JAK/STAT Signaling Pathway Regulates Nox1 and Nox4-Based NADPH Oxidase in Human Aortic Smooth Muscle Cells

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Objective—Oxidative stress mediated by Nox1- and Nox4-based NADPH oxidase (Nox) plays a key role in vascular diseases. The molecular mechanisms involved in the regulation of Nox are not entirely elucidated. Because JAK/STAT regulates many genes linked to inflammation, cell proliferation, and differentiation, we questioned whether this pathway is involved in the regulation of Nox1 and Nox4 in human aortic smooth muscle cells (SMCs).

Methods and Results—Cultured SMCs were exposed to interferon γ (IFNγ) for 24 hours. Using lucigenin-enhanced chemiluminescence and dihydroethidium assays, real-time polymerase chain reaction, and Western blot analysis, we found that JAK/STAT inhibitors significantly diminished the IFNγ-dependent upregulation of Nox activity, Nox1 and Nox4 expression. In silico analysis revealed the presence of highly conserved GAS elements within human Nox1, Nox4, p22phox, p47phox, and p67phox promoters. Transient overexpression of STAT1/STAT3 augmented the promoter activities of each subunit. JAK/STAT blockade reduced the Nox subunits transcription. Chromatin immunoprecipitation demonstrated the physical interaction of STAT1/STAT3 proteins with the predicted GAS elements from Nox1 and Nox4 promoters.

Conclusions—JAK/STAT is a key regulator of Nox1 and Nox4 in human vascular SMCs. Inhibition of JAK/STAT pathway and the consequent Nox-dependent oxidative stress may be an efficient therapeutic strategy to reduce atherogenesis. (Arterioscler Thromb Vasc Biol. 2010;30:105-112.)

Key Words: NADPH oxidase ▪ JAK/STAT ▪ oxidative stress ▪ atherosclerosis

Emerging clinical and experimental evidence demonstrate the role of oxidative stress in the development of cardiovascular disorders. Reactive oxygen species (ROS) are implicated in different cell processes associated to vascular plaque formation such as growth, proliferation, differentiation, and apoptosis of smooth muscle cells (SMCs). Deciphering the molecular mechanisms underlying the regulation of ROS production may lead to new therapeutic approaches.

NADPH oxidases (Nox) are regulated by a plethora of stimuli and represent a major source of ROS in the vasculature. Depending on the cell type, the vascular Nox comprises 3 distinct catalytic subunits (Nox1, Nox2, Nox4) and 6 cytosolic regulatory components (p47phox, p67phox, NoxO1, NoxA1, Rac1/2). The p22phox component is essential for Nox activity forming a bound complex with Nox1–4. SMCs express predominantly Nox1 and Nox4 isoforms, which are differentially distributed in the cellular compartments and direct several redox-dependent processes. It was postulated that Nox1 associates with and promotes SMCs proliferation, whereas Nox4 is required for the maintenance of differentiated phenotype.

Changes in gene expression of the Nox isoforms are critical for its function. In previous studies we showed that the expression of p22phox subunit and the ensuing superoxide production is regulated by NF-κB and AP-1 in human aortic SMCs. However, the transcriptional regulatory mechanisms of oxidase components are not entirely elucidated.

Activation of Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is an essential pathogenic mechanism leading to SMC hypertrophy and hyperplasia. It was shown that STAT1 and STAT3 are triggers of vascular remodeling leading to SMCs dysfunction in hypertension and diabetes. Also, activated STAT1/STAT3 was detected in atherosclerotic lesions.

The JAK/STAT pathway is stimulated by cytokines, vasoactive agents, hormones and growth factors and provides a direct mechanism to translate an extracellular signal into a transcriptional response. A redox-dependent mechanism of JAK/STAT activation by Nox-derived ROS was reported in SMCs. Activated STATs bind specific regulatory sequences, such as ISRE (interferon-stimulated response element) or GAS (γ-activated sequence) to induce or repress transcription of target genes.

