. CLG.979209, by Russian Basic Research Foundation grants No. 06-04-49303-a, No. 05-04-49108-a, No. 06-04-08138-ofi and state contracts with BSU No. 73/2006 and No. 60/2008. The authors are grateful for assistance with research of Drs. Oleg Viborov, Mikhail Menshikov, Marina Solomatina, Victoria Stepanova and Sergey Prokhorov.

References

1 Clowes AW, Reidy MA: Prevention of stenosis after vascular reconstruction: Pharmacologic control of intimal hyperplasia – a review. J Vasc Surg 1991;13:885–891.
2 Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK: A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86:III47–III52.
3 Schwartz RS: Pathophysiology of restenosis: interaction of thrombosis, hyperplasia, and/or remodelling. Am J Cardiol 1998;81:14E–17E.
4 Carmeliet P, Moons L, Herbert JM, Crawley J, Lupu F, Lijnen R, Collen D: Urokinase but not tissue plasminogen activator mediates arterial neointima formation in mice. Circ Res 1997;81:829–839.
5 Plekhanova OS, Parfyonova YV, Bibilashvily RS, Stepanova VV, Erne P, Bobik A, Tkachuk VA: Urokinase plasminogen activator enhances neointima growth and reduces lumen size in injured carotid arteries. J Hypertens 2000;18:1065–1069.
6 Collen D: Ham-Wasserman lecture: Role of the plasminogen system in fibrin-homeostasis and tissue remodelling. Hematology 2001;1:1–9.
7 Parfyonova Ye, Plekhanova O, Solomatina M, Naumov V, Bobik A, Berk BC, Tkachuk V: Contrasting effects of urokinase and tissue-type plasminogen activators on neointima formation and vessel remodeling early after arterial injury. J Vasc Res 2004;41:268–276.
8 Okamoto E, Couse T, De Leon H, Vinten-Johansen J, Goodman RB, Scott NA, Wilcox JN: Perivascular inflammation after balloon angioplasty of porcine coronary arteries. Circulation 2001;104:2228–2235.
9 Shah PK: Inflammation, neointimal hyperplasia, and restenosis: s the leukocytes roll, the arteries thicken. Circulation 2003;107:2175–2177.
10 Cipollone F, Marini M, Fazia M, Pini B, Iezzi A, Reale M, Paloscia L, Materazzo G, D'Annunzio E, Conti P, Chiarelli F, Cuccurullo F, Mezzetti A: Elevated circulating levels of monocyte chemoattractant protein-1 in patients with restenosis after coronary angioplasty. Arterioscler Thromb Vasc Biol 2001;21:327–334.
11 Plekhanova O, Parfyonova Y, Bibilashvily R, Domogatskii S, Stepanova V, Gulba DC, Agrotis A, Bobik A, Tkachuk V: Urokinase plasminogen activator augments cell proliferation and neointima formation in injured arteries via proteolytic mechanisms. Atherosclerosis 2001;159:297–306.
12 Brooks AI, Stein CS, Hughes SM, Heth J, McCray PM Jr, Sauter SL, Johnston JC, Cory-Slechta DA, Federoff HJ, Davidson BL: Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors. Proc Natl Acad Sci USA 2002;99:6216–6221.
13 Li C, Hung Wong W: Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. Genome Biol 2001;2:RESEARCH0032.
14 Welle S, Brooks AI, Thornton CA: Computational method for reducing variance with Affymetrix microarrays. BMC Bioinformatics 2002;3:23.
15 Hoffmann R, Seidl T, Dugas M: Profound effect of normalization on detection of differentially expressed genes in oligonucleotide microarray data analysis. Genome Biol 2002;3:RESEARCH0033.
16 Korshunov V, Nikonenko T, Tkachuk V, Brooks AI, Berk BC: Interleukin-18 and macrophage migration inhibitory factor are associated with increased carotid intima-media thickening. Arterioscler Thromb Vasc Biol 2006;26:295–300.

