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Forum Review

NADPH Oxidase-Mediated Oxidative Stress: Genetic Studies of the *p22phox* Gene in Hypertension

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ABSTRACT

Increased vascular production of reactive oxygen species, especially superoxide anion, significantly contributes to the oxidative stress associated with hypertension. An enhanced superoxide production causes an increased inactivation of nitric oxide that diminishes nitric oxide bioavailability, thus contributing to endothelial dysfunction and hypertrophy of vascular cells. It has been shown that NADPH oxidases play a major role as the most important sources of superoxide anion in phagocytic and vascular cells. Several experimental observations have described an enhanced superoxide generation as a result of NADPH oxidase activation in hypertension. Although these enzymes respond to stimuli such as vasoactive factors, growth factors, and cytokines, recent data suggest a significant role of the genetic background in the modulation of the expression of its different components. Several polymorphisms have been identified in the promoter and in the coding region of *CYBA,* the gene that encodes the essential subunit of the NADPH oxidase p22^{phox}, some of which seem to influence significantly the activity of these enzymes in the context of cardiovascular diseases. Among *CYBA* polymorphisms, genetic investigations have provided a novel marker, the −930$^{A/G}$ polymorphism, which determines the genetic susceptibility of hypertensive patients to oxidative stress. *Antioxid. Redox Signal.* 7, 1327–1336.

OXIDATIVE STRESS

THE TERM "OXIDATIVE STRESS" refers to the situation in which cells are exposed to high concentrations of molecular oxygen or its derivatives, also known as reactive oxygen species (ROS). ROS include free radicals, such as superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), nitric oxide ($\cdot NO$), and lipidic radicals, and other remarkably reactive molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (20, 45). Due to their aggressive oxidative action, ROS were considered to be mere toxic agents, as they oxidize macromolecules, including DNA, proteins, and lipids. In recent years, the pathophysiological role of ROS as second messengers has been highlighted in the literature.

The balance between redox homeostasis and oxidative stress is delicate. In physiological conditions, the changes in type and levels of ROS are finely regulated by the strict control of the enzymatic sources and by proper ROS-scavenging actions of antioxidant systems. Thus, it is due to the excess in ROS production and/or the lack of sufficient defense that ROS are notably increased in pathological situations. This redox imbalance leads to the plethora of disorders in which oxidative stress is known to be involved. ROS take part in aging and senescence, malignant processes, neurodegenerative diseases, rheumatoid arthritis, diabetes, and cardiovascular diseases, including atherosclerosis and hypertension (20).

The implication of oxidative stress is known in a wide number of models of cardiovascular disorders and human cardiovascular disease (29). For instance, H_2O_2 levels seem to be increased (49) and superoxide dismutase levels diminished (47) in human hypertension. Oxidative stress also participates in several of the mechanisms involved in atherosclerosis (34,

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48). Besides, clinical trials of antioxidants have had some success in the treatment of cardiovascular diseases (74, 80).

The cardiovascular system is a perfect example of the dual effect of ROS (30). At physiological levels, ROS participate in vascular and cardiac cell function, as they regulate processes ranging from gene control to cell-to-cell interactions, including vascular apoptosis, expression of adhesion molecules, matrix regulation, and migration (38, 81). Two of the key vascular processes regulated by oxidative stress in hypertension and atherosclerosis are the control of vessel tone and the control of vascular cell growth. Therefore, pathological levels of ROS lead to the imbalance of cell function and cause, among others, endothelial dysfunction and vascular hypertrophy.

The depletion of ·NO, the main vascular relaxing factor, by its reaction with $\cdot O_2^-$ leads to an impaired relaxation of the vessel, characteristic of endothelial dysfunction (10, 45). Besides, the $ONOO^-$ generated by their reaction is another potent oxidant (6). On the other hand, H_2O_2, product of the dismutation of $\cdot O_2^-$, constitutes a key signal for vascular hypertrophy (38, 55).

The sources of ROS are diverse, present in many cells and tissues. They include the mitochondrial electron transport system, cytochrome P450, lipoxygenase, cyclooxygenase, peroxidases, hemoproteins, xanthine oxidase, ·NO synthase, and NADPH oxidases (45). Nonetheless, in the last decade, the study of vascular oxidative stress has provided multiple evidences of the critical involvement of the NADPH oxidase system in the molecular basis of cardiovascular diseases, including hypertension (31, 93).