Because upregulated Nox and STATs are temporally and spatially colocalized in activated SMCs within atherosclerotic lesions, the JAK/STAT pathway is a potential therapeutic target for the treatment of atherosclerosis.
lesions, we searched for the involvement of JAK/STAT signaling itself in the regulation of oxidase expression and function in human aortic SMCs stimulated by IFNγ, a potent inducer of both Nox and JAK/STAT. We provide evidence that Nox1 and Nox4 isoforms, as well as p22phox, p47phox, and p67phox subunits, are regulated by STAT1 and STAT3 transcription factors.

**Methods**

**Materials**

Chemicals and reagents were obtained from Sigma unless stated otherwise.

**Cell Culture**

SMCs were isolated from the media of human 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 100 to 400 U/mL IFNγ in the presence or absence of JAK/STAT inhibitors: decoy oligodeoxynucleotides (ODN), AG490, S3I201, WP1066. Optimal concentration of inhibitors was established in transfection experiments employing pGAS/ISRE-luc control plasmid: 150 nmol/L ODN, 20 μmol/L of AG490 and S3I201, or 5 μmol/L WP1066.

**Measurement of NADPH Oxidase Activity**

Nox activity was measured by lucigenin-enhanced chemiluminescence, dihydriodihydrothidium, and cytochrome c reduction assays. The NADPH-dependent O₂⁻ production was expressed as arbitrary units.

**Real-Time PCR**

Quantification of Nox1, Nox4, manganese superoxide dismutase (MnSOD), and matrix metalloproteinase (MMP) 9 mRNA expression was done by amplification of cDNA using SYBR Green I chemistry. The relative quantification was performed by comparative Cₚ method and expressed as arbitrary units.

**Plasmid Construction**

The promoters (~1200 bp) of the human Nox1 (KpnI/SacI), Nox4 (XhoI/HindIII), p22phox (KpnI/SacI), p47phox (KpnI/SacI), and p67phox (KpnI/XhoI) genes were amplified by PCR from genomic DNA and inserted into the multicloning site of the pGL3 basic vector. Deletion mutants were generated by progressively removal of ~200 bp from Nox1 and Nox4 promoters and cloned in the same reporter vector.

**Transient Transfection**

Superfect reagent (Qiagen) was used as previously indicated. The promoter activity was calculated from the ratio of firefly luciferase to Renilla luciferase. Significant reporter activity was detected only after 24 hours in stimulated cells. Nox1 and Nox4 protein expression was significantly increased at 24-hour point. In each case, transfection of scrambled ODN did not affect considerably the IFNγ-stimulated Nox activity (Figure 1 and supplemental Figure II).

**Western Blot**

Protein analysis was performed as described. Quantification of Nox1 and Nox4 protein was done by normalization to β-actin protein and expressed as arbitrary units.

**Chromatin Immunoprecipitation**

DNA-protein interaction was evaluated using antibodies, reagents, and protocols from Santa Cruz Biotechnology as previously described. PCR was done using primers for Nox1 and Nox4 promoters flanking the GAS elements.

**Statistical Analysis**

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

**Results**

**IFNγ Increases Nox Activity in SMCs**

Stimulation of SMCs with IFNγ (100 to 400 U/mL) resulted in a dose- and time-dependent increase in Nox activity. Compared to control level, treatment with 100 U/mL IFNγ augmented significantly the NADPH-dependent O₂⁻ production at 4 hours (~40%) to a level that was maintained for 24 hours (~60%). Higher effect was detected for 200 U/mL and 400 U/mL of IFNγ at the 24-hour point (supplemental Figure I). The physiological relevant concentration of 100 U/mL IFNγ was used in all experiments.

**JAK/STAT Pathway Regulates Nox Activity in SMCs**

To determine whether JAK/STAT-dependent mechanism contributes to the regulation of Nox activity, SMCs were incubated for 4 or 24 hours with 100 U/mL IFNγ in the presence of STAT1/STAT3/Scrambled ODN, AG490, S3I201, or WP1066.

The results showed that chemical inhibition of JAK2 (AG490, S3I201, WP1066) significantly reduced the IFNγ-dependent upregulation of O₂⁻ production at either 4 or 24 hours. Conversely, oligodeoxynucleotides directed to the activated STAT1 and STAT3 diminished the upregulated Nox activity only at 24-hour point. In each case, transfection of scrambled ODN did not affect considerably the IFNγ-stimulated Nox activity (Figure 1 and supplemental Figure II).

