Cell Biology and Signaling

AP-1–Dependent Transcriptional Regulation of NADPH Oxidase in Human Aortic Smooth Muscle Cells
Role of p22phox Subunit

Adrian Manea, Simona A. Manea, Anca V. Gafencu, Monica Raicu, Maya Simionescu

Objective—NADPH oxidase (NADPHox) is the major source of reactive oxygen species in vascular diseases; the mechanisms of enzyme activation are not completely elucidated. AP-1 controls the expression of many genes linked to vascular smooth muscle cells (SMCs) dysfunction. In this study we searched for the role of AP-1 in the regulation of NADPHox expression and function in human aortic SMCs exposed to proinflammatory conditions.

Methods and Results—Cultured SMCs were exposed to either angiotensin II (Ang II) or tumor necrosis factor (TNF)-α.

The lucigenin-enhanced chemiluminescence assay and real-time polymerase chain reaction analysis revealed that AP-1 and mitogen-activated protein kinase inhibitors reduced both Ang II or TNF-α-dependent upregulation of NADPHox activity and mRNA expression (NOX1, NOX4, p67phox, p47phox, p22phox). Inhibitors of AP-1 significantly diminished the Ang II or TNF-α-stimulated p22phox promoter activity and protein level. Transient overexpression of c-Jun/c-Fos upregulated p22phox promoter activity. Transcription factor pull-down assay and chromatin immunoprecipitation demonstrated the physical interaction of c-Jun protein with predicted AP-1–binding sites in the p22phox gene promoter.

Conclusions—In SMCs exposed to Ang II or TNF-α, inhibition of AP-1–related pathways reduces NADPHox expression and the \( \mathrm{O}_2^− \) production. The physical interaction of AP-1 with p22phox gene promoter facilitates NADPHox regulation.

(Arterioscler Thromb Vasc Biol 2008;28:878-885)

Key Words: NADPH oxidase ■ AP-1 ■ hypertension ■ atherosclerosis
human aortic SMCs inhibition of AP-1 pathways reduces the NADPHox activity and expression and that AP-1 physically interacts with p22phox gene promoter. To our knowledge, there is no report regarding the role of AP-1 in the regulation of NADPHox subunits in vascular cells.

**Methods**

**Materials**

Standard chemicals and reagents were obtained from Sigma if not otherwise stated.

**Cell Culture**

SMCs were isolated from the media of fetal thoracic aorta and characterized as described. Confluent quiescent cells (at 8 to 12 passages) cultured 24 hours in serum-free Dulbecco modified Eagle Medium were exposed (up to 24 hours) to either 0.1 to 1 μmol/L Ang II or 10 to 20 ng/mL TNF-α in the presence or absence of AP-1 or MAPK inhibitors: decoy oligodeoxynucleotides, c-Jun siRNA, SP600125 (JNK), SB203580 (p38 MAPK), and U0126 (MEK-ERK1/2). Optimal concentrations of inhibitors were established in transfection experiments using p(5xAP-1)-luc control plasmid: 150 nmol/L ODN and 10 μmol/L of SP600125, SB203580, or U0126.

**Measurement of NADPH Oxidase Activity**

The lucigenin-enhanced chemiluminescence assay was used to determine the NADPHox activity in cell homogenates. The activity was expressed as mean light units (MLU)/μg of total protein.

**Real-Time PCR**

Quantification of NOX1, NOX4, p47phox, p67phox, p22phox, and matrix metalloproteinase (MMP) 9 mRNA expression was done by amplification of cDNA using SYBER Green. The relative quantification was performed by comparative Ct method and expressed as arbitrary units (AU).

**Plasmid Construction**

Proximal promoter of the human p22phox gene (GenBank AY 128666) and 9 5′ deletion mutants were amplified by PCR from genomic DNA and inserted into the KpnI/SacI cloning site of the pGL3 basic plasmid.

**Transient Transfection**

Superfect (Qiagen) was used as described. The promoter activity was calculated from the ratio of firefly luciferase to β-galactosidase levels and expressed as arbitrary light units (ALU).

**Western Blot**

Protein analysis was performed as described. Quantification of p22phox protein was done by normalization to β-actin protein and expressed as arbitrary units (AU).

**Biotin-Streptavidin Pull-Down Assay**

Nuclear protein extraction and transcription factor pull-down assay were done as previously indicated using a DNA-protein interaction detection system (DYNAL, Biotech, Invitrogen).

**Chromatin Immunoprecipitation**

DNA–protein interaction was evaluated using antibodies, reagents, and protocols from Santa Cruz Biotechnology. Real-time PCR was done using primers for p22phox promoter flanking the AP-1 binding sites. Input DNA was amplified for each sample in parallel, and amounts of sequence-specific immunoprecipitated DNA were expressed as the percentilce fraction of input DNA.

**Transfection of Decoy Oligodeoxynucleotide and siRNA**

Transfection of oligodeoxynucleotide (ODN) was performed using double-stranded DNA with sequences corresponding to the consensus AP-1 binding sites or scrambled. Scrambled (sc-37007), c-Jun (sc-29223), or p65 NF-kB (sc-29410) siRNA (Santa Cruz Biotechnology) were transfected into SMCs using Hiperfect reagent according to the manufacturer’s protocol (Qiagen).

**Statistical Analysis**

Data was expressed as means±SD. Statistical evaluation was done by 1-way ANOVA test; P<0.05 was considered statistically significant.

An extended methods section can be found in the online-only data supplement available at http://atvb.ahajournals.org.

