Functional Effect of the $p22^{phox} -930^{A/G}$ Polymorphism on $p22^{phox}$ Expression and NADPH Oxidase Activity in Hypertension

Gorka San José, María U. Moreno, Sara Oliván, Oscar Beloqui, Ana Fortuño, Javier Díez, Guillermo Zalba

Abstract—Oxidative stress induced by superoxide is implicated in hypertension. NADPH oxidase is the main source of superoxide in phagocytic and vascular cells, and the $p22^{phox}$ subunit is involved in NADPH oxidase activation. Recently we reported an association of $-930^{A/G}$.polymorphism in the human $p22^{phox}$ gene promoter with hypertension. This study was designed to investigate the functional role of this polymorphism in hypertension. We thus investigated the relationships between the $-930^{A/G}$ polymorphism and $p22^{phox}$ expression and NADPH oxidase–mediated superoxide production in phagocytic cells from 70 patients with essential hypertension and 70 normotensive controls. Genotyping of the polymorphism was performed by restriction fragment length polymorphism. NADPH oxidase activity was determined by chemiluminescence assays, and $p22^{phox}$ mRNA and protein expression was measured by Northern and Western blotting, respectively. Compared with hypertensive subjects with the AA/AG genotype, hypertensive subjects with the GG genotype exhibited increased ($P<0.05$) phagocytic $p22^{phox}$ mRNA (1.26±0.06 arbitrary unit [AU] versus 0.99±0.03 AU) and protein levels (0.58±0.05 AU versus 0.34±0.04 AU) and enhanced NADPH oxidase activity (1998±181 counts/s versus 1322±112 counts/s). No differences in these parameters were observed among genotypes in normotensive cells. Transfection experiments on vascular smooth muscle cells showed that the A-to-G substitution of this polymorphism produced an increased reporter gene expression in hypertensive cells. Nitric oxide production, as assessed by measurement of serum nitric oxide metabolites, was lower in GG hypertensive subjects than in AA/AG hypertensive subjects. In conclusion, these results suggest that hypertensive subjects carrying the GG genotype of the $p22^{phox} -930^{A/G}$ polymorphism are highly exposed to NADPH oxidase-mediated oxidative stress. *(Hypertension. 2004; 44:163-169.)*

Key Words: hypertension, arterial | oxidative stress | polymorphism

Oxidative stress induced by reactive oxygen species (ROS), including superoxide ($O_2^-$), is increased in arterial hypertension, and it has been proposed as a possible mechanism that predisposes to development of atherosclerosis in hypertension. Among other effects, oxidative stress may account for the decrease in the bioavailability of nitric oxide (NO), because it reacts with $O_2^-$. NO has been shown to regulate vascular tone and to inhibit platelet aggregation, thrombus formation, leukocyte adhesion, and vascular proliferation.

A plasma membrane-associated NADPH oxidase is present in endothelial cells, vascular smooth muscle cells (VSMCs), and fibroblasts and seems to be the most important source of $O_2^-$ in the vessel wall. A major component of NADPH oxidase is the $p22^{phox}$ protein, a critical subunit that plays an essential role in NADPH oxidase activation in vascular cells. Enhanced vascular NADPH oxidase activity has been shown to be associated with upregulation of $p22^{phox}$ mRNA in several models of hypertension, including the spontaneously hypertensive rat (SHR). Moreover, increased ROS generation has been found to be associated with augmented vascular NADPH oxidase activity in VSMCs from peripheral arteries in patients with hypertension. The human $p22^{phox}$ gene has several allelic variants, some of which are associated with cardiovascular disease. We recently reported the existence of functional polymorphisms in the $p22^{phox}$ gene promoter in SHR. In addition, we have described a novel genetic variation in the human $p22^{phox}$ gene promoter, the $-930^{A/G}$ polymorphism, associated with essential hypertension. We have hypothesized that the $G$ allele of the $-930^{A/G}$ polymorphism could be associated with higher $p22^{phox}$ expression and NADPH oxidase activity in patients...
with essential hypertension. Because NADPH oxidase present in phagocytes, including the p22\textsuperscript{phox} subunit, is structurally very similar to vascular NADPH oxidase,\textsuperscript{1} the studies reported here were performed in these cells. NO metabolites were determined in serum samples of the same subjects to evaluate the potential impact of \( \text{O}_2^- \) production on systemic generation of NO.\textsuperscript{17} Furthermore, promoter activity assays were performed in cultured VSMCs obtained from SHR and normotensive Wistar-Kyoto (WKY) rats to address the potential importance of the hypertensive stimulus on the functional activation of this polymorphism.