**JAK/STAT Signaling Regulates Nox1 and Nox4 Gene and Protein Expression**

Nox1 and Nox4 gene and protein expression was evaluated by real-time PCR and Western blot in SMCs exposed to 100 U/mL IFNγ (24 hours) in the presence or absence of JAK/STAT signaling pathway inhibitors: STAT1/STAT3/Scrambled ODN, AG490, S3I201, or WP1066.

The results showed that stimulation of SMCs with IFNγ caused a significant but differential increase in Nox1 and Nox4 mRNA. The Nox1 gene expression attained a maximum level at 6 hours after stimulation (~2-fold above the control level), and the level was sustained for 24 hours. In contrast, the upregulation of Nox4 message (~50% above the control) was detected only after 24 hours in stimulated cells. Nox1 and Nox4 protein expression was significantly augmented after 24-hour incubation with IFNγ; the values obtained were ~35% to 40% above the baseline (data not shown).

Inhibition of JAK/STAT signaling pathway by STAT1/STAT3 ODNs, AG490, S3I201, or WP1066 significantly diminished the enhanced Nox1 and Nox4 gene and protein expression in IFNγ-stimulated SMCs. In contrast, SC ODN failed to affect the IFNγ-dependent upregulation of mRNA and protein levels (Figure 2).
To validate our experimental design and to assess the IFNγ-induced oxidative stress and proinflammatory conditions in SMCs, the gene expression of MnSOD and MMP9 were measured in parallel experiments. The results showed that IFNγ determined a significant upregulation of both genes in a JAK/STAT-dependent manner (supplemental Figure III).

**Analysis of Human Nox1 and Nox4 Promoters**

In silico analysis (TRANSFAC) revealed the presence of typical GAS (STAT1/STAT3) elements in the promoters of human Nox1 [-277/−269 bp (III), −156/151 bp (II), −80/−72 bp (I)] and Nox4 [-859/−851 bp (III), −780/772 bp (II), −699/691 bp (I)]. The location of the nuclear factor consensus sequences were counted relative to the ATG codon (+1).

To identify the regions required for basic promoter activity and to determine the function of these binding sites, the promoters of human Nox1 and Nox4 genes and the corresponding deletion mutants were cloned into pGL3 basic vector (Figure 3A and 3E). The resulting plasmids were transfected into SMCs, and the expression of the luciferase reporter gene was analyzed as described.

**Nox1**

Maximal luciferase gene expression was directed by the proximal activating promoter elements of c4–7. Distal sequences between −1232 bp and −818 bp decreased the activity of the reporter gene. Deletion to −621 bp resulted in a significant increase of c4 promoter activity (≈80%) compared to c1. A further deletion of −60-bp fragment from c7 significantly downregulated the luciferase level to approximately 70% of the maximal level indicating the loss of essential cis-acting elements, including GAS (I), responsible for the basic promoter activity (Figure 3B).

**Nox4**

Sequences between −1163 bp and −377 bp acted as positive regulatory elements. Highest luciferase levels were directed by the promoters of c1–5. Deletion to −377 bp (c6) resulted in a significant decrease (≈50%) of the reporter gene activity compared to c5. A further removal of a ≈200-bp fragment from c6 decreased the luciferase signal to ≈50%. Nevertheless, the gene reporter activity directed by c7 promoter was significantly greater than those induced by the promoterless pGL3 basic vector. Removal of consecutively larger fragments almost proportionally decreased the Nox4 promoter activity (c5>c6>c7) indicating that GAS elements may have role but they are not essential for the basic Nox4 promoter activity (Figure 3F).

**Functional Analysis of GAS Sites**

To establish whether the above indicated putative binding sites mediate transcriptional activation of the Nox1 and Nox4 genes, we performed cotransfection experiments using 5’ deletion constructs (in which the GAS binding sites have been sequentially removed), and STAT1 or STAT3 expression vectors. In addition, inhibition studies were conducted using specific STAT1/STAT3 ODN and JAK2 inhibitors.