**Results**

The Concentration–Dependent Effect of Ang II or TNF-α on NADPH Oxidase Activity and Expression in SMCs

Incubation of SMCs with various concentrations of Ang II or TNF-α revealed that each agonist induced NADPHox activity and expression in a dose- and time-dependent manner. SMCs stimulation (up to 8 hours) with 100 nmol/L Ang II had a slight effect on NADPHox activity but induced an increase of NOX1, p22phox, p47phox, and p67phox mRNA expression over the baseline level; no significant effect on NOX4 was found. Incubation of SMCs with 10 ng/mL TNF-α had a minor effect on NADPHox activity and on p22phox, p47phox, and p67phox mRNA expression but had an effect on NOX1 and NOX4. A significant increase in oxidase activity and expression was detected when SMCs were exposed to either 1 μmol/L Ang II or 20 ng/mL TNF-α; therefore these concentrations were used in further experiments (for details please see supplemental materials).

**Role of AP-1 in the Regulation of NADPH Oxidase Activity**

The role of AP-1 pathway in the regulation of superoxide production was examined on quiescent SMCs exposed for 24 hours to 1 μmol/L Ang II or 20 ng/mL TNF-α in the presence or absence of AP-1/Scrambled ODN or SP600125 and of MAPK inhibitors (SB203580 or U0126). The results showed that in SMCs, either Ang II or TNF-α, induced a 1-fold increase of NADPHox activity over the controls. In each case AP-1 and MAPK inhibitors significantly reduced the upregulated NADPH-driven O2− production (n=5, *P<0.05). Conversely, transfection of scrambled ODN did not affect the stimulated NADPHox activity (Figure 1A).

**Modulation of NOX1, NOX4, p47phox, and p67phox mRNA Expression by AP-1**

The expression of NADPHox subunits was evaluated by real-time PCR. Because AP-1 is a key regulator of MMP9 gene expression in SMCs, its expression was used as positive control. The mRNA levels of MMP9, NOX1, NOX4, p47phox, and p67phox were quantified in SMCs stimulated up to 8 hours with either 1 μmol/L Ang II or 20 ng/mL TNF-α using different AP-1 or MAPK inhibitors: AP-1 ODN, SP600125, SB203580, or U0126. As shown in Figure 1 B through 1F, Ang II induced a significant increase above the control in
mRNA expression of MMP9 (≈4.5-fold), NOX1 (≈4-fold), NOX4 (≈3-fold), p47phox (≈1.5-fold), and p67phox (≈1.7-fold) in a time-dependent manner, peaking at 6 hours. Stimulation of SMCs with 20 ng/mL TNF-α augmented mRNA expression of MMP9 (≈4-fold), NOX1 (≈2-fold), NOX4 (≈4-fold), p47phox (≈1.5-fold), p67phox (≈1.5-fold) above the control. In each case the upregulated mRNA level was significantly reduced by the mentioned inhibitors (n=5, *P<0.05, **P<0.01) (Figure 1B through 1F).

**Regulation of p22phox Gene and Protein Expression by AP-1**

The role of AP-1 in the regulation of p22phox essential subunit of NADPHox was investigated in SMCs stimulated (up to 8
hours) with Ang II or TNF-α in the presence or absence of 150 nmol/L AP-1 ODN, 80 nmol/L siRNA, and 10 μmol/L of SP600125, SB203580, or U0126.

**The Promoter Activity**
Twelve hours after transfection, in stimulated SMCs, Ang II induced a ~1.5-fold increase of p22phox gene promoter activity over the control level and TNF-α had a similar effect. In both cases, AP-1 ODN, knockdown of c-Jun, SP600125, SB203580, or U0126 exposure significantly reduced the luciferase level directed by the p22phox promoter. In contrast, cotransfection of scrambled ODN/siRNA failed to affect p22phox transcriptional activity (n = 6, *P < 0.05, **P < 0.01).

**The mRNA Expression**
Analysis of p22phox mRNA expression assessed by q-PCR revealed that exposure of cells to either Ang II or TNF-α significantly augmented the p22phox message (~2.7-fold increase over the control, at 6 hours (Figure 2B). The inhibitors, AP-1 decoy ODN and c-Jun siRNA, downregulated the p22phox gene expression by ~50% (n = 6, *P < 0.05). The MAPK inhibitors, SP600125, SB203580, or U0126, diminished by ~70% the Ang II or TNF-α upregulated p22phox message. Conversely, transfection of scrambled ODN/siRNA had no effect on stimulated p22phox mRNA expression. (n = 6, *P < 0.5, **P < 0.01; Figure 2B).

**Protein Expression**
Western blot assays showed that in SMCs, Ang II or TNF-α significantly augmented the p22phox protein level (~70% above the control); AP-1 decoy ODN, c-Jun knockdown, SP600125, SB203580, or U0126 significantly reduced (~40%) the upregulated p22phox protein expression (n = 6, *P < 0.05). In contrast, scrambled ODN/siRNA failed to affect the upregulation of p22phox protein (Figure 2C). A representative immunoblot depicting the modulation of p22phox protein level in Ang II–exposed SMCs is presented in Figure 2D. Silencing of c-Jun was evaluated by RT-PCR and Western blot, 24 to 72 hours after siRNA transfection; maximal knockdown was detected between 36 to 48 hours.
Scrambled or p65 NF-kB siRNA failed to change c-Jun mRNA and protein levels (Figure 2E).