THE NADPH OXIDASE SYSTEMS

This membrane-associated enzyme system was first described in phagocytic cells, where it plays a role in immune protection due to its bactericidal activity (13). However, it is also present in vascular cells [reviewed by R. Touyz in this issue (82)], where its function is not well known, although it has been hypothesized to play a role in host defense, signal transduction, oxygen sensing, and metabolism (50). These phagocytic and vascular NADPH oxidases are related, but have structural and functional differences. The NADPH oxidase was well characterized in phagocytic cells, where there are two membrane-bound elements ($gp91^{phox}$/NOX-2 and $p22^{phox}$), three cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), and a low-molecular-weight G protein (rac1 or rac2) (13). The vascular NADPH oxidase is similar to the phagocytic oxidase, but there is controversy about the expression of several of its components, depending on cell type and species considered (Table 1). Besides, in the last years, several homologues have been discovered for these subunits (82). These structural differences among vascular and phagocytic NADPH oxidases could help explain their functional differences. In this way, phagocytic oxidases have an inducible activity with a large, extracellular $\cdot O_2^-$ production, whereas vascular oxidases are constitutive enzymes that produce small amounts of $\cdot O_2^-$ (52).

Several events take part in the phagocytic NADPH oxidase activation, such as phosphatidylinositol phosphates accumulation, interactions with cytoskeleton components, and phosphorylation of several subunits (13). The activation of vascular NADPH oxidases is not as well known as that of the phagocytic oxidase, but the mechanism is likely to be similar. In fact, $p47^{phox}$ phosphorylation is key to activation of phagocytic and vascular NADPH oxidases (13, 52). Several signaling pathways activate both the phagocytic and the vascular NADPH oxidases, such as protein kinase C activation, phospholipase D pathway, and phospholipase A_2 pathway (82).

Although vascular NADPH oxidases are constitutive enzymes, several stimuli, including humoral factors and hemodynamic forces, have been shown to regulate their activity (82). On the other hand, phagocytic NADPH oxidase is activated, among others, by nonopsonized zymosan, phorbol myristate acetate, tumor necrosis factor-α (TNF-α), interferon-γ, and interleukin-15 (22, 66).

NADPH OXIDASES IN HYPERTENSION

Nowadays, the central role of NADPH oxidases in cardiovascular diseases such as hypertension (53, 93), diabetes (33, 36), atherosclerosis (34), and cerebrovascular disease (65) is widely known. This role has been determined in experimental

TABLE 1. EXPRESSION OF NADPH OXIDASE COMPONENTS

Components	*Tissues or cell types*	*References*
Nox1	Colon, VSMCs, ECs, fibroblasts	42, 43, 78, 87
$Nox2/gp91^{phox}$	Neutrophils, macrophages, ECs, VSMCs, fibroblasts, cardiomyocytes	5, 13, 41, 46, 87
Nox 3	Fetal kidney, lung, spleen, and liver	3, 11
Nox 4	Kidney, heart, osteoclasts, pancreas, placenta, VSMCs	3, 11, 27, 42, 77, 78, 90
Nox 5	Testis, spleen, lymph nodes, ovary, placenta, pancreas, human VSMCs	2, 3, 11
Duox1	Thyroid	16, 21
Duox2	Thyroid, colon	16, 21
$p22^{phox}$	Neutrophils, macrophages, VSMCs, ECs, fibroblasts	5, 13, 24, 78, 84
$p47^{phox}$	Neutrophils, macrophages, VSMCs, ECs, fibroblasts	1, 4, 13, 64, 84
$p41^{nox}$/NOXO1	Colon	28
$p67^{phox}$	Neutrophils, macrophages	13, 54, 64, 84
$p51^{nox}$/NOXA-1	Colon	28
p40	Neutrophils, macrophages, VSMCs, ECs, fibroblasts	13, 54, 64, 84
rac	Neutrophils, macrophages, VSMCs, ECs	13, 54, 68

ECs, endothelial cells.

models of disease, as well as in human diseases. It is important to note that in cardiovascular diseases, not only the vascular oxidase, but also the phagocytic NADPH oxidase, plays an important role in $\cdot O_2^-$ production, because monocytes and lymphocytes can infiltrate cardiovascular tissues (73).