**Nox1**

Transient overexpression of STAT1 upregulated the c1, c5, c6, and c7 promoter activity (≈50% to 60%) over the control level (pDNA3.1). Likewise, compared to controls, overexpression of STAT3 significantly increased the promoter activity of the c1 (≈3-fold), c5 (≈4-fold), c6 (≈4.5-fold), and c7 (≈2.8-fold) constructs. In each case, the overexpression of STAT3 resulted in a much higher induction of promoter activity than STAT1. Moreover, overexpression of both STAT1 and STAT3 determined an even higher increase in luciferase activity (≈25% above the STAT3 level). The promoter activity of the construct c8 was not up-regulated by STAT1 or STAT3 overexpression (Figure 3C).

**Nox4**

Overexpression studies conducted with Nox4 constructs indicated that either STAT1 or STAT3 are able to activate the transcription of luciferase gene directed by the promoters of the c1, c2, c3, and c4 constructs. STAT1 overexpression upregulated the gene reporter activity by ≈50% over control, whereas STAT3 augmented luciferase level by ≈1.2-fold. Overexpression of STAT3 determined a higher induction of promoter activity than STAT1 in the tested constructs. Compared to STAT3 level, induction of both STAT1 and STAT3 had no additional effect on Nox4-derived promoter activity. The c5 promoter activity was not significantly affected by the STAT1/STAT3 overexpression (Figure 3G).
To further uncover the role of JAK/STAT signaling pathway in the regulation of Nox1 and Nox4 promoter activity we performed cotransfection experiments using sequence specific ODN or JAK2 inhibitors. The results demonstrated that blockage of STAT1 or STAT3 considerably decreased luciferase expression directed by each promoter. In contrast, the transcriptional activity was not significantly affected by the scrambled ODN. Furthermore, a negative regulation of IFNγ-stimulated Nox1 and Nox4 transcription was observed in AG490, S3I201 or WP1066 treated SMCs (Figure 3D and 3H). A schematic representation of GAS elements within Nox1 and Nox4 promoters is depicted in Figure 3A and 3E.

**Regulation of p22phox, p47phox, and p67phox Promoter Activity by JAK/STAT**

To test the role of JAK/STAT signaling pathway in the overall regulation of Nox transcription in SMCs, we performed additional experiments using p22phox, p47phox, or p67phox promoter-luciferase constructs and STAT1/STAT3 expression vectors. Compared to controls, transient overexpression of STAT1 induced a significant increase of p22phox (~80%), p47phox (~40%), and p67phox (~50%) promoter activity. STAT3 overexpression greatly induced the luciferase activity (~1.7-fold over the pcDNA3.1 level) directed by p47phox and p67phox promoters, but did not affect the p22phox transcription. Induction of both STAT1 and STAT3 had no additional effect on luciferase level directed by promoters of Nox subunits. Inhibition studies conducted in IFNγ-stimulated SMCs revealed that either p22phox, p47phox, or p67phox promoter activities were sensitive to STAT1/STAT3 ODN, AG490, S3I201 or WP1066 (supplemental Figure IV).

The overexpression of STAT1 and STAT3 and the specificity of the inhibitors were confirmed using pGAS/ISRE-luc control plasmid (Figure 4A and 4B).

**Physical Interaction of STAT1 and STAT3 With the Predicted GAS Elements**

To establish whether the GAS elements from Nox1 and Nox4 promoters are occupied by STAT1 or STAT3, we performed
Figure 3. A and E, Schematic representation of GAS sites (black boxes). B and F, Analysis of promoter-luciferase constructs in serum-stimulated SMCs. C and G, promoter activity in SMCs overexpressing STAT1/STAT3. D and H, Modulation of promoter activities by JAK/STAT inhibitors in IFN-α-treated SMCs; n=6, *P<0.05, **P<0.01, ***P<0.001. Probability values were taken in relation to the corresponding controls (pcDNA3.1 or promoter-luciferase constructs alone).
chromatin immunoprecipitation assay using antibodies directed against STAT1 and STAT3 transcription factors (Figure 5A and 5B). The specificity of the reaction was tested using, as positive control, one fragment containing the GAS element from human c-Myc gene promoter (R&D System). As negative control, similar experiments were done except that the STAT1/STAT3 antibodies were omitted. Input DNA was amplified for each sample in parallel experiments. Compared to control (quiescent cells), stimulation of SMCs with 100 U/mL IFNγ for 2 hours determined a specific enrichment of sequences surrounding the GAS elements. The association of STAT1 and STAT3 proteins with the Nox1 and Nox4 promoters was reduced by JAK2 inhibitor WP1066 (supplemental Figure V). As shown by agarose gel electrophoresis all primers pairs amplified a single band at the predicted molecular weight (Figure 5C).