Computer Analysis of Human p22phox Gene Promoter

In silico analysis of human p22phox promoter (TRANSFAC) revealed the presence of 5 putative binding sites for AP-1 [-871/-864 bp: AP-1(5), -711/-704 bp: AP-1(4), -595/-588 bp: AP-1(3), -552/-545 bp: AP-1(2), -418/-411 bp: AP-1(1)]. The location of the nuclear factor consensus sequences were counted relative to the ATG codon.

Functional Analysis of AP-1 Binding Sites

To determine whether the above mentioned putative binding sites mediate transcriptional activation of the p22phox gene, we performed cotransfection experiments using 5′ deletion constructs (in which the AP-1 binding sites have been sequentially removed), and c-Jun or c-Fos expression vectors. Transient overexpression of c-Jun/c-Fos upregulated the c1 promoter activity (≈2.5-fold) over the control level. Likewise, compared to controls, the overexpression of c-Jun/c-Fos significantly increased the promoter activity of the c3 (≈2.6-fold), c4 (≈2.0-fold), c5 (≈1.5-fold), and c6 constructs (≈1.7-fold; n=7, *P<0.001). The promoter activity of the construct c7 and c10 was not upregulated by c-Jun/c-Fos overexpression (Figure 3A).

The overexpression of c-Jun and c-Fos was confirmed using p5xAP-1-luc control plasmid (Figure 3B). A schematic representation of AP-1 putative elements in p22phox promoter is presented in Figure 3C.

Physical Interaction of AP-1 With the Predicted Binding Sites

To evaluate the actual binding of AP-1 proteins to their corresponding sequences derived from human p22phox gene promoter, biotin-streptavidin pull-down assay was performed. The results showed that c-Jun protein physically interacted with the fragments containing the AP-1(1, 3, 4, and 5) predicted sites. The protein bands corresponded to those obtained for AP-1 control oligonucleotides and nuclear extracts. Negative control performed with scrambled double-stranded oligonucleotides did not yield any product, demonstrating the lack of nonspecific binding (Figure 3D).

To determine whether these sites are occupied by AP-1 in vivo, we performed chromatin immunoprecipitation assay using antibodies directed against c-Jun (Figure 4A). The specificity of the reaction was assessed using, as positive control, 2 fragments containing the AP-1 binding sites (−79 bp and −533 bp) from human MMP9 gene promoter. As negative control, similar experiments were done except that the c-Jun antibody was omitted. All primer pairs amplified a single band at the expected molecular weight as shown by agarose gel electrophoresis (Figure 4B). In addition, compared to control (vehicle), Ang II or TNF-α stimulation of SMCs determined a specific enrichment of sequences surrounding the AP-1(1), AP-1(4), and AP-1(5) and to a lesser extent AP-1.2–3 There was no enrichment of sequences close to the transcriptional start site (Figure 4C).

Discussion

In vascular diseases, transcriptional regulation of NADPHox subunits is particularly important in the modulation of oxi-
dase activity. Vascular NADPHox are activated and regulated by vasoactive agents, hormones, cytokines, metabolic factors, and mechanical forces but the transcriptional mechanisms involved are not fully defined. Anrather et al demonstrated that in monocytic murine J774.A1 cell line the expression of NADPHox subunits, gp91phox, p47phox, and p22phox is regulated by NF-κB. These results are consistent with those reported on human phagocytes and vascular smooth muscle cells. Brewer et al reported that the NADPHox isoform, NOX1, is transcriptionally regulated by GATA-binding factors in human colon epithelial Caco-2 cells. Little is known about the effect of AP-1 on the regulation of NADPHox.

To investigate the involvement of AP-1 in the modulation of NADPHox complex in human aortic SMCs we performed initial computer analysis of NADPHox promoters. The program identified the presence of typical AP-1 elements in the promoters of the human “Phox” subunits (gp91phox, 4xAP-1, p22phox-5xAP-1, p40phox-2xAP-1, p47phox-3xAP-1, p67phox-2xAP-1), NOX1 (3xAP-1) and NOX4 (2xAP-1). We focused on the essential subunit p22phox because all NOX enzymes require this component for their activity.

Although it has been demonstrated that the expression of various NADPHox proteins and superoxide production is upregulated by Ang II or TNF-α in different cardiovascular cells, there are only few data on the mechanisms involved in this process.

To reveal the involvement of AP-1 pathway in the regulation of NADPHox expression and function in SMCs stimulated by either Ang II or TNF-α, different AP-1 or MAPK inhibitors were used. As positive control, we used the AP-1 regulated MMP9 gene expression, which reportedly is increased by both Ang II or TNF-α-stimulated vascular cells.

It has been previously demonstrated on rat SMCs that NOX1 and NOX4 are upregulated by renin-angiotensin system in vitro and in vivo, and the augmented expression correlates with increased vascular O2− production, SMCs hypertrophy, and fibrosis. Lassegue et al reported that stimulation of rat aortic SMCs with 100 nmol/L Ang II upregulate NOX1 mRNA and decrease NOX4 message. In our experiments, similar results were obtained when 100 nmol/L Ang II was used; by contrast 1 μmol/L Ang II upregulated the expression of both NOX1 and NOX4 mRNA.