Several works have established the role of the vascular NADPH oxidases in the development and progression of hypertension in numerous animals. Chronic infusion of angiotensin II (Ang II) in rats resulted in hypertensive animals, in correlation with an increased NADPH oxidase-derived $\cdot O_2^-$ generation and up-regulated p22phox mRNA levels (25). Enhanced $\cdot O_2^-$ production present in the aorta of DOCA-salt hypertensive rats is associated with increased NADPH oxidase activity and p22phox expression (7). Finally, we reported an enhanced NADPH oxidase-driven $\cdot O_2^-$ production associated with an up-regulated p22phox mRNA expression in the aorta of adult spontaneously hypertensive rats (SHR), which present endothelial dysfunction and vascular wall hypertrophy (91). In the same work, we demonstrated that activated NADPH oxidase in the aorta of SHR depended on humoral and hemodynamic factors.

Although there is little information concerning the role of NADPH oxidases in human hypertension, some studies have demonstrated its implication. Recently, Ang II-induced $\cdot O_2^-$ production has been described to be increased in vascular smooth muscle cells (VSMCs) isolated from hypertensive patients (83). Pettit *et al.* have shown that the NADPH oxidase-dependent $\cdot O_2^-$ production is increased in lymphoblasts derived from hypertensive subjects (69). Furthermore, we have described the increased $\cdot O_2^-$ production, dependent on NADPH oxidase, in stimulated mononuclear cells extracted from hypertensive patients (23).

Among the different components of the NADPH oxidase systems, p22phox emerges as an essential subunit for NADPH oxidase activity. Ushio-Fukai *et al.* demonstrated that the use of p22phox antisense technology prevented the increased NADPH oxidase-mediated $\cdot O_2^-$ production induced by Ang II (86). Interestingly, many of the stimuli that activate vascular and phagocytic NADPH oxidases also increase p22phox expression. Among humoral factors, Ang II, TNF-α, interferon-γ, and glucose are noteworthy (12, 17, 22, 25). Hemodynamic factors such as cyclic strain are also able to increase p22phox expression (59).

In summary, these studies suggest that p22phox overexpression may be a critical determinant of NADPH oxidase activation. It is important to note that, in addition to humoral and hemodynamic factors, the genetic background may also play an important role in the regulation of the NADPH oxidase systems (Fig. 1).

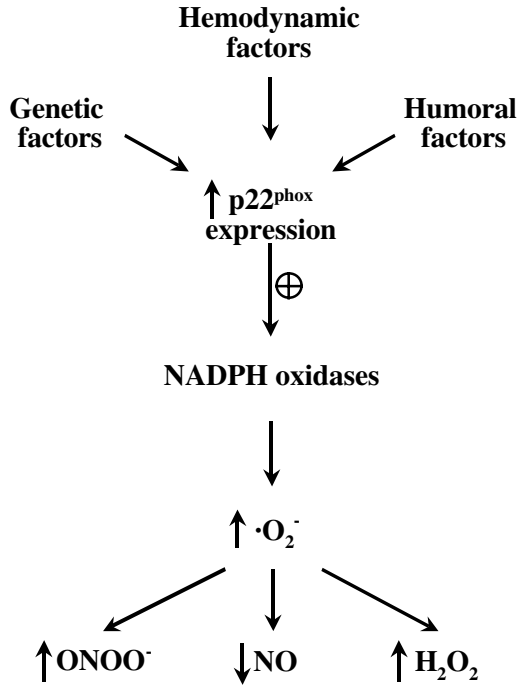

FIG. 1. NADPH oxidase activation and functional consequences in arterial hypertension. Besides multiple humoral agonists and hemodynamic forces, genetic changes may be involved in the activation of the NADPH oxidase systems by modulating the expression of NADPH oxidase subunits, including the p22phox protein. An enhanced $\cdot O_2^-$ anion production driven by NADPH oxidase activation is involved in endothelial dysfunction by decreasing ·NO bioavailability and by increasing $ONOO^-$ generation, and in media hypertrophy through the accumulation of H_2O_2.

