

NADPH Oxidase–Dependent Superoxide Production Is Associated With Carotid Intima-Media Thickness in Subjects Free of Clinical Atherosclerotic Disease

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

Objective—Oxidative stress plays a critical role in the pathogenesis of atherosclerosis. The NADPH oxidase constitutes the main source of superoxide in phagocytic and vascular cells. This study aimed to investigate the levels of NADPH oxidase–mediated superoxide production in phagocytic cells and the association between phagocytic superoxide production and carotid intima-media thickness (IMT), a surrogate marker of asymptomatic atherosclerosis.

Methods and Results—NADPH oxidase–mediated superoxide production was determined by a chemiluminescence assay using lucigenin and associated with IMT for 184 asymptomatic subjects free of overt clinical atherosclerotic disease. Compared with individuals in the lowest tertile of superoxide production, those in the upper tertile (>20 counts/sec) showed significantly higher IMT ($P<0.05$). In correlation analysis, a positive relationship was found between superoxide production and carotid IMT. Superoxide production also correlated positively ($P<0.05$) with body mass index (BMI). In multivariate analysis, the association of superoxide production with carotid IMT remained significant after adjustment for age, sex, systolic blood pressure, BMI, triglycerides, glucose, and smoking.

Conclusions—In a population sample of adults without clinically overt atherosclerotic disease, increased NADPH oxidase activity was associated with enhanced carotid IMT, suggesting a relationship between phagocytic NADPH oxidase–mediated oxidative stress and the development of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2005; 25:1452-1457.)

Key Words: atherosclerosis ■ carotid arteries ■ intima-media thickness ■ NADPH oxidase ■ superoxide

Oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the former, plays a significant role in the pathogenesis of atherosclerotic vascular disease.¹ Traditionally, it has been accepted that reactive oxygen species (ROS) are involved in low-density lipoprotein oxidation, a key step in the initiation and progression of atherosclerosis.² More recently, ROS have been also implicated in other pathological processes in the vessel wall, including endothelial dysfunction, activation of matrix metalloproteinases, and vascular smooth muscle cell (VSMC) migration, growth, and apoptosis.³ Taken together, ROS play an integral role in the initiation, the progression, and the end event of the atherosclerotic process.

The NADPH oxidase systems, which constitute the most important source of superoxide ($O_2^{\cdot-}$) in the vessel wall, are present in endothelial cells, VSMCs, and fibroblasts.⁴ The structure of vascular NADPH oxidases is similar, although differs structurally and biochemically, from the phagocytic NADPH oxidase, an oxidase originally identified in the defense against exogenous microorganisms. The phagocytic oxidase is a membrane-bound enzyme that

catalyzes the single electron reduction of molecular oxygen to form $O_2^{\cdot-}$. It consists of a membrane-associated cytochrome b_{558} , and 3 cytosolic components $p47^{phox}$, $p67^{phox}$, and $rac1/2$. Cytochrome b_{558} comprises a large subunit, $gp91^{phox}$ (also called Nox2), and a smaller subunit, $p22^{phox}$, and it functions as the final electron transporter from NADPH to molecular oxygen.⁵ Although intensive investigations have been conducted to identify the vascular NADPH oxidases, their molecular characterization still remains unclear. In the past years, 2 novel $gp91^{phox}$ /Nox2 homologues, Nox1 and Nox4, were identified in VSMCs and represent functional oxidases.^{6–9} More recently, it has been shown that Nox4 functions as the major catalytic component of endothelial NADPH oxidase.¹⁰

In the past years, several works have demonstrated a key role of vascular NADPH oxidase isoforms in the development of human atherosclerosis.^{11–15} Interestingly, it has been also reported the contributing role of the infiltrated monocyte NADPH oxidase in the development of the atherosclerotic lesion.^{13–15} It is traditionally accepted that ROS generation from infiltrated monocytes contributes to atherosclerotic le-

Original received December 1, 2004; final version accepted April 7, 2005.

From the Area of Cardiovascular Pathophysiology, Centre for Applied Medical Research (G.Z., G.S.J., M.U.M., A.F., J.D.), and the Departments of Internal Medicine (O.B.) and Cardiology and Cardiovascular Surgery (J.D.), University Clinic, School of Medicine, University of Navarra, Pamplona, Spain.

Correspondence to Dr Guillermo Zalba, Área de Fisiopatología Cardiovascular, Centro de Investigación Médica Aplicada, Avda Pío XII 55, 31008 Pamplona, Spain. E-mail gzalba@unav.es

© 2005 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000168411.72483.08

sion formation.¹⁶ With this background, it might be suggested that besides a major role of vascular NADPH oxidases, phagocytic NADPH oxidase could play a significant role in the development and progression of atherosclerotic lesion.