### Methods

An expanded Methods section is available in the online supplement at http://www.hypertensionaha.org.

### Subjects

The study was performed in individuals who were referred to our institution for routine medical examination. The hypertensive group comprised 70 subjects with systolic blood pressure \( >139 \text{ mm Hg} \), diastolic blood pressure \( >89 \text{ mm Hg} \), or both. Half of the patients were receiving antihypertensive treatment when they enrolled in the study. As a control group, 70 normotensive subjects were also studied. Clinical screenings were based on medical history, physical examination, and routine analytical tests. According to institutional guidelines, subjects were aware of the research nature of the study and agreed to participate. The study was carried out in accordance with the Helsinki Declaration, and the Ethical Committee of the University Clinic of Navarra approved the protocol.

### Preparation of Phagocytic Cells

Mononuclear phagocytic cells were isolated from blood samples with Lymphoprep.

### NADPH Oxidase Activity

Phagocytic cells (400,000 cells) were incubated (37°C for 30 minutes) in basal conditions and with phorbol myristate acetate (PMA; 3.2×10\textsuperscript{-6} mol/L), a protein kinase C (PKC) activator; \( \text{O}_2^- \) production was measured using lucigenin (10\textsuperscript{-3} mol/L). Chemiluminescence was determined every 10 seconds for 5 minutes in a Berthold luminometer. The area under the curve was used to quantify chemiluminescence. Data are expressed as counts per second. In previous experiments, basal and PMA-stimulated \( \text{O}_2^- \) production was completely abolished with apocynin (2.5×10\textsuperscript{-3} mol/L), a specific intracellular inhibitor of NADPH oxidase. Specificity of the lucigenin for \( \text{O}_2^- \) was verified because the addition of the \( \text{O}_2^- \) scavenger superoxide dismutase (SOD; 1000 U/mL) abolished the signal.

The lucigenin-based assay was validated against an independent measurement of \( \text{O}_2^- \) production using SOD-inhibitable ferricytochrome C reduction that closely correlates with lucigenin measurements \((n=5; r=0.92, P<0.001)\).

### NO Metabolites in Serum

Serum samples were obtained after overnight fasting to measure nitrate and nitrite (\( \text{NO}_3^-/\text{NO}_2^- \), \( \text{NOx} \)).\textsuperscript{18} \( \text{NOx} \) concentration was evaluated using a colorimetric assay based on Griess reaction.

### p22\textsuperscript{phox} mRNA Expression

p22\textsuperscript{phox} mRNA expression was determined by Northern blotting and by quantitative real-time reverse transcription–polymerase chain reaction. Western blotting with a mouse anti-p22\textsuperscript{phox} antibody (kindly provided by Professor D. Roos, University of Amsterdam, The Netherlands) was used to evaluate p22\textsuperscript{phox} protein abundance.

### Genotyping of the \(-930^{AG}\) Polymorphism of p22\textsuperscript{phox} Gene

The A-to-G substitution at position \(-930 \) in the p22\textsuperscript{phox} promoter was typed by restriction fragment length polymorphism as previously described.\textsuperscript{16}

### Directed Mutagenesis

Transient reporter gene expression assays with chimeric constructs containing A (c1-A) or G (c1-G) allelic p22\textsuperscript{phox} gene promoter and luciferase gene were performed as previously reported.\textsuperscript{15,16} Transfections were performed on VSMCs from the cell line A7r5 and on VSMCs obtained from SHR and WKY rats. In some experiments, we performed cotransfection of allelic constructs with the CCAAT enhancer-binding protein (C/EBP) \( \delta \) plasmid, generously provided by Professor S.L. McKnight (University of Texas Southwestern Medical Center, Dallas, Tex).

### Statistical Analysis

Data are expressed as mean±SEM. Statistical significance of differences in \( \text{O}_2^- \) generation, p22\textsuperscript{phox} expression, and \( \text{NOx} \) levels among groups was assessed by 1-way ANOVA. A Scheffe post hoc test was used to examine differences between groups when significance was achieved. Differences in the luciferase activity between allelic variants were assessed by Student \( t \) test.