p22phox GENE POLYMORPHISMS

During the past decade, the identification of NADPH oxidases as the major source of phagocytic and vascular $\cdot O_2^-$ in cardiovascular diseases has provoked a significant interest in the possible association of polymorphisms in genes encoding NADPH oxidase subunits with cardiovascular disease susceptibility. A particular interest has been shown for the gene encoding the p22phox subunit, which possesses a significant number of genetic polymorphisms within the promoter and coding sequence, some of which are able to influence gene expression and NADPH oxidase activity.

p22phox *gene in genetic hypertension*

Among the different studies performed in experimental hypertension, our group has demonstrated that increased p22phox expression correlated with activation of NADPH oxidase enzyme in the aorta of SHR (91). As SHR is a genetic model of hypertension, up-regulation of the oxidase p22phox subunit in SHR might be a consequence of alterations in the sequence of the *p22phox* gene (93). In line with this hypothesis, we characterized the 5′-end of the rat *p22phox* gene and identified five polymorphisms in this region of the SHR *p22phox* gene, one polymorphism located in the nontranslated region of exon 1 (+42), and four polymorphisms situated within the promoter region (−14, −166, −218, and −1,628). Interestingly, the higher promoter activity exhibited by the polymorphic promoter of the SHR *p22phox* gene evidenced a functional role for these allelic variants, thus suggesting that these polymorphisms might be involved in overexpression of the *p22phox* gene in the vascular wall of the SHR (92) (Fig. 2).

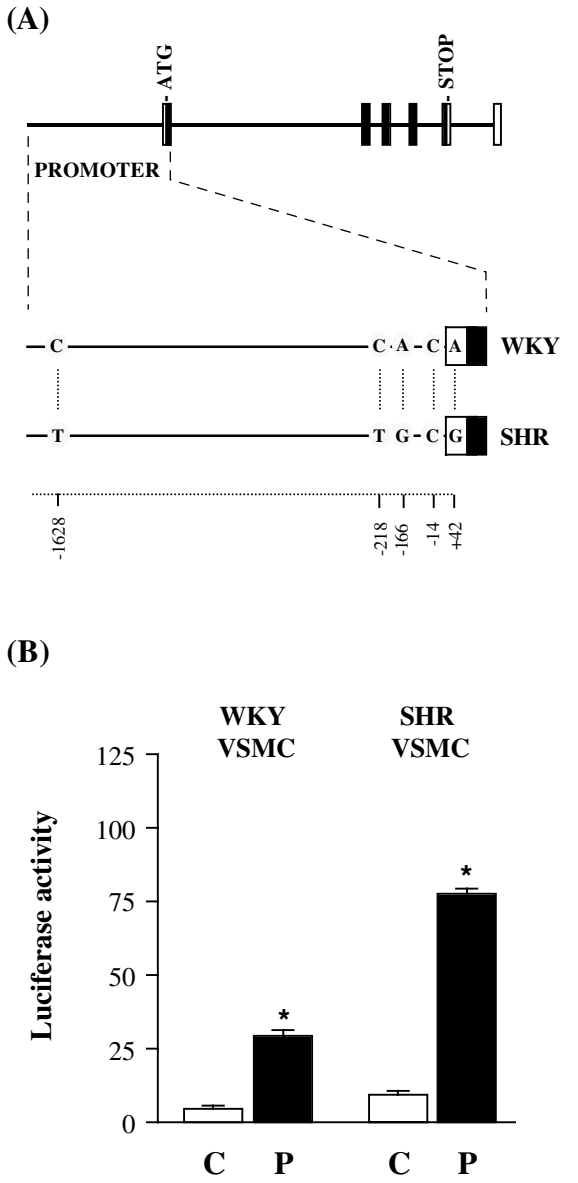

FIG. 2. Influence of gene polymorphisms on *p22phox* gene promoter activity. (**A**) Identification of the polymorphic variants in WKY and SHR *p22phox* gene promoters. ATG represents the translation initiation codon. (**B**) Transfection experiments with the SHR polymorphic promoter (P) and the WKY control promoter (C) into VSMCs from WKY and SHR. Histograms express relative luciferase activity of the p22phox promoter. $^{*}p < 0.05$ compared with WKY control promoter (adapted from reference 92).