Available evidence substantiates that carotid intima-media thickness (IMT), as measured by B-mode ultrasound, correlates with the presence of coronary atherosclerosis¹⁷ and represents an independent risk factor for atherothrombotic events (ie, coronary heart disease events, stroke, and transient cerebral ischemia).^{18–20} Thus, carotid ultrasound examination and IMT measurement can provide a useful surrogate marker for atherosclerotic disease.²¹ We have hypothesized that an association may exist between phagocytic NADPH oxidase activity and atherosclerosis in asymptomatic subjects. To test this hypothesis, we have investigated the relationship between NADPH oxidase-mediated $O_2^{\cdot-}$ production in phagocytic cells and carotid IMT in middle-aged adults with no past or current medical history of atherosclerotic disease.

Patients and Methods

The study was performed in 184 consecutive apparently healthy individuals (81% men, mean age 53.9 years) referred to our institution for global cardiovascular risk assessment. Subjects were free from clinically apparent atherosclerotic disease based on: (1) absence of history of coronary disease, stroke, or peripheral artery disease; and (2) normal electrocardiogram and chest-x-ray results. Coronary heart disease was defined by: (1) self-reported myocardial infarction, angina, or use of nitroglycerin; and (2) self-reported history of coronary angioplasty or coronary artery bypass surgery. Cerebrovascular disease was defined as self-reported stroke, transient ischemic attack, or carotid endarterectomy. Symptoms of intermittent claudication were queried in a questionnaire, together with the physician interview. Patients were also excluded if they had advanced carotid atherosclerosis according to IMT measurements (>1.7 mm). Additional exclusion criteria were the presence of severely impaired renal function, arteritis, collagenosis, and a history of alcohol abuse. Patients with significant acute infection, according to clinical criteria by the attending physician, were also excluded. Written informed consent was obtained from all subjects, the study was performed in accordance with the Declaration of Helsinki, and the local committee on human research approved of the study protocol.

Assessment of Cardiovascular Risk Factors

The presence of cardiovascular risk factors such as diabetes mellitus, arterial hypertension, dyslipidemia, obesity, and smoking habits were also assessed (available online at <http://atvb.ahajournals.org>).

Determination of $O_2^{\cdot-}$ Production

We measured $O_2^{\cdot-}$ production in peripheral mononuclear cells (lymphocytes and monocytes) isolated from blood samples with Lymphoprep, in response to stimulation with phorbol myristate acetate (PMA) (3.2×10^{-6} mol/L), and using $10 \mu\text{mol/L}$ lucigenin by a chemiluminescence method as previously described.²² In some experiments, the effect of $5 \mu\text{mol/L}$ diphenylene iodonium, a flavoprotein inhibitor, and 2.5×10^{-3} mol/L apocynin, a specific intracellular inhibitor of NADPH oxidase assembly, were studied. To verify the specificity of the lucigenin assay for $O_2^{\cdot-}$ generation in our model, the effect of superoxide dismutase (SOD) $10\,000$ U/mL, an enzymatic scavenger of $O_2^{\cdot-}$, was also examined. Although lucigenin concentration was low enough to avoid auto-oxidation, the measurements were validated against an independent measurement of $O_2^{\cdot-}$ production using SOD-inhibitable ferricytochrome *c* reduction (available online). The measurement of $O_2^{\cdot-}$ production using SOD-inhibitable ferricytochrome *c* reduction closely correlated with lucigenin measurements (Figure 1, available online at [\[ahajournals.org\]\(http://ahajournals.org\)\). In some subjects, phagocytic \$O_2^{\cdot-}\$ production was evaluated in response to angiotensin II and endothelin 1.](http://atvb.</p>
</div>
<div data-bbox=)

Measurement of Carotid IMT

Ultrasonography of the common carotid arteries was performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI). The measurement of IMT was made 1 cm proximal to the carotid bulb of each common carotid artery at plaque-free sites. For each individual, the IMT was determined as the average of near wall and far wall measurements of each common carotid artery. Subjects were examined by the same 2 certified sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in individuals who returned 2 weeks later for a second examination.²³ The intraobserver and interobserver coefficients of variation were 5% and 10%, respectively.

