Polymorphisms and Promoter Overactivity of the p22<sub>phox</sub> Gene in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

Guillermo Zalba, Gorka San José, Francisco J. Beaumont, María A. Fortuño, Ana Fortuño, Javier Díez

Abstract—In a previous study, we found that the p22<sub>phox</sub> subunit of the NADH/NADPH oxidase is overexpressed in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHRs) with enhanced vascular production of superoxide anion (O<sub>2</sub>·). Thus, we have investigated whether changes in the sequence or activity of the promoter region of p22<sub>phox</sub> gene are present in SHRs. To carry out this analysis, first of all, we characterized the rat gene structure and promoter region for the p22<sub>phox</sub> subunit. The p22<sub>phox</sub> gene spans ~10 kb and contains 6 exons and 5 introns. Primer extension analysis indicated the transcriptional start site 100 bp upstream from the translational start site. The immediate promoter region of the p22<sub>phox</sub> gene does not contain a TATA box, but there are a CCAC box and putative recognition sites for nuclear factors, such as SP1, γ-interferon, and nuclear factor-κB. Using reporter-gene transfection analysis, we found that this promoter was functional in VSMCs. Furthermore, we observed that p22<sub>phox</sub> promoter activity was significantly higher in VSMCs from SHRs than from normotensive Wistar-Kyoto rats. In addition, we found that there were 5 polymorphisms in the sequence of p22<sub>phox</sub> promoter between Wistar-Kyoto rats and SHRs and that they were functional. The results obtained in this study provide a tool to explore the mechanisms that regulate the expression of p22<sub>phox</sub> gene and in the degree of activation of VSMCs are responsible for upregulated expression of p22<sub>phox</sub> in SHRs. (circ res. 2001;88:217-222.)

Key Words: NADH/NADPH oxidase ■ gene promoter ■ vascular smooth muscle cells ■ superoxide anion

Oxidative stress induced by vascular superoxide anion (O<sub>2</sub>·) has been implicated in the development of hypertension and atherosclerosis. The enzyme NADH/NADPH oxidase plays a major role as the most important source of superoxide anion in the vessel wall. Although vascular NADH/NADPH is similar to the neutrophil NADPH oxidase, recent studies suggest that it represents a novel family of oxidative enzymes. Recent investigations show that p22<sub>phox</sub>, a component of the NADH/NADPH oxidase, is expressed in vascular smooth muscle cells (VSMCs) and plays an essential role in O<sub>2</sub>· generation in these cells.

Recently, we reported that enhanced NADH/NADPH oxidase–driven O<sub>2</sub>· production in the aorta of adult spontaneously hypertensive rats (SHRs) was associated with upregulation of p22<sub>phox</sub> mRNA. Our data pointed to VSMCs as the potential source for both p22<sub>phox</sub> mRNA overexpression and O<sub>2</sub>· overproduction in the aorta of SHRs. Thus, we hypothesized that p22<sub>phox</sub> mRNA upregulation observed in VSMCs from SHRs could be a consequence of either modifications in the p22<sub>phox</sub> gene-promoter sequence or differences in its activation degree. Thus, the first goal of this study was to perform the structural and functional characterization of the p22<sub>phox</sub> gene. Second, we compared the p22<sub>phox</sub> promoter sequences from normotensive Wistar-Kyoto (WKY) rats and SHRs. Finally, we analyzed the p22<sub>phox</sub> promoter activity in VSMCs from the 2 strains of rats.

Materials and Methods

Exon Mapping

Rat genomic DNA was isolated from peripheral blood leukocytes by standard methods. The intron positions were determined by polymerase chain reaction (PCR) amplification of rat genomic DNA using oligonucleotides on the basis of rat p22<sub>phox</sub> cDNA sequence (p1: 5′-GGCAGATCGAGTGGGCCATGTG-3′; p2: 5′-AGGTA-GATCACACTGGAATG-3′; p3: 5′-CATTCGCGAATGTGTGAC-3′; p4: 5′-GTTGATGATGGCTCAACATCTG-3′). The resultant products were cloned into pCR (Invitrogen) and sequenced.

Cloning of the 5′-Flanking Region

The 5′-flanking region of the gene was amplified using the Promoter Finder System from Clontech. This kit contains pools of uncloned, adaptor-ligated genomic DNA fragments. A first amplification was performed between an outer adapter primer (ap1, 5′-GTAATACGACCTCTAGAGCC-3′) and a p22<sub>phox</sub> cDNA-specific primer (sp1, 5′-CACGGATGCCAAGCCTTGTGGT-3′). A second round of PCR was done using the nested adapter primer.
primer (ap2, 5'-ATCATAGGCGAGCCGTGTTG-3') and a nested p22<sup>phox</sup>-specific primer (sp2, 5'-TGCCCACATGGCCACCTCGA-TCTG-3'). The amplifications were carried out according to the supplier's protocol. The two rat p22<sup>phox</sup> cDNA-specific primers were localized in the exon 1. This protocol detected a single clear band. This band was ~2.5 kbp and was subcloned into pCR plasmid for additional sequencing reactions.