Furthermore, upregulation of the enzyme activity associated with increases in p22phox, p40phox, p47phox, and p67phox mRNA and protein levels was reported. Our data on human vascular SMCs extend these reports. We found that sustained stimulation of SMCs with Ang II or TNF-α resulted in a significant increase of enzyme activity and oxidase expression. Moreover, inhibition of AP-1 pathway greatly reduced the stimulated NADPHox activity and expression. Modulation of p67phox mRNA level by an AP-1-dependent mechanism is in harmony and extends previous observation that demonstrated that AP-1 is essential for human p67phox gene promoter activity. The correlation
between increased NOX1, NOX4, p47phox, p67phox, and p22phox expression and enhanced O₂⁻ production in Ang II or TNF-α-stimulated cells, as well as the inhibition of AP-1 pathway, suggests that the increased enzyme activity may be a result of transcriptional upregulation of NADPHox subunits via AP-1 pathway. However, other AP-1–independent mechanisms cannot be excluded.

The role of other MAPK members in the regulation of oxidase complex was investigated using inhibitors of p38 MAPK (SB203580) or ERK1/2 (U0126); both inhibitors significantly decreased the stimulated NADPHox activity and expression. These data confirm and extend previous studies on the regulation of enzyme complex.22 It is worth noting that ERK1/2 mediates many of its biological effects by regulating transcription factors, such as c-Fos and Elk-1. Moreover, p38 MAPK enhances the function of AP-1 mainly through other transcription factors (Elk-1, ATF-2, or CREB).7 Because p38 MAPK and ERK1/2 also activates NF-κB, Ets1, STAT, Elk1, other transcription factors may also be involved in the regulation of NADPHox. It was reported that Ets1, the downstream target of p38 MAPK, is a critical regulator of ROS and p47phox expression in Ang II–exposed human aortic SMCs.47 Our data regarding the modulation of NADPH-dependent O₂⁻ production and p47phox mRNA expression by SB203580 corroborate well and extend these observations.

The transcriptional regulation of the p22phox gene is a mechanism to control the NADPHox activity.15 Azumi et al.18 demonstrated that p22phox is more abundant in advanced atherosclerotic plaques than in nonatherosclerotic arteries, suggesting a correlation between p22phox expression, superoxide production, and the severity of atherosclerosis. In a previous study, we found that NF-κB has an important role in the regulation of p22phox promoter in human aortic SMCs.36 To further uncover the function of the putative AP-1 cisacting elements in the human p22phox gene promoter, we performed cotransfection experiments using 5’ deletion constructs and c-Jun or c-Fos expression vectors. Transient overexpression of c-Jun or c-Fos induced a significant increase of luciferase level directed by p22phox gene promoter indicating the presence of functionally AP-1–binding sites.

To emphasize the function of the predicted binding sites, we analyzed the nuclear factor binding activities in vitro and in vivo and found that the oligonucleotides corresponding to AP-1(1), AP-1(3), AP-1(4), and AP-1(5) predicted binding sites formed a bound complex with the c-Jun protein. The deletion analyses and DNA-protein interaction assays indicate that human p22phox gene promoter contains AP-1 positive regulatory elements.

Because AP-1 is a redox-sensitive transcription factor, we can safely assume the existence in SMCs of a positive feedback mechanism whereby ROS, generated by the NADPHox, may be important for the persistent superoxide production. This hypothesis is also supported by previous data concerning the redox-regulation of the NADPHox subunit p22phox in endothelial cells.49 Because p22phox gene is regulated by both NF-κB36 and AP-1 (this study), one can assume that other proinflammatory redox-sensitive transcription factors such as C/EBP, Elk-1, STAT1, or HIF-1 may be important in the overall regulation of NADPHox subunits.

To our knowledge, this is the first report demonstrating the role of AP-1 related pathway in the regulation of NADPHox in vascular cells, and in particular in human aortic SMCs. The role of AP-1–dependent transcriptional regulation of NADPHox in cardiovascular disorders remains to be further characterized. One can predict that members of this transcription factor family may become important therapeutic targets in the treatment of cardiovascular diseases such as hypertension, atherosclerosis, or heart failure.

Acknowledgments

We acknowledge the skillful assistance of Floarea Georgescu, Ioana Manolescu, and Constanta Stan.

Sources of Funding

This work was supported by grants from Romanian Academy, the Romanian Ministry of Education and Research, and the contract 16873/2005 of the European Community.

Disclosures

None.

References


AP-1–Dependent Transcriptional Regulation of NADPH Oxidase in Human Aortic Smooth Muscle Cells: Role of \textit{p22phox Subunit}

Adrian Manea, Simona A. Manea, Anca V. Gafencu, Monica Raicu and Maya Simionescu

\textit{Arterioscler Thromb Vasc Biol.} 2008;28:878-885; originally published online February 28, 2008;
doi: 10.1161/ATVBAHA.108.163592

\textit{Arteriosclerosis, Thrombosis, and Vascular Biology} is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/5/878

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/04/18/ATVBAHA.108.163592.DC1

\textbf{Permissions:} Requests for permissions to reproduce figures, tables, or portions of articles originally published in \textit{Arteriosclerosis, Thrombosis, and Vascular Biology} can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

\textbf{Reprints:} Information about reprints can be found online at:
http://www.lww.com/reprints

\textbf{Subscriptions:} Information about subscribing to \textit{Arteriosclerosis, Thrombosis, and Vascular Biology} is online at:
http://atvb.ahajournals.org//subscriptions/
Data supplement

Materials

Standard chemicals and reagents were obtained from Sigma-Aldrich (Germany), Gibco-Life Technologies GmbH (Germany), Promega (Germany), Qiagen (Germany), and Invitrogen Life Technologies (Austria). Antibodies, siRNA, and chromatin immunoprecipitation (ChIP) reagents were purchased from Santa Cruz Biotechnology (USA). The c-Jun, c-Fos, green fluorescent protein (GFP) expression vectors and p(5xAP-1)-luc control plasmid were generously provided by Professor Didier Picard (University of Geneva, Switzerland).