CYBA *gene in human hypertension*

CYBA, the human gene that encodes the p22phox protein, is situated on chromosome 16q24 (18, 67), and possesses several polymorphic regions both in the promoter and in the coding sequence (14). Some of these polymorphic sites (Fig. 3) have been associated with a number of cardiovascular diseases, such as hypertension, coronary artery disease (CAD), and myocardial infarction, all of which associate with an impaired endothelium-dependent vasodilation (45). It has been suggested that polymorphisms in the *CYBA* gene reduced the ability of p22phox subunit to anchor gp91phox, thus conditioning the stability of the b558 cytochrome and altering the NADPH oxidase activity (35).

A640G polymorphism. The A640G gene polymorphism is located in the 3′-untranslated region of *CYBA* with no frank amino acid substitution (15, 71). It has been suggested that this genetic variant might modify mRNA processing and stability, thus influencing p22phox biosynthesis.

Gardemann *et al.* showed that the association of the A640G polymorphism with the presence and extent of CAD was stronger in hypertensives than in normotensives (26). Thus, a role for *CYBA* polymorphisms in NADPH oxidase activity can be hypothesized, contributing to the development of atherosclerosis in essential hypertension.

C242T polymorphism. The C242T polymorphism is located in exon 4, at position 242, of *CYBA* (18). It generates a CAC-TAC codon change, which results in a nonconservative substitution of histidine-72 by a tyrosine residue in the heme-binding site of the p22phox protein. It has been suggested that this replacement leads to a loss of oxidative function and to a reduced production of ROS and oxidative stress in the vasculature (88).

The association of the *CYBA* C242T polymorphism with atherosclerosis has been studied previously, although the results have been conflicting. Inoue *et al.* found that the T allele conferred protection against atherosclerosis in a Japanese population (37). However, other studies either did not find any association of the C242T polymorphism with atherosclerotic disease or even showed the opposite effect, that the T allele is significantly associated with progression of atherosclerosis (9) and cerebrovascular disease (39).

In the past decade, a functional role for C242T polymorphism in NADPH oxidase activity in physiological and pathophysiological conditions has been suggested. Guzik *et al.* demonstrated that 242T allele is associated with reduced vas-

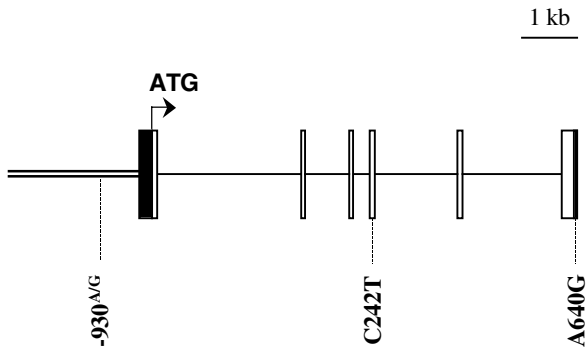

FIG. 3. Identification of the *CYBA* polymorphisms associated with cardiovascular diseases. Boxes represent exons, single lines indicate introns, and the double line corresponds to the promoter region. An arrow points to the translation initiation ATG codon in exon 1. Black and white boxes indicate the nontranslated and the translated regions of exons, respectively.

cular NADPH oxidase activity in saphenous veins of CAD patients, independently of other clinical risk factors (32). More recently, Wyche *et al.* have shown that the NADPH oxidase-dependent phagocytic respiratory burst activity in homozygous healthy individuals with the T allele is significantly lower than that of wild-type carriers and heterozygous healthy individuals (89).

In agreement with these data, further studies have elucidated a significant impact of the C242T polymorphism on oxidative stress and endothelial function. Recently, Stanger *et al.* found no significant difference among genotypes in serum concentrations of malondialdehyde, an end product of lipid peroxidation (79). In contrast, Nakano *et al.* have demonstrated a diminished circulating concentration of oxidized high-density lipoprotein in CT/TT genotypes compared with CC genotypes in type 2 diabetic patients (63). Moreover, Schächinger *et al.* showed that carriers of the CC genotype revealed a significantly blunted endothelium-dependent dilator response, which was independent of other risk factors or atherosclerosis (76). Collectively, the association of the C242T polymorphism with NADPH oxidase activity and endothelial function implies this genetic variant might be a relevant clinical marker of CAD.

On the other hand, Raijmakers *et al.* reported a lack of association between the C242T polymorphism and preeclampsia (72). Nevertheless, in a recent study, we have found a significant association of the C242T polymorphism with essential hypertension in a Spanish population (95). Moreover, we have found that phagocytic NADPH oxidase activity was significantly higher in CC than in TC/TT hypertensives, whereas no differences were found between genotypes in the normotensive control population, suggesting that the effects of C242T polymorphism may become evident in the presence of cardiovascular risk factors.