Statistical Analysis

Data are expressed as mean \pm SEM. Differences in the baseline characteristics of subjects classified according to tertiles of $O_2^{\cdot-}$ production, in the $O_2^{\cdot-}$ production of subjects classified according to quartiles of carotid IMT, and in the $O_2^{\cdot-}$ production of phagocytes in response to different agonists were evaluated by ANOVA followed by Tukey B post hoc test. The χ^2 analysis was used to search for differences for qualitative variables. Pearson correlation test was used to assess correlations between $O_2^{\cdot-}$ production and all continuous variables. Multivariate linear regression analysis was performed to evaluate factors related to carotid IMT and the possibility of interactions. All variables that were significantly correlated with carotid IMT in the univariate analysis were included in the model. The statistical analysis was performed with SPSS 11.0 for Windows.

Results

NADPH Oxidase-Dependent $O_2^{\cdot-}$ Production

The PMA-stimulated $O_2^{\cdot-}$ production was inhibited by diphenylene iodonium, a flavoprotein inhibitor, and by apocynin, a potent intracellular inhibitor of NADPH oxidase system more specific than diphenylene iodonium (Figure 1). Apocynin impedes the assembly of the p47^{phox} and p67^{phox} subunits with cytochrome b₅₅₈, and it does not have known inhibitory effects on the other potential enzymatic sources of ROS.²⁴ Furthermore, SOD, a scavenger of $O_2^{\cdot-}$, completely abolished the chemiluminescence induced by PMA stimulation. These results demonstrate that the enzymatic source of $O_2^{\cdot-}$ in phagocytic cells was the NADPH oxidase complex.

Angiotensin II ($0.1 \mu\text{mol/L}$) and endothelin 1 ($0.01 \mu\text{mol/L}$) significantly stimulated the phagocytic NADPH oxidase-mediated $O_2^{\cdot-}$ production (basal, 1.00 ± 0.04 counts/sec; angiotensin II, 1.78 ± 0.09 counts/sec; endothelin 1, 1.65 ± 0.06 counts/sec) (Figure 1).

$O_2^{\cdot-}$ Production: IMT Associations

The mean level of phagocytic $O_2^{\cdot-}$ production was 18.6 counts/sec (range, 0.1 to 130 counts/sec). Table 1 shows the demographic and clinical characteristics of subjects stratified by tertiles of phagocytic NADPH oxidase-mediated $O_2^{\cdot-}$ production. Subjects in the upper tertile of phagocytic $O_2^{\cdot-}$ production exhibited an increased carotid IMT compared with subjects in the medium and the lowest tertiles ($P < 0.05$). Moreover, subjects in the upper tertile also had higher systolic blood pressure, body mass index, glucose, and triglycerides than subjects in the lowest tertile ($P < 0.05$). The percentage of men was also significantly elevated in the

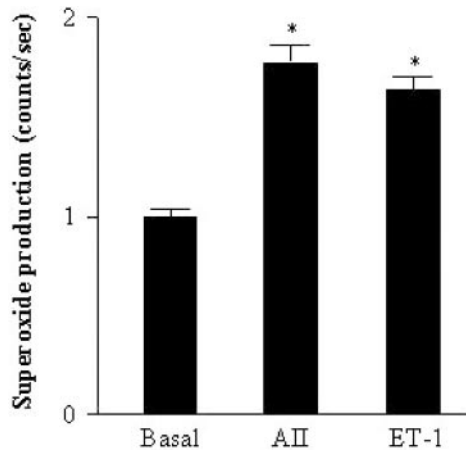


Figure 1. Effects of angiotensin II and endothelin 1 on the phagocytic NADPH oxidase-mediated $O_2^{\cdot-}$ production. Baseline and angiotensin II (0.1 $\mu\text{mol/L}$) and endothelin 1 (0.01 $\mu\text{mol/L}$)-stimulated $O_2^{\cdot-}$ generation in mononuclear cells obtained from subjects. Data are expressed as mean \pm SEM (n=14). * P <0.05 vs baseline.

upper tertile. No differences among tertiles were found for the remaining characteristics tested.