### Results

### Clinical Characteristics of the Subjects Studied

The demographic and clinical characteristics of normotensive and hypertensive subjects are shown in Table 1. Hypertensive subjects exhibited significantly higher values of age, body mass index, and blood pressure than normotensive subjects.

Each subject was genotyped for the \(-930^{AG}\) polymorphism. To enhance statistical power, and given the low allelic frequency of the A allele, AA homozygote subjects were added to AG heterozygous subjects. Thus, homogeneous normotensive and hypertensive groups were composed of 35 subjects with GG genotype and 35 subjects with AA/AG genotype (10 individuals AA and 25 individuals AG).

### Relation Between the \(-930^{AG}\) Polymorphism and Phagocytic NADPH Oxidase Activity and p22\textsuperscript{phox} Expression

Although baseline NADPH oxidase activity was similar in hypertensive and normotensive groups (167±21 counts/s versus 148±17 counts/s, \( P=\text{NS} \)), PMA-stimulated NADPH oxidase activity was higher in hypertensive than in normotensive subjects (1660±129 counts/s versus 1125±109 counts/s, \( P<0.01 \)). Whereas baseline NADPH oxidase activity was similar in hypertensive subjects with AA/AG and GG genotype (168±22 counts/s versus 166±31 counts/s, \( P=\text{NS} \)), PMA-stimulated NADPH oxidase activity was higher in GG than in AA/AG hypertensive subjects (1998±181 counts/s versus 1322±112 counts/s, \( P<0.05 \)) (Figure 1). No differences in NADPH oxidase activity were observed between AG and AA hypertensive subjects (data not shown). Finally, no differences in PMA-stimulated NADPH oxidase activity were found between AA/AG and GG genotypes in normotensive subjects (1092±108 counts/s versus 1158±149 counts/s, \( P=\text{NS} \)).

p22\textsuperscript{phox} mRNA levels were higher in GG than in AA/AG hypertensive subjects (1.26±0.06 AU versus 0.99±0.03 AU, \( P<0.05 \)) (Figure 2). No differences in p22\textsuperscript{phox} expression were
found between AG and AA hypertensive subjects (data not shown). No differences in p22phox expression were found between AA/AG and GG normotensive subjects (1.00±0.03 AU versus 1.01±0.05 AU, *P=NS*). These Northern blot data were confirmed by quantitative real-time polymerase chain reaction. In fact, whereas increased p22phox mRNA levels were observed in GG hypertensive subjects compared with AA/AG hypertensive subjects (0.26±0.04 AU versus 0.24±0.04 AU, *P=NS*), no differences were found between GG and AA/AG normotensive subjects (0.94±0.03 AU versus 1.00±0.04 AU, *P=NS*).

The differences in p22phox expression and NADPH oxidase activity between the 2 hypertensive subgroups were not related to age, gender, body mass index, blood pressure, and metabolic factors because these potentially confounding factors were similar in the 2 subgroups (Table 2). In addition, no differences were found in the frequency of antihypertensive drugs and other cardiovascular medication (eg, statins) between the 2 hypertensive subgroups.

### Interaction of the −930AG Polymorphism With Cell Phenotype

To investigate whether the A-to-G substitution may influence gene expression depending on cell phenotype, transfection experiments were carried out on the cell line A7r5 and on VSMCs obtained from WKY and SHR rats, which possess a characteristic normotensive and hypertensive phenotype, respectively. As shown in Figure 3A, the A-to-G substitution increased the reporter gene expression by 30% in hypertensive cells. This allelic change did not modify the reporter gene expression in normotensive cells and in A7r5 cells (Figure 3B and 3C).

Because the −930 polymorphic site lies on a potential binding site for C/EBP transcription factors, additional experiments were carried out on A7r5 cells. Cotransfection of p22phox allelic constructs with C/EBPα increased the p22phox promoter activity (Figure 4A). This effect was significantly