−930$^{A/G}$ polymorphism. The −930$^{A/G}$ polymorphism is located in the promoter region of *CYBA*, and it has been speculated to have a functional effect on the modulation of *CYBA* transcriptional activity.

The association of the *CYBA* −930$^{A/G}$ polymorphism with hypertension has been studied recently. Moreno *et al.* found that the −930$^{A/G}$ polymorphism was associated with hypertension in a Spanish population (61). Frequencies of the GG, GA, and AA genotypes were 0.34, 0.43, and 0.23 for the normotensive group and 0.41, 0.47, and 0.12 for the hypertensive group. In line with these results, San José *et al.* have recently demonstrated that the −930^{GG} genotype is associated with increased p22^{phox} expression and NADPH oxidase activity in phagocytic cells of hypertensive patients (Fig. 4) (75). In contrast, no dif-

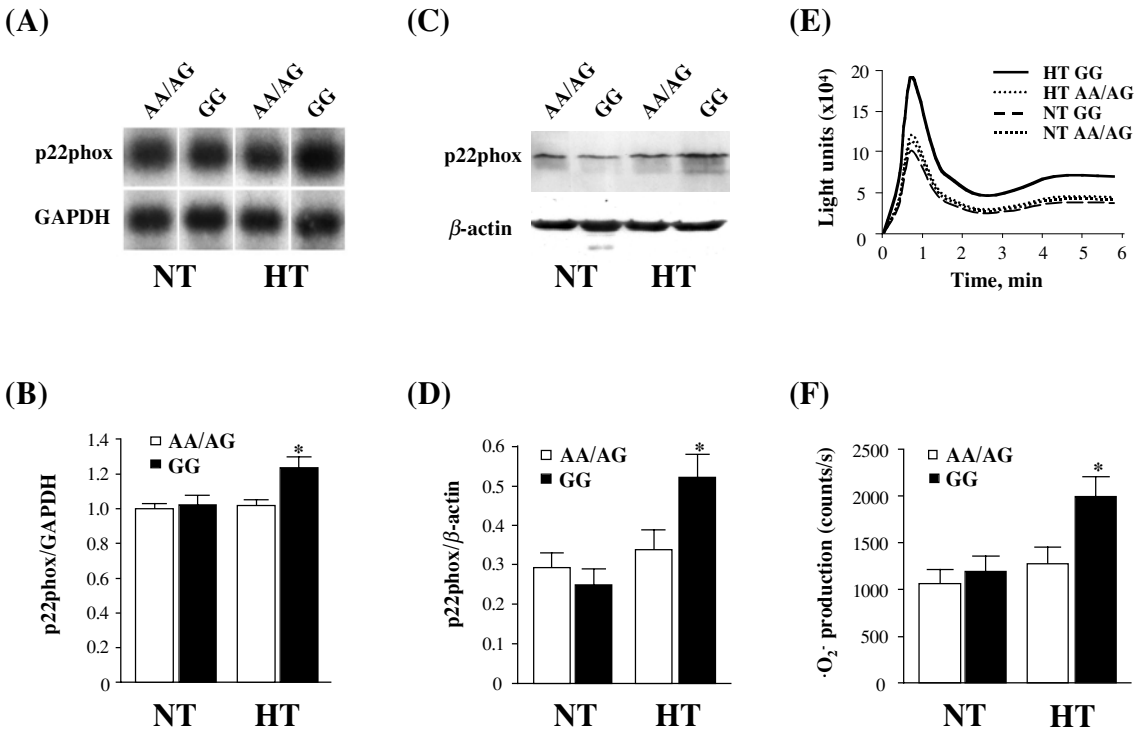

FIG. 4. Effect of the −930$^{A/G}$ polymorphism on p22^{phox} expression and NADPH oxidase activity. (**A**) The expression of phagocytic p22^{phox} mRNA was adjusted for the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**B**) Estimation of the p22^{phox} mRNA expression. $^{*}p < 0.05$ compared with AA/AG hypertensives (HT) and with AA/AG and GG normotensives (NT). (**C**) The expression of phagocytic p22^{phox} protein was adjusted for the expression of the housekeeping gene β-actin. (**D**) Estimation of the p22^{phox} protein expression. $^{*}p < 0.05$ compared with AA/AG hypertensives and with AA/AG and GG normotensives. (**E**) $\cdot O_2^-$ production was determined by lucigenin chemiluminescence after phorbol myristate acetate stimulation. (**F**) Estimation of the NADPH oxidase activity. $^{*}p < 0.05$ compared with AA/AG hypertensives and with AA/AG and GG normotensives (adapted from reference 75).