17 Loppnow H, Westphal E, Buchhorn R, Wessel A, Werdan K: Interleukin-1 and related proteins in cardiovascular disease in adults and children. Shock 2001;16(suppl 1):3–9.
18 Li H, Liang J, Castrillon DH, DePinho RA, Olson EN, Liu ZP: FoxO4 regulates tumor necrosis factor alpha-directed smooth muscle cell migration by activating matrix metalloproteinase 9 gene transcription. Mol Cell Biol 2007;27:2676–2686.
19 Kubica J, Kozinski M, Krzewina-Kowalska A, Zbikowska-Gotz M, Dymek G, Sukiennik A, Piasecki R, Bogdan M, Grzesk G, Chojnicki M, Dziedziczko A, Sypniewska G: Combined periprocedural evaluation of CRP and TNF-alpha enhances the prediction of clinical restenosis and major adverse cardiac events in patients undergoing percutaneous coronary interventions. Int J Mol Med 2005;16:173–180.
20 Menshikov M, Plekhanova O, Cai H, Chalupsky K, Parfyonova Y, Bashtrikov P, Tkachuk V, Berk BC: Urokinase plasminogen activator stimulates vascular smooth muscle cell proliferation via redox-dependent pathways. Arterioscler Thromb Vasc Biol 2006; 26:801–807.
21 Solomatina MA, Plekhanova OS, Menshikova MY, Ratner EI, Tkachuk VA, Parfyonova YV: Urokinase increases the content and activity of matrix metalloproteinases 2 and 9 during in vivo constrictive arterial remodeling. Bull Exp Biol Med 2005;139:283–286.
22 Pawlak K, Pawlak D, Mysliwiec M: Serum matrix metalloproteinase-2 and increased oxidative stress are associated with carotid atherosclerosis in hemodialyzed patients. Atherosclerosis 2007;190:199–204.
23 Haj-Yehia A, Nassar T, Sachais BS, Kuo A, Bdeir K, Al-Mehdi AB, Mazar A, Cines DB, Higazi AA: Urokinase-derived peptides regulate vascular smooth muscle contraction in vitro and in vivo. FASEB J 2000;14:1411–1422.
24 Mori E, Komori K, Yamaoka T, et al: Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. Circulation 2002;105:2905–2910.
25 Boyle JJ: Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. Curr Vasc Pharmacol 2005; 3:63–68.
26 Levine B, Kalman J, Mayer L, Fillit HM, Packer M: Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 1990;323:236–241.
27 Böse D, Leineweber K, Konorza T, Zahn A, Bröcker-Preuss M, Mann K, Haude M, Erbel R, Heusch G: Release of TNFα during stent implantation into saphenous vein aortocoronary bypass grafts and its relation to plaque extrusion and restenosis. Am J Physiol Heart Circ Physiol 2007;292:H2295–H2299.
28 Seki N, Bujo H, Jiang M, Shibasaki M, Takahashi K, Hashimoto N, Saito Y: A potent activator of PPARalpha and gamma reduces the vascular cell recruitment and inhibits the intimal thickning in hypercholesterolemic rabbits. Atherosclerosis 2005;178:1–7.
29 Ono H, Ichiki T, Fukuyama K, Iino N, Masuda S, Egashira K, Takeshita A: AMP-response element-binding protein mediates tumor necrosis factor-alpha-induced vascular smooth muscle cell migration. Arterioscler Thromb Vasc Biol 2004;24:1634–1639.
30 Selzman CH, Meldrum DR, Cain BS, Meng X, Shames BD, Ao L, Harken AH: Interleukin-10 inhibits postinjury tumor necrosis factor-mediated human vascular smooth muscle proliferation. J Surg Res 1998;80: 352–356.
31 Voisard R, Voglic S, Baur R, Susa M, Koenig W, Hombach V: Leukocyte attack in a 3D human coronary in-vitro model. Coron Artery Dis 2001;12:401–411.
32 Overall CM, Lopez-Otin C: Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer 2002;2: 657–672.
33 Siwik DA, Colucci WS: Regulation of matrix metalloproteinases by cytokines and reactive oxygen/nitrogen species in the myocardium. Heart Fail Rev 2004;9:43–51.
34 Kuzuya M, Iguchi A: Role of matrix metalloproteinases in vascular remodeling. J Atheroscler Thromb 2003;10:275–282.
35 Korshunov VA, Berk BC: Flow-induced vascular remodeling in the mouse: a model for carotid intima-media thickening. Arterioscler Thromb Vasc Biol 2003;23:2185–2191.
36 Berk BC: Redox signals that regulate the vascular response to injury. Thromb Haemost 1999;82:810–817.
37 Alexander RW. Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. Hypertension 1995;25:155–161.
38 Berk BC: Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 2001;81:999–1030.
39 Nunes GL, Robinson K, Kalynych A, King SB III, Sgoutas DS, Berk BC: Vitamins C and E inhibit O_2– production in the pig coronary artery. Circulation 1997;96:3593–3601.
40 Cao D, Mizukami IF, Garni-Wagner BA, Kindzelskii AL, Todd RF 3rd, Boxer LA, Petty HR: Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. Possible roles of complement receptor type 3 and calcium. J Immunol 1995;154:1817–1829.
41 Sitrin RG, Pan PM, Harper HA, Todd RF 3rd, Harsh DM, Blackwood RA: Clustering of urokinase receptors (uPAR; CD87) induces proinflammatory signaling in human polymorphonuclear neutrophils. J Immunol 2000;165:3341–3349.
42 Nakano H, Nakajima A, Sakon-Komazawa S, Piao JH, Xue X, Okumura K: Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. Cell Death Differ 2006;13: 730–737.
43 Nishikawa T, Araki E: Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Signal 2007;9:343–353.
44 Ungvari Z, Wolin MS, Csiszar A: Mechanosensitive production of reactive oxygen species in endothelial and smooth muscle cells: role in microvascular remodeling? Antioxid Redox Signal 2006;8:1121–1129.