There was a significant positive bivariate correlation between phagocytic $O_2^{\cdot-}$ production and carotid IMT ($r=0.293$, $P<0.001$) in all the subjects. As shown in Table 2, the association between the 2 parameters remained highly significant ($r=0.248$, $P=0.001$) after controlling for age and sex. Phagocytic $O_2^{\cdot-}$ production was also significantly associated with triglycerides ($r=0.159$, $P<0.05$) and body mass index

TABLE 1. Baseline Characteristics of the Studied Population Stratified by Tertiles of NADPH Oxidase-Dependent $O_2^{\cdot-}$ Production

	Superoxide Production		
	<8 Counts/s (n=62)	8–20 Counts/s (n=66)	>20 Counts/s (n=56)
Age, y	52.3 \pm 1.5	54.4 \pm 1.4	55.3 \pm 1.7
Sex, M/F	49/13	58/8*	48/8*
BMI, kg/m ²	27.9 \pm 0.5	28.5 \pm 0.8	30.3 \pm 0.8*
SBP, mm Hg	124 \pm 2	136 \pm 2*	133 \pm 2*
DBP, mm Hg	81 \pm 2	83 \pm 1	82 \pm 1
Subjects who smoke, %	29	32	32
Hypertensive subjects, %	44	61	62
Diabetic subjects, %	11	12	32*†
Obese subjects, %	24	30	44
Glucose, mg/dL	98 \pm 2	98 \pm 2	106 \pm 3*†
Total cholesterol, mg/dL	223 \pm 6	225 \pm 5	223 \pm 6
HDL cholesterol, mg/dL	47 \pm 1	45 \pm 1	45 \pm 1
LDL cholesterol, mg/dL	152 \pm 5	152 \pm 5	151 \pm 5
Triglycerides, mg/dL	100 \pm 5	106 \pm 5	117 \pm 5*
Carotid IMT, mm	0.67 \pm 0.01	0.66 \pm 0.01	0.72 \pm 0.02*†

BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure.

* P <0.05 compared with the lowest tertile of $O_2^{\cdot-}$ production.

† P <0.05 compared with the middle tertile of $O_2^{\cdot-}$ production.

TABLE 2. Correlation Between $O_2^{\cdot-}$ Production and All Other Parameters Evaluated

	R	P	Rc	Pc
Age, y	0.119	0.108	—	—
BMI, kg/m ²	0.202	0.009	0.167	0.032
SBP, mm Hg	0.141	0.059	0.102	0.172
DBP, mm Hg	-0.024	0.747	-0.047	0.528
Glucose, mg/dL	0.133	0.072	0.096	0.195
Total cholesterol, mg/dL	-0.056	0.447	-0.064	0.388
HDL cholesterol, mg/dL	-0.096	0.202	-0.089	0.235
LDL cholesterol, mg/dL	-0.082	0.270	-0.086	0.248
Triglycerides, mg/dL	0.159	0.043	0.140	0.077
Carotid IMT, mm	0.293	<0.001	0.248	0.001

R and P values for bivariate correlations. Rc and Pc values of partial correlations after controlling for age and sex.

($r=0.202$, $P<0.01$), with the latter also remaining statistically significant after controlling for age and sex.

Carotid IMT significantly correlated with age ($r=0.404$, $P<0.001$), systolic blood pressure ($r=0.159$, $P<0.05$), glucose ($r=0.278$, $P<0.001$), body mass index ($r=0.240$, $P<0.005$), and triglycerides ($r=0.169$, $P<0.05$), with the 3 latter also remaining statistically significant after controlling for age and sex (Table 3).

Because of the associations found of phagocytic $O_2^{\cdot-}$ production and IMT with some cardiovascular risk factors, a further multivariate analysis was performed to assess the relationship between phagocytic $O_2^{\cdot-}$ production and carotid IMT after adjusting for these potential confounding factors (Table 4). A significant association between phagocytic $O_2^{\cdot-}$ production and IMT ($P=0.001$) remained after adjusting for all the potential confounding factors, with the $O_2^{\cdot-}$ production explaining up to 14.3% of the IMT variance after adjusting for the effects of common risk factors. Besides this association, we found that age also associated with IMT ($P=0.001$) independently of $O_2^{\cdot-}$ production.

The distribution of $O_2^{\cdot-}$ production by quartiles of carotid IMT showed a significant difference in $O_2^{\cdot-}$ production ($P<0.05$) between the quartiles of IMT (Figure 2); moreover,

TABLE 3. Correlation of the Carotid IMT With All Other Variables Investigated

	R	P	Rc	Pc
Age, y	0.404	<0.001	—	—
BMI, kg/m ²	0.240	0.002	0.200	0.010
SBP, mm Hg	0.159	0.032	0.139	0.854
DBP, mm Hg	0.088	0.234	0.056	0.455
Glucose, mg/dL	0.278	<0.001	0.193	0.009
Total cholesterol, mg/dL	-0.051	0.494	-0.074	0.320
HDL cholesterol, mg/dL	0.016	0.837	-0.013	0.859
LDL cholesterol, mg/dL	-0.092	0.214	-0.105	0.156
Triglycerides, mg/dL	0.169	0.031	0.159	0.044
$O_2^{\cdot-}$ production, counts/s	0.293	<0.001	0.248	0.001

P values for bivariate correlations. R and Pc values of partial correlations after controlling for age and sex.