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**Table 1. Clinical Characteristics of Normotensive and Hypertensive Subjects**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensives</th>
<th>Hypertensives</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male/female</td>
<td>56/14</td>
<td>58/12</td>
<td>NS</td>
</tr>
<tr>
<td>Age, years</td>
<td>56±1</td>
<td>60±1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.8±0.7</td>
<td>29.3±0.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>26 (37)</td>
<td>21 (30)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetics, n (%)</td>
<td>9 (13)</td>
<td>8 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>121±2</td>
<td>149±2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>78±1</td>
<td>91±1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>93±2</td>
<td>110±1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>43±1</td>
<td>57±2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>100±3</td>
<td>103±3</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>224±7</td>
<td>233±5</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>48±2</td>
<td>51±2</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>155±5</td>
<td>160±3</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>108±6</td>
<td>111±6</td>
<td>NS</td>
</tr>
<tr>
<td>Granulocytes, cells/mm³</td>
<td>4099±181</td>
<td>4300±195</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes, cells/mm³</td>
<td>1960±91</td>
<td>1853±96</td>
<td>NS</td>
</tr>
<tr>
<td>Monocytes, cells/mm³</td>
<td>302±23</td>
<td>318±21</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.
higher on the promoter activity of the G than of the A allelic construct. These results were associated with upregulated C/EBPα protein expression, which was nearly absent in C/EBPα-nontransfected control A7r5 cells (Figure 4B).

**Association of the −930AG Polymorphism With NO Production**

NOx levels were lower in hypertensive than in normotensive subjects (4.8±0.2 μmol/L versus 6.2±0.3 μmol/L, *P*<0.05). In

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AA/AG</th>
<th>GG</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male/female</td>
<td>29/6</td>
<td>29/6</td>
<td>NS</td>
</tr>
<tr>
<td>Age, years</td>
<td>61±2</td>
<td>60±2</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29.4±0.8</td>
<td>29.7±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>12 (34)</td>
<td>9 (26)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetics, n (%)</td>
<td>5 (14)</td>
<td>3 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>149±2</td>
<td>147±3</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>90±2</td>
<td>91±2</td>
<td>NS</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>110±2</td>
<td>110±3</td>
<td>NS</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>57±3</td>
<td>57±3</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>105±3</td>
<td>107±4</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>230±6</td>
<td>239±9</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>50±2</td>
<td>51±3</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>158±5</td>
<td>164±7</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>104±9</td>
<td>121±10</td>
<td>NS</td>
</tr>
<tr>
<td>Granulocytes, cells/mm³</td>
<td>4316±247</td>
<td>4017±287</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes, cells/mm³</td>
<td>1707±144</td>
<td>1990±164</td>
<td>NS</td>
</tr>
<tr>
<td>Monocytes, cells/mm³</td>
<td>295±26</td>
<td>353±51</td>
<td>NS</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor, n (%)</td>
<td>8 (23)</td>
<td>7 (20)</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin II type-1 receptor antagonist, n (%)</td>
<td>5 (14)</td>
<td>4 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Other antihypertensives, n (%)</td>
<td>5 (14)</td>
<td>6 (17)</td>
<td>NS</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>6 (17)</td>
<td>8 (23)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.
addition, NOx levels were lower in GG than in AA/AG hypertensive subjects (4.2 ± 0.3 μmol/L versus 5.4 ± 0.4 μmol/L, P < 0.05) (Figure 5). No differences in NOx levels were found between the AA/AG and GG genotypes in normotensive subjects (6.3 ± 0.5 μmol/L versus 6.2 ± 0.4 μmol/L, P = NS).

Discussion

The main finding of the present study is that the −930AG polymorphism of the p22phox gene was associated with p22phox expression and NADPH oxidase activity in phagocytic cells from patients with essential hypertension. In particular, hypertensive patients carrying the GG genotype exhibited greater p22phox mRNA and protein levels and NADPH oxidase activity than AA/AG hypertensive patients. These associations were not affected by other confounding variables that are known to influence NADPH oxidase expression and activity (ie, blood pressure, cholesterol, glucose).19–21 Therefore, these findings suggest that the −930AG polymorphism is functional in essential hypertension.