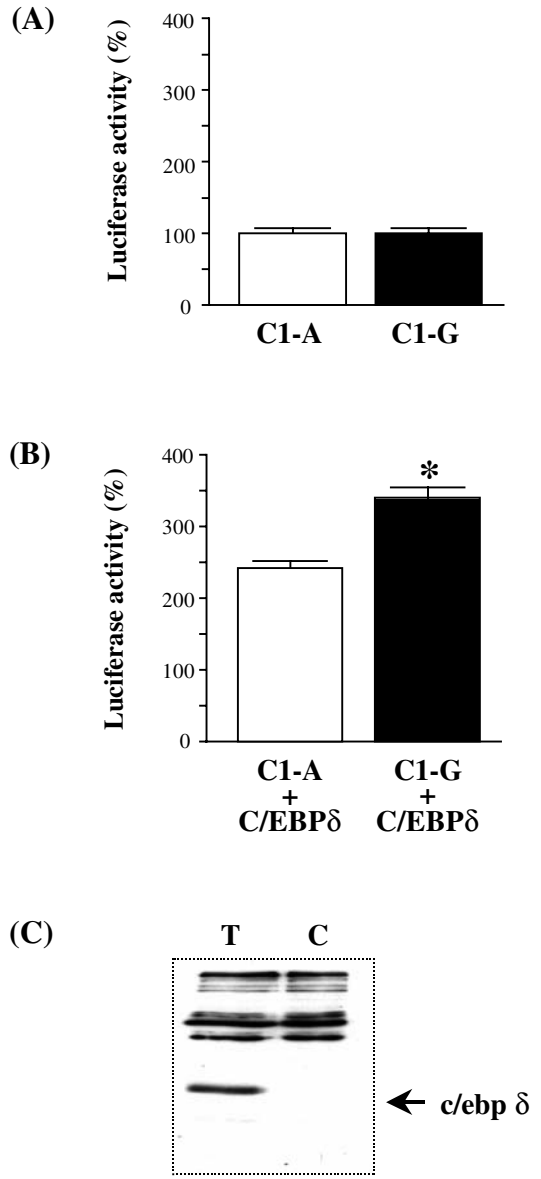

FIG. 5. Reporter gene expression assays with *CYBA* promoter allelic constructs. (**A**) Changes in luciferase levels induced by the A-to-G substitution at the −930 polymorphic site in A7r5 cells. C1-A, A allelic construct; C1-G, G allelic construct. (**B**) Effect of C/EBPδ cotransfection on luciferase levels of $p22^{phox}$ promoter allelic constructs in A7r5 cells. $^{*}p < 0.05$. (**C**) Western blot experiment shows a positive immunodetection of C/EBPδ protein in C/EBPδ-transfected A7r5 cells. C, control A7r5 cells; T, C/EBPδ-transfected A7r5 cells. An arrow points to the expressed protein band of 35 kDa (adapted from reference 75).

ferences were found in the NADPH oxidase expression and activity between genotypes within the normotensive group. In accordance with these *in vivo* measurements, *in vitro* experiments showed that the A-to-G substitution results in an increased *CYBA* gene promoter activity in hypertensive, but not in normotensive, VSMCs. In agreement with this, Wyche *et al.* have shown no differences in NADPH oxidase-dependent phagocytic respiratory burst activity between $-930^{A/G}$ polymorphism genotypes in healthy young individuals (89). Collectively, these data suggest that the $-930^{A/G}$ polymorphism may be functionally relevant in the control of the *CYBA* gene expression 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 *$p22^{phox}$* gene. In this regard, an analysis of the promoter sequence shows that the −930 polymorphic site lies on a potential binding site for CCAAT enhancer-binding protein (C/EBP) transcription factors. Furthermore, our results showing the effect of C/EBPδ overexpression on higher transcriptional activity of the G than of the A allelic $p22^{phox}$ promoter construct allow us to suggest the involvement of C/EBPs in the hypertensive phenotype (Fig. 5). Several findings that underlie the role of C/EBPs in hypertension support this possibility. C/EBPδ expression is nearly absent in Wistar Kyoto rat (WKY) VSMCs, whereas it is abnormally increased in SHR VSMCs (44). Changes in angiotensinogen mRNA expression associated with the $-217^{A/G}$ polymorphism of this gene are regulated by several members of the C/EBP family (40). Inflammatory cytokines up-regulate C/EBP expression (70), and cytokine levels have been reported to be increased in phagocytic cells in hypertensives (19).