**Discussion**

Upregulation of Nox activity is commonly associated with the progression of cardiovascular diseases and correlates with increases in Nox1 and Nox4 expression. Stimuli-induced Nox1 plays a significant role in vascular response to injury.22 Unlike Nox1, which requires regulatory subunits for its activity, Nox4 produces ROS constitutively and changes in mRNA level directly affect the Nox4 activity.23 Thus, regulation and the rate of transcription of Nox4 represent an important mechanism for its function. Numerous proinflammatory and growth-related agents including serum, angiotensin II, and tumor necrosis factor α24–26 induce Nox4.

To establish whether JAK/STAT signaling cascade is involved in the regulation of oxidase transcription we initially performed computer analysis of the Nox promoters. The program identified the presence of highly conserved GAS (STAT1/STAT3) elements \[\text{NTT(C/A)(C/T)N(T/G)AA, where N is any nucleotide}\] within Nox1 (3GAS), Nox4 (3GAS), p22phox (1GAS), p47phox (4GAS), p67phox (3GAS), NoxO1 (2GAS), NoxA1 (1GAS), Rac1 (1GAS), and Rac2 (1GAS) promoters. Because Nox1- and Nox4-derived ROS mediate processes underlying SMCs dysfunction we focused on these essential Nox isoforms.

The role of JAK/STAT signaling in the regulation of Nox was investigated using different inhibitors: doubled-stranded decoy ODN directed to the activated STAT1 or STAT3, and upstream pharmacological inhibitors of JAK2 namely, AG490, S3I201, WP1066. The latter is a potent inhibitor of JAK2 phosphorylation, but unlike AG490 and S3I201, also degrades JAK2 protein thereby blocking more effectively its downstream STAT activation.27

The reported upregulation of oxidative stress and inflammation-related genes in atherogenesis, such as MnSOD and MMP9, represent important biomarkers of vascular injury.28 Therefore, to validate our experimental design and to confirm that IFNγ induces oxidative stress and proinflamma-
tory conditions in SMCs, the gene expression of MnSOD and MMP9 were evaluated as positive controls.

Using lucigenin chemiluminescence and dihydroethidium assays, we found that IFNγ increased the Nox activity in a JAK/STAT-dependent manner. Chemical inhibition of JAK2 significantly reduced the IFNγ-dependent upregulation of O2− production at 4 and 24 hours. Conversely, oligodeoxynucleotides directed to the activated STAT1 and STAT3 diminished the upregulated Nox activity only at 24 hours. At 4 hours, the upregulated Nox activity was insensitive to STAT1 and STAT3 ODN suggesting the existence of a transcription-independent mechanism of oxidase regulation. Thus, it is necessary to differentiate between the direct activation of Nox by IFNγ and the increase in enzyme activity attributable to a transcriptional upregulation.

STAT1 and NF-kB are crucial regulators of cytokine-induced transcription of gp91phox (Nox2) in phagocytes. Although important, the molecular mechanisms responsible for the increased expressions of Nox1- and Nox4-based NADPH oxidase in vascular cells are scantily defined.

Nox1 is transcriptionally regulated by GATA-binding factors in Caco-2 cell line.Less is known about the regulation of human Nox1 promoter in human SMCs. Cevik et al showed that AP-1 is essential for the rat Nox1 promoter activity. These results are consistent with our previous findings on human aortic SMCs regarding the modulation of Nox expression by AP-1/MAPK-related pathways. The role of JAK/STAT in SMCs is unknown.