For comparing experiments between WKY and SHR p22<sup>phox</sup> promoter sequences, the 2500-bp fragment corresponding to p22<sup>phox</sup> full promoter was amplified using Pfu polymerase from WKY and from SHR genomic DNA cloned into pCR plasmid and sequenced.

**Primer Extension Analysis**

Primer extension was carried out using the antisense oligonucleotide PE, (5'-GCCGGACGCCTGCCTGCTGTTG-3'), corresponding to a sequence located in exon 1. The oligonucleotide was end-labeled with [γ<sup>32</sup>P]ATP, hybridized to 5 μg of mRNA extracted from the rat kidney, and extended using Moloney murine leukemia virus reverse transcriptase. The primer-extended product was separated on a 7 mol/L urea 6% polyacrylamide gel, dried, and exposed to generate the corresponding autoradiography.

**Plasmid Construction**

A 2.5-kb fragment containing the p22<sup>phox</sup> 5'-untranslated region and including the codon ATG of exon 1 was cloned in pCR. Serial deletion fragments from the p22<sup>phox</sup> promoter were generated by PCR using Pfu polymerase and 7 nested sense primers. Sense and antisense primers were designed for containing HindIII restriction sites. After digestion with HindIII, products were cloned into HindIII-digested pGL3 basic (Promega). Insert orientation was determined by sequencing of the fusion sites.

To compare the p22<sup>phox</sup> promoter activity between WKY and SHR sequences, the 2500-bp fragments corresponding to the WKY and SHR p22<sup>phox</sup> full promoters were cloned in pGL3 basic plasmid.

**Cell Culture**

Primary VSMCs were obtained from the thoracic aorta and cultured as previously reported. VSMCs were cultured in DMEM with 10% FCS. The rat aortic smooth muscle cell line A7r5 was cultured in DMEM with 10% FCS supplemented with sodium pyruvate. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. For studies with VSMCs from WKY rats and SHRs, cells were obtained from the aortas of 30-week-old SHRs and WKY rats.

**Transfection Experiments and Luciferase Activity**

A7r5 cells (2.5 × 10<sup>4</sup> cells) and VSMCs (5 × 10<sup>4</sup> cells) were plated 24 hours before transfection into 60-mm tissue-culture dishes. Transient transfection was performed by Superfect method (Qiagen) with 2 μg of DNA. Transfection was performed with an antisense oligonucleotide PE, (5'-GCCGGACGCCTGCCTGCTGTTG-3'), corresponding to a sequence located in exon 1. The oligonucleotide was end-labeled with [γ<sup>32</sup>P]ATP, hybridized to 5 μg of mRNA extracted from the rat kidney, and extended using Moloney murine leukemia virus reverse transcriptase. The primer-extended product was separated on a 7 mol/L urea 6% polyacrylamide gel, dried, and exposed to generate the corresponding autoradiography.

**Inspection of the Sequence of the 5'-flanking region** indicated that although there was no typical TATA box in close proximity to the transcriptional start site, there was a CCAC box. Furthermore, there were multiple transcription factor binding sites, such as SP1, AP1, AP4, GATA, γ-interferon, and nuclear factor-κB (NF-κB). Inspection of the sequence of the 5'-flanking region indicated that although there was not typical TATA box in close proximity to the transcriptional start site, there was a CCAC box. Furthermore, there were multiple transcription factor binding sites, such as SP1, AP1, AP4, GATA, γ-interferon, and nuclear factor-κB (NF-κB). (Figure 2), that might transcriptionally regulate p22<sup>phox</sup> gene expression. A primer extension experiment was performed with an antisense oligonucleotide that mapped in the first exon close to the translation initiation codon ATG. The results clearly indicate the existence of a predominant site for transcription initiation, pointed to a C located 100 nucleotides upstream of the codon ATG.

**Promoter Function of the p22<sup>phox</sup> Gene**

To determine whether the putative promoter region is functional, a 2500-bp fragment containing the complete promoter was subcloned into a luciferase reporter plasmid (p22c1) (Figure 3) and transfected into A7r5 cells and VSMCs. As shown in Figure 4A, remarkable expression was observed in both types of cells. Relative expression of p22c1 to that of the pGL3 promoter was higher (P<0.05, Student’s unpaired t test) in A7r5 cells than in VSMCs.