Cell culture and experimental design

Human aortic smooth muscle cells (SMCs) were isolated by explantation from the media of foetal thoracic aorta and characterized as a pure cell line devoid of any cell type contaminants. By phase-contrast microscopy, cultured SMCs displayed an elongated spindle-shaped morphology; upon confluence the cultured cells exhibited a characteristic ‘hills and valleys’ pattern. Transmission electron microscopy revealed the presence of bundles of myofilaments in the cytoplasm and numerous caveolae at the periphery of SMCs. Fluorescence immunocytochemistry revealed the positive reaction for smooth muscle α-actin and negative reaction for Von Wilebrand factor. The cells (at 8-12 passages) were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) with 5 mmol/l glucose, supplemented with essential and non-essential amino acids, sodium
selenite, ascorbic acid, 10 % fetal bovine serum (FBS) (v/v) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml neomycin).

Cell viability was measured spectrophotometrically, using MTT [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan]TOX-7 kit. To establish whether the DNA/siRNA concentrations used in transfection experiments affect the cell viability, MTT kit was employed. The results showed that at concentration used, the plasmids (2 μg/ml), oligodeoxynucleotides (150 nM) and siRNA (80 nM) were not cytotoxic (0.872 ± 0.01 versus 0.825 ± 0.01 arbitrary units, n=5, p > 0.05). Similarly, SP600125, SB203580, U0126 had no effect on cell viability (0.89 ± 0.01 versus 0.87 ± 0.01 arbitrary units, n=5, p > 0.05).

Measurement of NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine the NADPH oxidase activity in cell homogenates. In order to minimize artifactual O₂⁻ production due to redox cycling, a low concentration of lucigenin (5 μM) was used. The reaction mixture comprised 50 mM phosphate buffer containing 1mM EGTA, pH 7.0, 5 μM lucigenin and 100 μM NADPH. The reaction was started by the addition of cell homogenate (100-150 μg of protein) and the light emission was recorded every second for 15 min in a luminometer (Berthold). To verify the specificity of the reaction, different O₂⁻ scavengers and enzyme inhibitors were used: 300 U/ml superoxide dismutase (SOD), 1 mM tiron, 10 μM diphenylene iodonium (DPI), 100 μM allopurinol, 100 μM N⁰-nitro-L-arginine methyl ester (L-NAME), and 50 μM rotenone. The activity was expressed as mean light units (MLU)/μg of total protein.
Tiron and SOD inhibited $O_2^-$ production by 75 % and 70 % respectively. The flavoprotein inhibitor (DPI) attenuated the lucigenin signal by 48 %, whereas the xanthine oxidase inhibitor allopurinol, NO synthase inhibitor L-NAME or mitochondrial complex I inhibitor, had no effect on NADPH-dependent $O_2^-$ production.

Cytochrome c reduction assay was employed to evaluate the NADPH-dependent superoxide production in cell homogenates. The reaction mixture comprised 50 mmol/l phosphate buffer containing 0.1 mmol/l EDTA, pH 7.8, 50 mmol/l cytochrome c and 100 mmol/l NADPH, in the presence or absence of 300 units/ml SOD. The reaction was started by the addition of cell homogenate (100–150 μg of protein) and cytochrome c reduction was monitored for 30 min at 550 nm in a microplate reader (GENios, Tecan). The superoxide production was expressed as nmol of $O_2^-$/mg of total protein. The results showed that in the absence of NADPH, a very low level of $O_2^-$ was detected. Addition of NADPH (100 μM) induced a significant increase in superoxide production (~ 5 fold increase above the baseline).

**Real Time PCR**

Total cellular RNA was isolated from cultured SMCs using GenElute® Mammalian Total RNA kit (Sigma). First-strand cDNA synthesis was performed employing 1 μg of total RNA and MMLV reverse transcriptase, according to the manufacturer’s protocol (Invitrogen). Quantification of NOX1, NOX4, p47phox, p67phox, p22phox, and MMP9 mRNA expression was done by amplification of cDNA using an Opticon 2 DNA Engine real-time thermocycler (MJ Research) and SYBER Green chemistry. The primer sequences are shown in Table I. Optimized amplification conditions were 0.2 μM of each
primer, 2.5 mM MgCl₂, annealing at 58 ºC and extension at 72 ºC for 40 cycles. GAPDH gene was used as internal control. The relative quantification was done using the comparative C₇ method and expressed as arbitrary units (AU)⁵.

**Plasmid construction**

Proximal promoter of the human p22 phox gene (GenBank® accession number AY 128666) was amplified by PCR from genomic DNA using primers designed to contain Kpn I (sense) and Sac I (antisense) restriction sites incorporated into their 5’ ends. After digestion, the ~ 1200 bp fragment containing the whole promoter was inserted into the Kpn I / Sac I cloning site of the pGL3 basic plasmid, resulting the construct c1. Nine 5’ deletion mutants were generated from c1 by progressively removal of ~ 100 bp and cloned in the same reporter vector. Correct orientation and sequence of the p22 phox promoter in all constructs were confirmed by restriction enzyme analysis, polymerase chain reaction and DNA sequencing.