TABLE 4. Correlation of the Carotid IMT With NADPH Oxidase-Dependent $O_2^{\cdot-}$ Production in Multiple Linear Regression Analysis

Independent Variables	<i>b</i>	<i>P</i> *	Partial R^2 , %
$O_2^{\cdot-}$ production, counts/sec	0.0019	0.001	14.3
Age, y	0.0046	0.001	12.1
Sex, F/M	-0.0289	0.268	0.9
BMI, kg/m ²	0.0033	0.167	0.6
Triglycerides, mg/dL	0.0003	0.251	0.2
SBP, mm Hg	-0.0002	0.761	0
Glucose, mg/dL	0.0002	0.635	0
Smokers, no/yes	0.0357	0.109	0

*Adjusted for age, sex, BMI, SBP, triglycerides, glucose, and smoking. R^2 for the total population was 28.1%

$O_2^{\cdot-}$ production was significantly increased in the third and the fourth quartiles compared with the first (quartile 1: 13.6 ± 1.5 counts/sec, $N=43$; quartile 2: 17.4 ± 2.1 counts/sec, $N=49$; quartile 3: 20.1 ± 2.5 counts/sec, $N=45$; quartile 4: 23.1 ± 3.9 counts/sec, $N=47$).

Discussion

In a population sample of middle-aged adults free of clinically evident atherosclerotic disease, the main finding of this study is that phagocytic NADPH oxidase-dependent $O_2^{\cdot-}$ production showed a significant correlation with carotid IMT, an index of subclinical atherosclerosis, independently of a wide range of important confounding variables, including some conventional cardiovascular risk factors. Oxidative stress mediated by NADPH oxidase systems plays an unfavorable effect on the thickening of the arterial wall, contributing to the initiation and development of the atherosclerotic lesion. Despite not having determined other sources of oxidative stress, our data suggest that phagocytic NADPH oxidase may account for part of these effects.

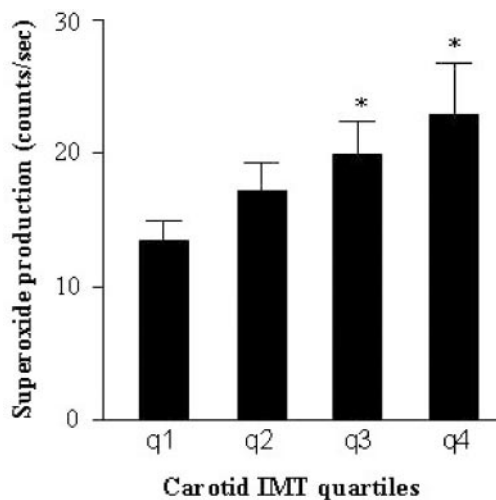


Figure 2. Phagocytic NADPH oxidase-dependent $O_2^{\cdot-}$ production according to the quartiles of carotid IMT. q1, IMT <0.6 mm; q2, IMT \geq 0.6 and <0.7 mm; q3, IMT \geq 0.7 and <0.8 mm; q4, IMT \geq 0.8 mm. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with q1; q indicates quartile.

Carotid IMT indicates the status of the vascular wall and is a useful method to study atherosclerotic disease. Carotid IMT has been correlated with cardiovascular risk factors, such as age, hypertension, hypercholesterolemia, and smoking, associated with the development of atherosclerosis in any vascular bed.²¹ Available evidence substantiates that carotid IMT correlates positively with coronary and peripheral atherosclerosis. Craven et al¹⁷ demonstrated that a measure of carotid IMT was strongly and independently associated with coronary artery disease in patients older than 50 years. Besides, carotid IMT represents an independent risk factor for atherothrombotic events.^{18–20} Moreover, an increase of 1 standard deviation in IMT measurement was associated with a 1.36 relative risk for the combined end point of myocardial infarction or stroke.²⁵ Taken together, these studies demonstrate that carotid IMT correlates with atherosclerosis and represents an independent risk factor for coronary heart disease events, stroke, and transient cerebral ischemia, thus providing a useful surrogate marker for atherosclerotic disease.²¹