To define the regions required for promoter activity, we cloned a series of progressively deleted DNA fragments of the putative promoter directly upstream of the firefly luciferase reporter gene (Figure 3). The resulting plasmids were transiently expressed in A7r5 cells. As shown in Figure 4B, constructs p22c6 and p22c7 produced relatively strongest signals. This identified positive regulatory elements involved in basal promoter activity in the proximal part of the p22<sup>phox</sup> promoter between ~402 bp and +1 bp, such as AP1, AP4, GAGA, and NF-κB.

**Figure 1.** A. Organization of the rat p22<sup>phox</sup> gene. Boxes denote exons, and lines denote introns and 5'-flanking region. B, Genomic PCR products obtained with the pair of primers p1/p2, p3/p4, and sp2/ap2. sp2 primer is an adapter-specific primer used for amplification of p22<sup>phox</sup> promoter from adaptor-ligated genomic DNA fragments by using the Promoter Finder System.

**TABLE 1. Exon-Intron Organization of the Rat p22<sup>phox</sup> Gene**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Intron</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGGCCCTGA</td>
<td>gtggagtgaagtg (intron 1, ~4800 bp) cacctctcactttag tagcctcactttag</td>
</tr>
<tr>
<td>TACTCTCTAT</td>
<td>gtatctctctac (intron 2, ~390 bp) ttcctctctcactttag TGTTGACAG</td>
</tr>
<tr>
<td>GAGGCGGTG</td>
<td>gtagtcatctc (intron 3, ~190 bp) ttgcctctcactttag TGAGGCAAGA</td>
</tr>
<tr>
<td>CACCTTACT</td>
<td>gtgtggtgtct (intron 4, ~800 bp) ttgcctctcactttag GCTGTCG</td>
</tr>
<tr>
<td>ACCTGCTG</td>
<td>gaagtaggtagt (intron 5, ~1100 bp) ttcctctcactttag GAGCCCAT</td>
</tr>
</tbody>
</table>
To determine whether the upregulation of p22\textsuperscript{phox} gene expression is dependent of the level of cell activation, the full promoter (construct p22c1) was transfected into VSMCs from WKY rats and SHRs. As shown in Figure 5, relative expression of p22c1 to that of the pGL3 promoter was 2-fold higher (P<0.05) in cells from SHRs than in cells from WKY rats.

To determine the functional significance of putative binding sites for NF-κB transcription factor, we performed experiments to know whether deletion of the NF-κB sites abrogated the difference in promoter activity observed between WKY and SHR VSMCs. Transfection experiments were performed with constructs p22c2, p22c3, p22c4, and p22c5.
performed with construct p22c7, without these NF-κB sites, on VSMCs from WKY rats and SHRs. As shown in Figure 5, deletion of NF-κB does not abrogate the difference in promoter activity observed between SHR and WKY VSMCs.

**p22**<sup>phox</sup> Promoter Sequences in WKY Rats and SHRs

To determine whether the upregulation of p22<sup>phox</sup> gene expression in SHRs was the consequence of differences in the p22<sup>phox</sup> promoter sequence, a 2500-bp fragment corresponding to the p22<sup>phox</sup> full promoter was amplified from WKY rats and SHR genomic DNA and sequenced as described above. The comparison of the WKY rats and SHR p22<sup>phox</sup> promoters by computer analysis revealed that the sequence of the WKY promoter does not match completely the SHR promoter sequence. In fact, we found 5 polymorphisms: 4 in the upstream region of the gene at positions −1628, −218, −166, and −14 from the first transcribed nucleotide and 1 in the nontranslated region at position +42 (Table 2).

Table 2. Variants Identified in the p22<sup>phox</sup> Gene

<table>
<thead>
<tr>
<th>Location</th>
<th>Position</th>
<th>Substitution WKY→SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>−1628</td>
<td>C→T</td>
</tr>
<tr>
<td>5'</td>
<td>−218</td>
<td>C→T</td>
</tr>
<tr>
<td>5'</td>
<td>−166</td>
<td>A→G</td>
</tr>
<tr>
<td>5'</td>
<td>−14</td>
<td>C→T</td>
</tr>
<tr>
<td>5'</td>
<td>+42</td>
<td>A→G</td>
</tr>
</tbody>
</table>

To test whether there is any functional significance to the promoter polymorphisms, transfection experiments on A7r5 cells and on VSMCs from WKY rats and SHRs were performed with the WKY full promoter and with the SHR full promoter. As shown in Figure 6, the activity of the SHR polymorphic construct was higher (P<0.05, Student’s t test) than the activity of WKY construct in all cell types.