**Transient transfection**

Twenty-four hours before transfection, exponentially growing SMCs were seeded at 1.0x10⁵ cells/well (~ 80 % confluence) into 12-well tissue culture plates. Transient transfection was performed according to manufacturer’s protocol using Superfect reagent (Qiagen), DMEM supplemented with 10 % FBS (v/v), 1μg of luciferase construct and 0.5 μg pSV-β-galactosidase vector⁶. In some experiments luciferase constructs were cotransfected with 0.5 μg of c-Jun , c-Fos expression vectors or pcDNA 3.1 empty vectors (control). The DNA/superfect ratio was 1: 7.5 (wt/wt). The promoter activity was
calculated from the ratio of firefly luciferase to β-galactosidase levels and expressed as arbitrary light units (ALU). For comparison, transfection experiments were performed either in human aortic smooth muscle cells, bovine aortic endothelial cells, and HepG2 cell line. The results showed a comparable profile of promoter/deletion mutants activity and a similar mechanism of gene regulation.

**Western-blot analysis**

Cultured cells were washed twice in ice-cold PBS before lysis in 2x Laemmli’s electrophoresis sample buffer and boiled for 10 min. Protein concentration was quantified by the Amido Black method. Equal amounts of protein (70 µg) were run on 12% (p22phox) or 10% (c-Jun) SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were exposed to TBS Blotto A, and then incubated overnight at 4 °C with the primary antibodies against p22phox (rabbit polyclonal, sc-20781), β actin (mouse monoclonal, sc-47778) or c-Jun (rabbit polyclonal, sc-45) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG-HRP). The protein bands were detected using chemiluminescence substrate solution and images were taken with a gel analyzer system (ImageMaster VDS, Pharmacia Biotech). The quantification of the p22phox protein was determined by normalization to β actin protein and expressed as arbitrary units (AU).

**Nuclear protein extraction**

The nuclear extract was prepared as previously described. Briefly, confluent SMCs were collected, rinsed with PBS, resuspended in hypotonic buffer (10 mM Hepes, pH 7.9, 10
mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.5% Nonidet P-40), and incubated on ice for 10 min. After centrifugation (15 min, 10,000 x g), insoluble pellets were resuspended in high salt buffer (25% glycerol, 20 mM Hepes, pH 7.9, 0.6 M KCl, 1.5 mM MgCl2, 0.2 mM DTT, 0.5 mM PMSF), and placed on ice with gentle agitation for 40 min. After centrifugation at 14,000 x g for 10 min, the supernatant (nuclear extract) was collected for further analysis.

**Biotin-streptavidin pull-down assay**

Transcription factor pull-down assay was performed as previously described using a DNA-protein interaction detection system (DYNAL Biotech, Invitrogen). Briefly, 5’ biotin labeled sense oligonucleotides containing the putative AP-1 binding sequences were annealed to their respective complementary strands. Typical AP-1 and scrambled oligonucleotides were used as positive or negative control (Table 2). The resulting double-stranded oligonucleotides were gel-purified (Qiagen Gel Extraction Kit) and used. One microgram of each biotinylated DNA fragments were immobilized on streptavidin-coupled Dynabeads according to manufacturer’s protocol and incubated with 300 μg of nuclear proteins at 25 °C for 30 min in the binding buffer (12% glycerol, 12 mM Hepes, pH 7.9, 4 mM Tris, pH 7.9, 150 mM KCl, 2.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 10 μg poly(dI-dC) competitor). The protein-DNA-streptavidin-magnetic beads complex was washed five times with the binding buffer and loaded onto SDS gel. Detection of c-Jun protein was performed by Western blot.
**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed using antibodies, reagents and protocols from Santa Cruz Biotechnology. Briefly, $10^7$ cells were cross-linked with 1% formaldehyde (v/v). Samples were processed using 0.125 M glycine, cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % NP-40, protease inhibitor cocktail) and nuclear lysis buffer (1xPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail), followed by sonication and centrifugation. This led to DNA fragments spanning from 0.3 to 1.5 kb as analyzed by agarose gel electrophoresis. The supernatant was precleared with protein A/G agarose, incubated with c-Jun antibodies (rabbit polyclonal, sc-45) overnight and with beads for 2 hours at 4 °C. Complexes were washed (100 mM Tris pH 8.0, 500 mM LiCl, 1% NP-40, 1% deoxycholate) and eluted (1% SDS, 0.1 M NaHCO$_3$). Immunoprecipitated and input DNA was purified with QIAquick purification columns (Qiagen). Real time PCR was performed with primers for the p22$^{phox}$ promoters flanking the AP-1 binding sites. Two fragments containing the AP-1 binding sites (-79 bp and -533 bp) from human MMP9 gene promoter were amplified from immunoprecipitated chromatin and served as positive control. ‘No-antibody’ and negative controls from genomic regions which do not contain predicted AP-1 binding sites (AP-1 primer set 5) were employed. The specificity of PCR products was analyzed by gel electrophoresis and melting curve. Input DNA was amplified for each sample in parallel and amounts of sequence-specific immunoprecipitated DNA were expressed as the percentile fraction of input DNA.
Transfection of decoy oligodeoxynucleotide and siRNA

Transfection of decoy oligodeoxynucleotides (ODN)\textsuperscript{10} was performed using double-stranded DNA with sequences corresponding to the consensus AP-1 binding sites or scrambled (Table II). Each pair of single-stranded ODN was annealed for 2 h, during which the temperature was reduced from 90 °C to 25 °C. The resulting double-stranded oligonucleotides were gel-purified (Qiagen Gel Extraction Kit) and used. Transfection of SMCs was performed as described above employing 150 nM of decoy ODN. We first verified that double-stranded ODN tagged with FITC at their 5’end could be introduced efficiently into SMCs nuclei using the dendrimer-based transfection reagent. Twenty-four hours after transfection, FITC-labeled ODN were detected in the nuclei of ~ 60 % of the cells. No FITC signal was seen in untransfected cells. In the absence of dendrimers, FITC-labeled ODN were not taken up significantly by SMCs. The uptake efficiency was evaluated by fluorescence microscopy using an inverted fluorescence microscope (Nikon) and FITC-labeled ODN (Figure I).