Kuwano et al demonstrated that IFNγ induces Nox1 expression and O2− production in Caco-2 cells, and this upregulation may be in part mediated by a distal (−4.3 kb) STAT1 element. Our data on SMCs confirm and extend these observations. Stimulation of SMCs with IFNγ led to a marked upregulation of Nox activity and Nox1 mRNA and protein expression through a JAK/STAT-dependent mechanism. In addition, we predicted 3 novel GAS (STAT1/STAT3) elements in the Nox1 proximal promoter. Of particular interest is that the GAS (III) element is located in the core promoter in the proximity of TATA box, suggesting the essential role of JAK/STAT signaling in the upregulation of Nox1 isoform in response to proinflammatory or growth-related stimuli.

To identify the regions required for the basic transcription activity, the luciferase gene expression directed by the human Nox1 promoter was analyzed in SMCs. The results showed a similar pattern of Nox1 promoter activity, as previously found in Caco2 cell line, characterized by distal negative regulatory sequences and proximal positive acting elements.

Nox4 is highly expressed in many cell types, including SMCs and endothelial cells, indicating that Nox4 may have a fundamental role in promoting redox-dependent cellular processes. Because changes in mRNA level directly affect the Nox4-derived ROS production, regulation and the rate of transcription of Nox4 is of particular interest. There are few data concerning the Nox4 promoter regulation. A first analysis of the mouse Nox4 promoter was done by Zhang et al in A7r5 cells and primary mouse aortic SMCs demonstrating that growth-promoting transcription factor E2F physically interact with Nox4 promoter. Using human Nox4 promoter-luciferase constructs in SMCs, we obtained a similar pattern of Nox4 promoter regulation like those indicated for the mouse Nox4 promoter. In addition, we identified 3 highly conserved GAS elements that might regulate transcriptionally the human Nox4 gene.

To investigate the function of the GAS elements within human Nox1 and Nox4 promoters, we performed cotransfection experiments using 5′ deletion constructs and STAT1 or STAT3 expression vectors. Transient overexpression of STAT1 or STAT3 induced a significant increase of luciferase level directed by the promoters of either Nox1 or Nox4 genes indicating the presence of functionally STAT1 and STAT3 binding sites.

To further uncover the function of GAS elements, we analyzed the nuclear factor binding activities and found that sequences corresponding to the predicted GAS form a bound complex with STAT1 or STAT3 proteins. The overexpression analysis and DNA–protein interaction assays indicated that human Nox1 and Nox4 gene promoter contains STAT1 and STAT3 positive-acting regulatory elements.

To test the involvement of JAK/STAT pathway in the overall Nox transcription, overexpression and inhibition studies were conducted using luciferase-constructs carrying the human p22phox, p47phox, or p67phox promoters. IFNγ and STAT1 play a role in the upregulation of Nox regulatory subunits in several cell types. Our results extend these observations to human SMCs. In addition, we found that STAT3 also might be an important regulator of p47phox or p67phox transcription in human aortic SMCs.

To our knowledge, this is the first report providing evidence on the regulation of human Nox1 and Nox4 in SMCs, highlighting the mechanism whereby JAK/STAT pathway is involved in the modulation of Nox expression and function. The novel data emphasize the crossway between inflammation and oxidative stress in the development of vascular disorders.

Inhibition of JAK/STAT pathway is already used in cancer therapy. Because the JAK/STAT system is also a key regulator of vascular cell response to insults, “regulation of the regulator” may represent a new pharmacological target in vascular diseases, such as atherosclerosis.

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Disclosures

None.

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JAK/STAT signaling pathway regulates Nox1 and Nox4-based NADPH oxidase in human aortic smooth muscle cells

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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 and chromatin immunoprecipitation (ChIP) reagents were purchased from Santa Cruz Biotechnology (USA). The STAT1 and STAT3 expression vectors were from Thermo Scientific/Open Biosystems (USA). pGAS/ISRE-luc control plasmid was purchased from Stratagene (Germany).

Cell culture and experimental design

Human aortic smooth muscle cells (SMCs) were isolated by explantation from the media of fetal 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 Willebrand 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).

Confluent quiescent cells (at 8-12 passages) were exposed (up to 24 hours) to 100-400 U/ml IFNγ in the presence or absence of JAK/STAT inhibitors: decoy oligodeoxynucleotides (ODN), AG490, S3I201, WP1066. Optimal concentration of inhibitors was established in transfection experiments employing pGAS/ISRE-luc control plasmid: 150 nM ODN, 20 μM of AG490 and S3I201 or 5 μM WP1066.