### Discussion

In the present study, we established the genomic structure of the rat p22<sup>phox</sup> gene, which contains 6 exons and 5 introns and spans a region of 10 kb. We found that the rat exon-intron genomic structure is very similar to that of the human p22<sup>phox</sup> gene. In addition, this is the first study that characterizes the promoter region of a p22<sup>phox</sup> gene. The p22<sup>phox</sup> promoter possesses no typical TATA in the appropriate positions, but there is a CCAC box. Furthermore, sequence analysis of the p22<sup>phox</sup> promoter region revealed several potential consensus sequences for transcriptional factors.

p22<sup>phox</sup> is a common component of vascular and phagocytic NADH/NADPH oxidases. Despite their similarities, vascular and phagocytic NADH/NADPH oxidases posses enzymatic differences. Thus, the vascular oxidase system prefers NADH to NADPH as substrate for its activity and has much lower activity in contrast to phagocytic oxidase. Recent...
studies have reported the significance of p22phox overexpression gene in cardiovascular diseases. Vascular p22phox is expressed at low levels in normal vessels and is upregulated in atherosclerosis and hypertension and in response to trophic factors, such as angiotensin II, and cytokines, such as tumor necrosis factor-α.

From previous findings, we proposed a significant role for upregulation of VSMC p22phox mRNA in NADH/NADPH-driven O₂⁻ overproduction found in the aorta from adult SHRs. A possible origin of p22phox upregulation would be some difference in the p22phox promoter sequence. We identified 5 polymorphisms in the 5’ region of the p22phox gene, 1 polymorphism located in nontranslated region (+42), and 4 polymorphisms located in the promoter region (−14, −166, −218, and −1628 bp). Interestingly, we have found that these polymorphisms possess functional significance, suggesting that they may be involved in overexpression of the p22phox gene. This is additionally reinforced by the observation that 4 of these 5 polymorphisms are situated in the first 250 bp, where it seems that maximal basal promoter activity of the p22phox gene is localized.

Another finding of this study is that p22phox promoter activity was higher in VSMCs from SHRs compared with VSMCs from WKY rats, suggesting that in vivo variations in the expression of the p22phox gene might be the result of differences in the level of activation of cells from the 2 strains of rats. For instance, stimulation of VSMCs from SHRs with angiotensin II results in an amplified activation of p22phox expression. In addition, exaggerated production of angiotensin II and enhanced expression of both AT₁ receptor and angiotensin-converting enzyme have been reported in vessels of SHRs compared with WKY rats. Thus, the possibility exists that angiotensin II can be involved in changes in cell activation that, in turn, influence the expression of p22phox gene in SHR VSMCs. Although additional studies are necessary to test this hypothesis, some arguments are in accordance with it. First, angiotensin II has been found to stimulate p22phox expression and NAD(P)H-driven O₂⁻ production in the rat aorta. This effect was inhibited by treatment with losartan, suggesting that it was mediated by the interaction of angiotensin II with AT₁ receptors. Second, we reported previously that chronic blockade of AT₁ receptors with irbesartan decreased p22phox expression and O₂⁻ production in the aorta of SHR despite a noncomplete normalization of blood pressure.

It has been shown recently that angiotensin II activates NF-κB in VSMCs. Furthermore, NF-κB has been implicated in the transcription of several vascular genes. Nevertheless, from our data with p22c7 construct, it is unlikely that upregulated p22phox expression seen in VSMCs from SHRs is mediated by a NF-κB–dependent pathway. In fact, experiments on luciferase activity with the deleted promoter construct show that deletion of NF-κB sites does not abrogate the difference in p22phox promoter activity between SHR and WKY VSMCs (Figure 5). Similarly, it is unlikely that other sites (ie, AP1 and AP4) also absent in the p22c7 construct are important for the observed differences. In contrast, the GATA and MZF1 sites are more likely to mediate promoter activity, because they are retained in p22c7.

In summary, we have characterized the genomic structure of the rat p22phox gene promoter, providing a tool to explore the mechanisms regulating the expression of this gene in VSMCs. Our results suggest that besides changes in activation degree of VSMCs associated with the development of hypertension in SHRs, the presence of several polymorphisms in the promoter region of the p22phox gene may contribute to enhanced p22phox promoter activity in SHRs. Thus, the findings reported here provide a potential explanation for the upregulation of p22phox in the vessel wall of SHRs. The significance of these experimental results is underlined by clinical data, indicating the occurrence of increased O₂⁻ production in humans with essential hypertension and the existence of an association between a p22phox gene polymorphism and NAD(P)H oxidase–mediated O₂⁻ production in the vascular wall of patients with atherosclerosis.

References


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