Scrambled (sc-37007), c-Jun (sc-29223) or p65 NF-kB (sc-29410) siRNA (Santa Cruz Biotechnology) was transfected into SMCs using Hiperfect reagent according to the manufacturer’s protocol (Qiagen). In order to disrupt aggregates formed during lyophilization, siRNA was incubated at 90 °C for 1 min and then at 37 °C for 60 min prior to transfection procedure. Knockdown of c-Jun was evaluated by RT-PCR (sc-29223-PR) and western blot 24 to 72 hours after siRNA transfection. Maximal knockdown of c-Jun was observed between 36 to 48 hours. The c-Jun gene knockdown was transient and increased 72 hours after transfection. Scrambled or p65 NF-kB siRNA failed to affect c-Jun mRNA and protein levels.
**The concentration–dependent effect of Ang II or TNFα on NADPH oxidase activity and expression in SMCs**

Incubation of SMCs with various concentrations of Ang II or TNFα showed that each agonist induced NADPHox activity and expression in a dose- and time-dependent manner. SMCs stimulation (up to 8 hours) with 100 nM Ang II had a slight effect (~0.25 fold) on NADPHox activity (Figure IIA) but induced a considerable increase of NOX1 (~2.5 fold), p22\textsuperscript{phox} (~ 1.5 fold), p47\textsuperscript{phox} (~ 1 fold), and p67\textsuperscript{phox} (~ 0.8 fold) mRNA expression over the baseline level; no significant effect on NOX4 was found (Figure II B-E). Incubation of SMCs with 10 ng/ml TNFα had a minor effect on NADPHox activity (~0.25 fold increase over the baseline) as well as on p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox} mRNA expression but a significant effect on NOX1 and NOX4 namely ~1 fold and ~0.5 fold respectively, over the baseline (Figure II A-F). A significant increase in oxidase activity and expression was detected when SMCs were exposed to either 1 μM Ang II or 20 ng/ml TNFα. The effect of both pro-inflammatory factors was gradual, peaking at 6 hours of cell treatment, and the effect was constant for at least 24 hours (Figure II). Based on these data, 1 μM Ang II or 20 ng/ml TNFα were employed in further experiments.
Tables and figures legends

Table I
Sequences and GenBank® accession number of oligonucleotide primers used for real time PCR.

Table II
Oligonucleotide sequences used for biotin-streptavidin pull down assay. Nonbiotinylated oligonucleotides were used for decoy ODN assay (AP-1, Scrambled). The nuclear factor consensus sequences are underlined.

Figure I
FITC-labeled ODN uptake in SMCs. Twenty-four hours after transfection FITC- labeled ODN signal was detected both in cytoplasm and the nuclei the cells. (A) Fluorescence microscopy, (B) Corresponding phase contrast microscopy.

Figure II
The concentration-dependent effect of Ang II or TNFα on NADPH oxidase activity and mRNA expression. SMCs were stimulated for the indicated periods with either Ang II (0.1-1 μM), TNFα (10-20 ng/ml) or medium alone (control). Oxidase activity was assessed by lucigenin-chemiluminiscence and the mRNA expression by real time PCR. n=5, * p < 0.05, ** p < 0.01.
### Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank® Accession Number</th>
<th>Sequences of Oligonucleotide Primers</th>
<th>Predicted size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>NM_013955</td>
<td>S: 5’-CACAAGAAAAATCCCTTGTTGCTAA-3’</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-GACAGCAGATTGCAGACACA-3’</td>
<td></td>
</tr>
<tr>
<td>NOX1</td>
<td>NM_016931</td>
<td>S: 5’-TGGCTGCCCATCGTGGAATG-3’</td>
<td>281</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-CA6CAGGCCCTCCTGAAACATGC-3’</td>
<td></td>
</tr>
<tr>
<td>p47&lt;sup&gt;pos&lt;/sup&gt;</td>
<td>NM_000265</td>
<td>S: 5’-GTCACCCACCCACATATG-3’</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-CTTCGGCTTTCCTGCTCTCG-3’</td>
<td></td>
</tr>
<tr>
<td>p67&lt;sup&gt;pos&lt;/sup&gt;</td>
<td>NM_000433</td>
<td>S: 5’-ATGCCCTTCAGTGTCGTCCAG-3’</td>
<td>423</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-TGCTTCCAGACACACTCCATCG-3’</td>
<td></td>
</tr>
<tr>
<td>p22&lt;sup&gt;pos&lt;/sup&gt;</td>
<td>NM_000101</td>
<td>S: 5’-GTGGTGTGCGCTGCTTGAGT-3’</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-TG6CGCGCTGCTTGATGCT-3’</td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>NM_004894</td>
<td>S: 5’-GGAGCGGCTCTCCAAAGAGCTTG-3’</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-CTTCCTCCITTCCCTCCAGAACAGAA-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>S: 5’-ACCACACAG7CCATGAGATC-3’</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A: 5’-TCCTACACCCCTGTTGCTGTA-3’</td>
<td></td>
</tr>
</tbody>
</table>