The NADPH oxidase systems constitute the most important source of $O_2^{\cdot-}$ in the cells of the vessel wall.⁴ Vascular NADPH oxidase isoforms are similar, although they differ from the phagocytic NADPH oxidase system. In the past years, it has been reported a significant involvement of vascular NADPH oxidases in the pathophysiology of vascular wall. Overexpression of Nox homologues in fibroblasts suggests that Nox1 has mitogenic activity and is growth-promoting,⁶ whereas Nox4 decreased the rate of cell proliferation and is implicated in cellular senescence.⁷ In VSMC, Nox1 is upregulated by proliferative stimuli, such as angiotensin II and platelet-derived growth factor, whereas Nox4 is downregulated by these agonists²⁶ and by proinflammatory mediators, such as IL-1 β and thrombin.²⁷ In endothelial cells, Nox4 is upregulated by serum removal and downregulated by addition of serum.¹⁰ In a model of restenosis, the expression of Nox1, Nox2, and p22^{phox} is elevated early after injury, whereas Nox4 increases later,²⁸ coinciding with a reduction in the rate of VSMC proliferation. Inhibition of Nox2-dependent NADPH oxidase suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery.²⁹ Collectively, Nox1 and Nox2 are involved in acute response to injury or to proliferative stimulation, whereas Nox4 is involved in maintaining the quiescent phenotype. In contrast to Nox2 and Nox4, the importance of Nox1 in human vessels is less clear, because very low levels of expression have been detected in coronary arteries,¹⁴ and in mammary artery and saphenous vein.⁹

During the past decade, it has been shown an essential role of NADPH oxidases in the development of atherosclerosis. Guzik et al¹¹ showed that vascular NADPH oxidases are major sources of $O_2^{\cdot-}$ in human vessels and showed an association between enzymatic activity and clinical risk factors in atherosclerosis. Besides, this increase in NADPH oxidase activity impaired endothelium-dependent vasodilation in atherosclerotic patients. Azumi et al¹² found that the severity of atherosclerotic lesion correlated with p22^{phox} overexpression in coronary arteries. Sorescu et al¹⁴ demonstrated that Nox4 was increased in early lesions during the

atheroma stage of the plaque and decreased in more advanced stages of lesions in coronary arteries, whereas Nox2 and p22^{phox} were greatly increased along the progression of human atherosclerotic plaques, thus suggesting a possible causal link between the classic NADPH oxidase and the development of lesions. Interestingly, the contribution of Nox2 to lesion progression was caused almost entirely by infiltrated monocytes.^{13,14} In fact, preactivated monocytes exhibit higher cytokine production and greater adherence to the vascular wall,^{30,31} and play a role in the oxidative stress-mediated pathogenesis of atherosclerosis.³² Moreover, NADPH oxidase-derived O₂⁻ production from infiltrated monocytes may contribute to atherosclerotic lesion formation.¹⁶ Thus, our study showing a substantial increase in NADPH oxidase-dependent O₂⁻ formation in phagocytic cells from subjects with higher values of IMT adds new insights into the contributing role of the phagocytic NADPH oxidase system in the initiation and progression of human atherosclerosis. The significance of our data are underlined by a study showing that ROS production spatially associated with the distribution of p22 phox and oxidized low-density lipoprotein in atherosclerotic human coronary arteries.¹⁵ Although VSMCs and fibroblasts participated in ROS generation, these species were mainly generated by infiltrated inflammatory cells.¹⁵ Moreover, ROS production was significantly higher in unstable versus stable angina pectoris, which suggests that ROS might also modulate plaque stability.¹⁵ Collectively, these studies support the possibility that both phagocytic and vascular NADPH oxidase systems may play an essential role in the development and progression of atherosclerotic lesion. This hypothesis is underlined by a genetic study showing that deletion of the NADPH oxidase subunit p47^{phox} gene reduces the area of atherosclerosis in the descending aorta of apolipoprotein E^{-/-} knockout mice.³³

Our findings showing that phagocytic NADPH oxidase-dependent O₂⁻ production explained up to 14.3% of the carotid IMT variance after adjusting for the effect of common risk factors, suggest that vascular NADPH oxidase isoforms may be also participating in the atherosclerotic process.¹¹⁻¹⁵ Furthermore, we should take into account that several enzymatic origins have been proposed as sources of ROS in atherosclerotic process other than NADPH oxidases, such as lipoxygenase, xanthine oxidase, and nitric oxide synthase.³⁴ It has also been highlighted the impact of age as a major determinant of carotid IMT.³⁵ Likewise, the influence of age on carotid IMT was similarly strong (12.1%) in our survey, even after adjusting for phagocytic NADPH oxidase-mediated O₂⁻ production.