In addition to the JAK/STAT inhibitors we have also used different MAPK inhibitors (10 μM SB203580 – p38 MAPK, 10 μM U0126 – ERK1/2, 20 μM SP600125 – JNK). The results showed that MAPK inhibition slightly reduced the STAT transcriptional activity.

In addition to the classical JAK/STAT pathway, IFNγ is also a potent inducer of the MAPK signaling. Moreover, it has been demonstrated that MAPK itself are able to activate STATs under mitogenic and pro-inflammatory conditions in several cell types. It has been shown that p38 MAPK, ERK1/2, and also JNK can activate different STAT isoforms including STAT1 and STAT3. In this context, the crosstalk between JAK/STAT and MAPK signaling pathways should be considered.
Inhibition studies were conducted in transfected SMCs treated with 100 U/ml IFNγ for 24 hours. Addition of IFNγ in the culture medium increased (≈ 40 to 50 %) the luciferase activity directed by the Nox(s) constructs or GAS/ISRE elements from the control reporter vector.

The study was performed in accordance with the ethical principles for medical research involving human subjects (World Medical Association Declaration of Helsinki), and the local committee on human research approved of the study protocol.

**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 O$_2^-$ 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 O$_2^-$ 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 N$^G$-nitro-L-arginine methyl ester (L-NAME), and 50 µM rotenone. The activity was calculated from the ratio of mean light units to total protein level and expressed as arbitrary units.

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.

The generation of superoxide in intact SMCs was measured by dihydroethidium (DHE) fluorescence technique$^5$. After loading with 5 μmol/l DHE for 30 min in the dark at 37°C, the cells were scraped and resuspended in Hapes-buffered saline solution, pH 7.4, containing (in mmol/l): 145 NaCl, 5 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 1 Na$_2$HPO$_4$, 5 glucose, 25 Hapes. The cells were distributed at 10$^4$/well into a 96-well microplate reader (Tecan). DHE fluorescence emission was detected at 610 nm with an excitatory wavelength of 530 nm. The O$_2^-$ production was calculated from the ratio of relative fluorescence units to total protein level and expressed as arbitrary units.

Cytochrome c reduction assay$^6$ 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, MnSOD, and MMP9 mRNA expression was done by amplification of cDNA using an Opticon 2 DNA Engine real-time thermocycler (MJ Research) and SYBR Green I 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 Cₜ method and expressed as arbitrary units⁷.

**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 0.1 μg pSV-β-galactosidase vector⁸. In some experiments luciferase constructs were cotransfected with 0.3 μg of STAT1 , STAT3 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 units.
**Western-blot analysis**

Cultured cells were washed twice in ice-cold PBS before lysis in 2 x Laemmli’s electrophoresis sample buffer and boiled for 10 min. Protein concentration was quantified by the Amido Black method\(^9\). Equal amounts of protein (70 µg) were run on 10 % 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 Nox1 (rabbit polyclonal, sc-25545), Nox4 (rabbit polyclonal, sc-30141) or β actin (mouse monoclonal, sc-47778), 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 Nox1 and Nox4 proteins was determined by normalization to β actin protein and expressed as arbitrary units.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed using antibodies, reagents and protocols from Santa Cruz Biotechnology. Briefly, 10⁷ 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 STAT1 (rabbit polyclonal, sc-
592) or STAT3 (rabbit polyclonal, sc-7179) antibodies 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 NaHCO₃). Immunoprecipitated and input DNA was purified with QIAquick purification columns (Qiagen). PCR was performed with primers for the Nox1 and Nox4 promoters flanking the GAS sites. A fragment containing the GAS element from human c-Myc gene promoter were amplified from immunoprecipitated chromatin and served as positive control (R&D Systems). ‘No-antibody’ and negative controls from genomic regions which do not contain predicted GAS elements were employed. The specificity of PCR products was analyzed by gel electrophoresis and melting curve. Input DNA was amplified for each sample in parallel experiments.