Whereas p22phox mRNA levels were increased in GG hypertensive subjects compared with AA/AG hypertensive subjects, no differences were found in this parameter between the 2 subgroups within the normotensive group. In agreement with this, we found increased luciferase activity driven by the A-to-G substitution in hypertensive but not in normotensive VSMCs. Collectively, these data suggest that the −930AG polymorphism may be functionally relevant in the control of the p22phox gene expression only under hypertensive conditions. Although the mechanisms that underlie this control remain unclear, one possibility is that the presence of the G allele modulates the transcription of the p22phox gene. In this regard, an analysis of the promoter sequence shows that the −930 polymorphic site lies on a potential binding site for C/EBP transcription factors. Furthermore, our results showing a higher effect of C/EBPδ overexpression in the transcriptional activity of the G rather than of the A allelic p22phox promoter constructs, enable us to suggest the involvement of C/EBPs in the hypertensive phenotype. Several findings showing a relevant role of C/EBPs in hypertension support this possibility. C/EBPδ expression is nearly absent in WKY VSMCs, whereas it is abnormally increased in SHR VSMCs.22 Changes in angiotensinogen mRNA expression associated with the 217AG polymorphism of this gene are regulated by several members of the C/EBP family.23 Inflammatory cytokines upregulate C/EBP expression,24 and cytokine levels have been reported to be increased in phagocytic cells in hypertensive patients.25 Alternatively, the G allele may be in linkage disequilibrium with other genetic variants that influence the transcriptional activity. In this regard, we have identified other polymorphisms in the p22phox promoter,26 although their implication in hypertension remains to be established.
Recent studies support a role for p22phox overexpression in hypertension.27 An increased p22phox mRNA expression leading to greater NAD(P)H oxidase activity accompanied by diminished NO availability and endothelial dysfunction has been reported in several models of hypertension.9–11 Furthermore, angiotensin II–induced generation of ROS by NADPH oxidase is augmented in VSMCs from peripheral arteries of hypertensive patients.12 Finally, enhanced NADPH oxidase–dependent production of ROS in lymphoblasts from subjects with hypertension is associated with a greater p22phox subunit abundance, but not with changes in the other NADPH oxidase subunits.28 Therefore, p22phox overexpression may be a critical determinant of NADPH oxidase overactivity in GG hypertensive subjects. Moreover, the higher O2•− generation observed in GG hypertensive subjects was associated with diminished production of systemic NO. The potential relevance of this finding is further supported by research demonstrating that NADPH oxidase is involved in the uncoupling of the endothelial NO synthase, which favors a diminished NO generation in hypertensive animals.29 Nevertheless, we cannot exclude the existence of oxidative stress in AA/AG hypertensive subjects, which may be mediated by the triggering of other oxidative systems, including uncoupled endothelial NO synthase and xanthine oxidase.3,30 In addition, oxidative stress in hypertensive subjects may be also the consequence of a decrease in the activity of antioxidant mechanisms.31

Preactivated monocytes in hypertensive patients exhibit a higher production of cytokines and a greater adherence to the vascular wall25,32 and may play a key role in the oxidative stress–mediated pathogenesis of atherosclerosis.33 Thus, our data showing increased NADPH oxidase expression and activity in phagocytic mononuclear cells, including monocytes, from GG hypertensive patients suggest this genotype may constitute an important proatherogenic feature in these patients. This possibility is supported by findings demonstrating that the severity of atherosclerosis correlates with p22phox mRNA overexpression34 and that p22phox mutations are able to regulate NADPH oxidase activity in atherosclerosis.35 Further clinical studies are necessary to ascertain whether the −930A/G polymorphism is actually involved in the development of atherosclerosis in hypertensive patients.

Some limitations of the study should be acknowledged. First, the study was performed in a small population, so we are aware that additional studies, including larger numbers of subjects, should be performed to confirm the current results. Second, because PKC activity is upregulated in the vasculature in hypertension,12,21 it may be suggested that the NADPH oxidase–activity results in response to PMA, an agonist of PKC, had been provoked by the use of this stimulus. However, the values of NADPH oxidase activity in AA/AG hypertensive subjects were almost identical to those measured in AA/AG normotensive subjects. Thus, we can discard any significant contribution resulting from the stimulus used on the increased NADPH oxidase activity observed in GG hypertensive subjects. Third, although our findings show a greater impact of GG genotype on p22phox protein abundance than on p22phox mRNA levels in hypertensive subjects, it may be suggested that genetically determined small changes in p22phox mRNA levels may play a relevant role in the p22phox protein expression. In accordance with this, it has been reported that changes in p22phox phagocytic expression are regulated by posttranscriptional mechanisms.36

**Perspectives**

The findings presented here suggest that the p22phox gene −930A/G polymorphism determines p22phox expression and NADPH oxidase activity in phagocytic cells from patients with essential hypertension. Increased NADPH oxidase O2•− production might lead to a diminished NO production in GG hypertensive patients. Therefore, the interaction between GG genotype and factors linked to the hypertensive phenotype may be involved in cardiovascular damage mediated by NADPH oxidase–dependent oxidative stress in hypertensive patients.

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