Alternatively, the G allele may be in linkage disequilibrium with other genetic variants that could influence the transcriptional activity of *CYBA*. In this regard, we have identified other genetic variants in the $p22^{phox}$ promoter. One of them, the $-675^{A/T}$ polymorphism, is associated with hypertension, although its functional implication in NADPH oxidase activity remains to be elucidated (62).

The impact of the $-930^{A/G}$ polymorphism on oxidative stress and endothelial function is currently being elucidated. Recent research demonstrated the involvement of the NADPH oxidases in the uncoupling of the endothelial ·NO synthase,

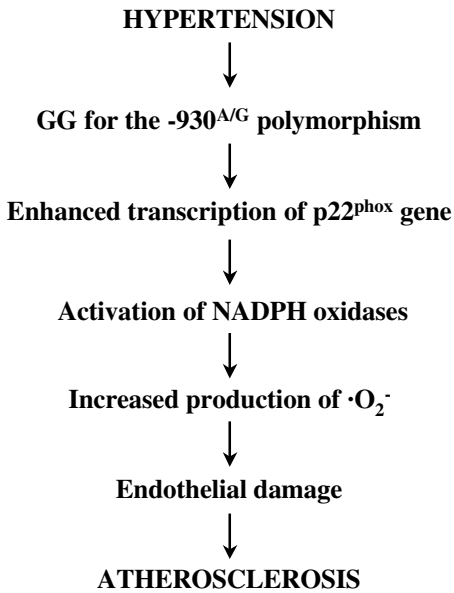

FIG. 6. The $-930^{A/G}$ polymorphism as a genetic marker of oxidative stress in hypertension.

which favors the diminished ·NO generation in hypertensive animals (51). Previous studies reported reduced levels of ·NO metabolites (nitrate and nitrite) in the hypertensive population (57, 85). Interestingly, the higher $\cdot O_2^-$ generation observed in -930^{GG} hypertensives associated with a diminished production of systemic ·NO. Finally, GG hypertensives associated with higher plasma levels of von Willebrand factor (94). As increased levels of von Willebrand factor have been found to be associated with the extent of damage in the vascular endothelium and increased subsequent occurrence of atherosclerotic cardiovascular events (8, 56, 58, 60), these findings suggest that the $-930^{A/G}$ polymorphism may be a novel genetic marker of endothelial damage and of risk of atherosclerosis in essential hypertension (Fig. 6).

CONCLUSION AND PERSPECTIVES

Increased $\cdot O_2^-$ generation is an important feature of the vascular phenotype in human hypertension, as it affects ·NO bioavailability, $ONOO^-$ generation, and redox-sensitive cell-signaling pathways. The NADPH oxidases are important sources of $\cdot O_2^-$ in human vessels. Genetic factors, including polymorphic variants in the genes encoding the NADPH oxidase subunits, modulate $\cdot O_2^-$ production, influencing hypertension and the progression of cardiovascular disease. The identification of functional polymorphisms in *CYBA*, associated with a higher NADPH oxidase-dependent $\cdot O_2^-$ production, could represent another criterion to bear in mind for the adequate characterization of patients with a genetically increased susceptibility to oxidative stress, which would constitute an appropriate population for therapeutic interventions with antioxidants.

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ABBREVIATIONS

Ang II, angiotensin II; CAD, coronary artery disease; C/EBP, CCAAT enhancer-binding protein; H_2O_2, hydrogen peroxide; ·NO, nitric oxide; $\cdot O_2^-$, superoxide anion; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells; WKY, Wistar Kyoto rats.

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