An important number of potential stimulating factors of the NADPH oxidase system may be related to increased phagocytic O₂⁻ production in the setting of cardiovascular diseases. Several humoral factors, including angiotensin II, endothelin 1, and some cytokines, stimulate the phagocytic NADPH oxidase activity.³⁶⁻³⁹ Likewise, our study reported that angiotensin II and endothelin 1 stimulated O₂⁻ production in phagocytic cells. In addition, several polymorphisms of *CYBA*, the human gene that encodes the p22^{phox} subunit, play a functional role on the NADPH oxidase activity.^{22,40,41}

Overall, our findings suggest that increased phagocytic NADPH oxidase activity may represent a potential indicator of oxidation occurring in the vascular wall of asymptomatic subjects. Because the present study does not provide direct evidence of the potential role of the vascular NADPH oxidase isoforms, it is important to point out that the increased phagocytic NADPH oxidase-dependent O₂⁻ production might not necessarily be the main culprit of the increased carotid IMT but may be simply a harbinger of the activation of vascular NADPH oxidase isoforms. Moreover, our data showing that phagocytes respond to the same circulating factors that are also directly stimulating the vascular NADPH oxidase homologues lead us to consider the possibility that phagocytic NADPH oxidase activation might be the reflection of the oxidation occurring in the vasculature.

One limitation of the study is that the cross-sectional analysis and the small sample size restrict our interpretation of the results, which represent a potential bias for complex multifactorial analysis. Because few women and no elderly individuals were included, results cannot be extrapolated to such populations at risk of cardiovascular disease. Another limitation is the lack of standardized ultrasound protocols for the measurement and interpretation of carotid IMT.²³ To obtain accurate measurements of carotid IMT, the same 2 certified sonographers performed ultrasound examination along our study. Finally, the unavailability of vascular tissue biopsies that would allow us to identify which NADPH oxidase system is in fact involved in the thickening of vascular wall constitutes another limitation.

In summary, we found that increased phagocytic NADPH oxidase-dependent O₂⁻ production is associated with enhanced carotid IMT in subjects without clinically overt atherosclerotic disease. Because carotid IMT correlates with atherosclerosis, our study supports that activation of phagocytic NADPH oxidase significantly associates with the atherosclerotic process in these subjects. The relevance of our findings is underlined by the significant role that oxidative stress plays in the initiation, progression, and the end event of the atherosclerotic process.

Acknowledgments

We gratefully acknowledge technical assistance by Raquel Ros and Ana Montoya. This project was funded through the agreement between FIMA and "UTE project CIMA," Foundation MMA, 55/2002 and 56/2002 from Department of Health of Government of Navarra, SAF2004-07910 from Ministry of Education and Science, and RECAVA (C03/01)/FIS/ISC from Ministry of Health of Spain.

References

- Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol.* 2003;91(3A):7A-11A.
- Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115-126.
- Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension.* 2003;42:1075-1081.
- Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase. Role in cardiovascular biology and disease. *Circ Res.* 2000;86:494-501.
- Bokoch GM, Knaus UG. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem Sci.* 2003;28:502-508.
- Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. Cell transformation by the superoxide-generating oxidase mox1. *Nature.* 1999;401:79-82.