S, sense; A, antisense
<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Oligonucleotide sequences</th>
</tr>
</thead>
</table>
| AP-1(1)       | S: 5’-Biotin-CCGGGCTGTTAGGGGTGACTCCCCCAACCCCAAGGCTC-3’  
A: 5’-GACCTTTGGGAGTGGGAGTCACCCCTACAGCCGG-3’ |
| AP-1(2)       | S: 5’-Biotin-GGCCCGCTGACCTGCTGACGCGAGGGCAAGCTG-3’  
A: 5’-GCTGCAAGCATGCCCTGGTCAAGACTGAGAGGGG-3’ |
| AP-1(3)       | S: 5’-Biotin-CCCTCCCTCGAAGTGCTCAAGTTCAGTGACGCTG-3’  
A: 5’-CAAGTCAGCTAAGCTGAGACTGAGCTGAGGAGG-3’ |
| AP-1(4)       | S: 5’-Biotin-GTCCTCCATGGGCTGGCTCAGCAGGGGGAGGACG-3’  
A: 5’-GCTGGAAGGCTGGGGTGGAAGCCGAGCGGGGGAGAG-3’ |
| AP-1(5)       | S: 5’-Biotin-CGCGGCGGCTCGTCTGAGTCGAGGCCGTGAAGCCGCC-3’  
A: 5’-ACAGTGCGAGGCTCAGCAACAGGACGCGGG-3’ |
| AP-1          | S: 5’-Biotin-TTCGGCTGATGTCATCAAGCCTG-3’  
A: 5’-CCCTTGGATGACTGAGGCCGAA-3’ |
| Scrambled     | S: 5’-Biotin-ATGACGCGCATGACGACATG-3’  
A: 5’-CATGTCATGCTGGCTCAT-3’ |

S, sense; A, antisense

AP-1 consensus sequence: TGAC/GTC/AA
Figure I
Figure II

A

B

C

D

E

F
Detailed Figure Legends - Manuscript

Figure 1
AP-1 modulates NADPH oxidase activity (A) and the mRNA expression of MMP9 (B), NOX1 (C), NOX4 (D), p47\(^{phox}\) (E), and p67\(^{phox}\) (F) in Ang II or TNF-\(\alpha\) stimulated SMCs. Cells were treated for 8 hours with either vehicle (control), 1\(\mu\)M Ang II or 20 ng/ml TNF\(\alpha\) in the presence/absence of the indicated AP-1 or MAPK inhibitors (150 nM ODN, 10 \(\mu\)M SP600125, 10 \(\mu\)M SB203580, 10 \(\mu\)M U0126). NADPH dependent \(O_2^-\) production was evaluated by lucigenin-enhanced chemiluminescence assay. mRNA level was quantified by real time PCR. \(n=5\), * \(p < 0.05\), ** \(p < 0.01\).

Figure 2
Regulation of p22\(^{phox}\) essential subunit by AP-1 in Ang II or TNF-\(\alpha\) treated SMCs. The cells were stimulated for 8 hours with 1\(\mu\)M Ang II or 20 ng/ml TNF\(\alpha\) in the presence/absence of 150 nM AP-1 ODN, 80 nM c-Jun siRNA or 10 \(\mu\)M of SP600125, SB203580, and U0126. Scrambled ODN and siRNA were used as negative control. p22\(^{phox}\) promoter activity (A), mRNA (B), and protein expression (C) was performed by luciferase reporter assay, real time PCR and Western blotting analysis. (D) Representative immunoblot showing the modulation of p22\(^{phox}\) and \(\beta\)-actin (loading control) protein expression by Ang II. (E) Evaluation of c-Jun knockdown by RT-PCR and western blot 48 hours after siRNA transfection. Scrambled and p65 NF-kB siRNA were used as negative controls. A representative immunoblot depicting the p22\(^{phox}\)
protein down-regulation in c-Jun or p65 NF-kB siRNA transfected SMCs is presented. C: control (non-transfected cells), SC: scrambled (n=6, * p < 0.05, ** p < 0.01).

**Figure 3**
Functional analysis of AP-1 binding sites in human p22phox gene promoter. (A) p22phox gene promoter activity in c-Jun or c-Fos overexpressing SMCs; (B) Validation of c-Jun/c-Fos overexpression using p(5xAP-1)-luc control plasmid (n=7, * p<0.001); (C) Schematic representation of AP-1 putative elements; (D) Representative immunoblot depicting the physical interaction of c-Jun protein with the fragments containing the AP-1 predicted sites.

**Figure 4**
Chromatin immunoprecipitation analysis of AP-1 elements in the human p22phox gene promoter. (A) Schematic drawing of p22phox gene promoter. Only putative AP-1 binding sites are indicated. Positions are relative to the first exon. The location and amplicons and primer sets used in ChIP experiments are shown. (B) Representative agarose gel electrophoresis depicting the expected molecular weight of the PCR products. Two fragments containing the -79 and -533 AP-1 binding sites within human MMP9 promoter were amplified from immunoprecipitated chromatin and served as positive control. ‘No-antibody’ and negative controls from genomic regions, which do not contain predicted AP-1 binding sites (AP-1 primer set 5), were employed. (C) Real time PCR analysis of AP-1 sites in Ang II or TNFα stimulated cells. Quiescent SMCs were stimulated for 8 hours with 1µM Ang II, 20 ng/ml TNFα or medium alone (control). Soluble chromatin
was processed using c-Jun antibodies. The enrichment of sequences surrounding AP-1 sites was assessed by real time PCR. n=5, * p < 0.05, ** p < 0.01.

References