**Transfection of decoy oligodeoxynucleotide**

Transfection of decoy oligodeoxynucleotides (ODN)¹⁰ was performed using double-stranded DNA with sequences corresponding to the consensus STAT1 or STAT3 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.
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 decoy ODN assay (Santa Cruz Biotechnology). The nuclear factor consensus sequences are underlined.

Figure I
Time- and dose-dependent effect of IFNγ on NADPH oxidase activity in SMCs. SMCs were treated with IFNγ (100 U/ml, 200 U/ml or 400 U/ml) for the indicated times. The NADPH-dependent superoxide production was measured by lucigenin-enhanced chemiluminescence assay; n=5, *P<0.05, **P<0.01, ***P<0.001. P-values were taken in relation to the corresponding controls.

Figure II
The effect of JAK/STAT inhibition on Nox activity in normal and 100 U/ml IFNγ-exposed SMCs. O2− production was measured by dihydroethidium assay at (A) 4- and (B) 24-hour point; n=5, *P<0.05, **P<0.01, ***P<0.001. P-values were taken in relation to IFNγ-stimulated cells.
Figure III

Regulation of MnSOD (A) and MMP9 (B) mRNA by JAK/STAT. SMCs were treated for 24 hours with 100 U/ml IFNγ in the presence/absence of JAK/STAT inhibitors. Quantification of mRNA level was done by real time PCR; n=5, * P < 0.05, ** P < 0.01, *** P < 0.001. P-values were taken in relation to the IFNγ-treated cells.

Figure IV

Regulation of p22phox, p47phox, and p67phox promoter activities by STAT1/STAT3 overexpression (A) and JAK/STAT inhibition (B); n=6, *P<0.05, **P<0.01, ***P<0.001. P-values were taken in relation to the corresponding controls (pcDNA3.1 or promoter-luciferase constructs alone).

Figure V

Chromatin immunoprecipitation analysis of GAS elements. Quiescent SMCs were treated with vehicle (control) or 100 U/ml IFNγ for 2 hours in the presence/absence of JAK2 inhibitor WP1066. A, B: Schematic drawing of Nox1 and Nox4 promoters depicting the relative position of the GAS sites (black boxes); C: Representative agarose gel electrophoresis illustrating the predicted molecular weight of the PCR products; n=4.
Table I

<table>
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<tr>
<th>Gene</th>
<th>GeneBank® Accession Number</th>
<th>Sequences of Oligonucleotide Primers</th>
<th>Predicted size (bp)</th>
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<tr>
<td>Nox1</td>
<td>NM_013955</td>
<td>S: 5'-CACAAAGAAATGCTTTGAGTCA-3' A: 5'-GACGAGCAATTGGGCAAC-3'</td>
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Table II

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<th>Fragment name</th>
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<tr>
<td>STAT1</td>
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<tr>
<td>STAT3</td>
<td>S: 5'-GATCTGGATCTCTGAAATCGCTGAT-3' A: 5'-GATCTGGATCTCTGAAATCGCTGAT-3'</td>
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<td>Scrambled</td>
<td>S: 5'-ATGAGGCAGGCGACTGACATG-3' A: 5'-CATGCTGCTCAGTCGCTC-3'</td>
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</tbody>
</table>

S, Sense; A, Antisense
Figure I

![Graph showing O₂ production over time](image)

Figure II

**A** Dihydroethidium assay-4h

![Graph showing O₂ production in A](image)

**B** Dihydroethidium assay-24h

![Graph showing O₂ production in B](image)
Figure V

A  
Nox1 (Nq22)  
(+) TTCCTGAA  
(-) TAAAGGGTC  
(-) TTCCTGAA

B  
Nox4 (11q14.2-q21)  
(+) TTCCTGAA  
(-) AAGTCATT  
(-) AATCCCAT

C  
ChIP STAT1  
ChIP STAT3  
Input DNA

D  
ChIP STAT1  
ChIP STAT3  
Input DNA

E  
ChIP STAT1  
ChIP STAT3  
Input DNA

1. DNA ladder  
2. c-Myc  
3. Primer set (P1)  
4. Primer set (P2)  
5. Primer set (P3)  
6. Primer set (P4)  
7. Primer set (P5)  
8. Primer set (P6)  

Nox1  
Nox4
References