7. Geiszt M, Kopp JB, Várnai P, Leto TL. Identification of Renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A*. 2000;97:8010–8014.
8. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2004;24:677–683.
9. Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, Korbut R, Channon KM. Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. *Arterioscler Thromb Vasc Biol*. 2004;24:1614–1620.
10. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation*. 2004;109:227–233.
11. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res*. 2000;86:E85–E90.
12. Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M. Expression of NADH/NADPH oxidase p22^{phox} in human coronary arteries. *Circulation*. 1999;100:1494–1498.
13. Kalinina N, Agrotis A, Tararak E, Antropova Y, Kanellakis P, Ilyinskaya O, Quinn MT, Smirnov V, Bobik A. Cytochrome b558-dependent NAD(P)H oxidase-phox units in smooth muscle and macrophages of atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2002;22:2037–2043.
14. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation*. 2002;105:1429–1435.
15. Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris. Important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2002;22:1838–1844.
16. Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2004;24:23–28.
17. Craven TE, Ryu JE, Espeland MA, Kahl FR, McKinney WM, Toole JF, McMahan MR, Thompson CJ, Heiss G, Crouse JR 3rd. Evaluation of the associations between carotid artery atherosclerosis and coronary artery stenosis. A case-control study. *Circulation*. 1990;82:1230–1242.
18. Bots ML, Hoes AW, Kondstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam study. *Circulation*. 1997;96:1432–1437.
19. Chambless LE, Folsom AR, Clegg LX, Sharrett AR, Shahar E, Nieto FJ, Rosamond WD, Evans G. Carotid wall thickness is predictive of incident clinical stroke: the Atherosclerosis Risk in Communities (ARIC) study. *Am J Epidemiol*. 2000;151:478–487.
20. Longstreth WT Jr., Shemanski L, Lefkowitz D, O'Leary DH, Polak JF, Wolfson SK Jr. Asymptomatic internal carotid artery stenosis defined by ultrasound and the risk of subsequent stroke in the elderly. The Cardiovascular Health Study. *Stroke*. 1998;29:2371–2376.
21. Mancini GB, Dahlof B, Diez J. Surrogate markers for cardiovascular disease: structural markers. *Circulation*. 2004;109(Suppl IV):22–30.
22. San Jose G, Moreno MU, Oliván S, Beloqui O, Fortuno A, Diez J, Zalba G. Functional effect of the p22^{phox}-930A/G polymorphism on p22^{phox} expression and NADPH oxidase activity in hypertension. *Hypertension*. 2004;44:163–169.
23. Martínez-Vila E, Páramo JA, Beloqui O, Irimia P, Colina I, Monreal I, Benito A, Barba J, Zubietta JL, Diez J. Independent association of fibrinogen with carotid intima-media thickness in asymptomatic subjects. *Cerebrovasc Dis*. 2003;16:356–362.
24. Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*. 1994;11:95–102.
25. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med*. 1999;340:14–22.
26. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res*. 2001;88:888–894.
27. Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc Res*. 2005;65:495–504.
28. Szocs K, Lassegue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol*. 2002;22:21–27.
29. Jacobson GM, Dourron HM, Liu J, Carretero OA, Reddy DJ, Andrzejewski T, Pagano PJ. Novel NAD(P)H oxidase inhibitor suppresses angioplasty-induced superoxide and neointimal hyperplasia of rat carotid artery. *Circ Res*. 2003;92:637–643.
30. Dörrfel Y, Lättsch C, Stuhlmüller B, Schreiber S, Scholze S, Burmester GR, Scholze J. Preactivated peripheral blood monocytes in patients with essential hypertension. *Hypertension*. 1999;34:113–117.
31. Hilgers KF. Monocytes/macrophages in hypertension. *J Hypertens*. 2002;20:593–596.
32. Dörrfel Y, Franz S, Pruss A, Neumann G, Rohde W, Burmester GR, Scholze J. Preactivated monocytes from hypertensive patients as a factor for atherosclerosis? *Atherosclerosis*. 2001;157:151–160.
33. Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET, Runge MS. p47^{phox} is required for atherosclerotic lesion progression in ApoE(−/−) mice. *J Clin Invest*. 2001;108:1513–1522.
34. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol*. 2005;25:29–38.
35. O'Leary DH, Polak JF, Kronmal RA, Kittner SJ, Bond MG, Wolfson SK Jr., Bommer W, Price TR, Gardin JM, Savage PJ. Distribution and correlates of sonographically detected carotid artery disease in the Cardiovascular Health Study. The CHS Collaborative Research Group. *Stroke*. 1992;23:1752–1760.
36. El Bekay R, Alvarez M, Monteseirin J, Alba G, Chacon P, Vega A, Martin-Nieto J, Jimenez J, Pintado E, Bedoya FJ, Sobrino F. Oxidative stress is a critical mediator of the angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF- κ B. *Blood*. 2003;102:662–671.
37. Liu J, Yang F, Yang XP, Jankowski M, Pagano PJ. NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. *Arterioscler Thromb Vasc Biol*. 2003;23:776–782.
38. Cassatella MA, Bazzoni F, Amezaga MA, Rossi F. Studies on the gene expression of several NADPH oxidase components. *Biochem Soc Trans*. 1991;19:63–67.
39. Fortuño A, Oliván S, Beloqui O, San José G, Moreno MU, Díez J, Zalba G. Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide production in essential hypertension. *J Hypertens*. 2004;22:2169–2175.
40. Moreno MU, San Jose G, Orbe J, Paramo JA, Beloqui O, Diez J, Zalba G. Preliminary characterisation of the promoter of the human p22^{phox} gene: identification of a new polymorphism associated with hypertension. *FEBS Lett*. 2003;542:27–31.
41. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Functional effect of the C242T polymorphism in the NADPH oxidase p22^{phox} gene on vascular superoxide production in atherosclerosis. *Circulation*. 2000;102:1744–1747.