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Arterioscler. Thromb. Vasc. Biol. 2007;27;587-593; originally published online Dec 28, 2006;
DOI: 10.1161/01.ATV.0000256467.25384.c6

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 75214
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Phagocytic NADPH Oxidase-Dependent Superoxide Production Stimulates Matrix Metalloproteinase-9 Implications for Human Atherosclerosis

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Objective—Data suggest that matrix metalloproteinase-9 (MMP-9) has a role in atherosclerosis. The phagocytic NADPH oxidase has also been associated with atherosclerosis. This study aimed to investigate the association between phagocytic NADPH oxidase and MMP-9 in human atherosclerosis.

Methods and Results—In vitro experiments performed in human monocytes showed that NADPH oxidase activation enhanced MMP-9 secretion and activity, determined by enzyme-linked immunosorbent assay and zymography, respectively. Immunohistochemical study showed that phagocytic NADPH oxidase localized with MMP-9 in endarterectomies from patients with carotid stenosis. In addition, a positive relationship (P<0.001) was found between phagocytic NADPH oxidase-dependent superoxide production determined with lucigenin and plasma MMP-9 levels in 188 asymptomatic subjects free of overt clinical atherosclerosis. In multivariate analysis, this association remained significant after adjustment for cardiovascular risk factors. Interestingly, subjects in the upper quartile of superoxide production exhibited the highest values of MMP-9, oxidized low-density lipoprotein, nitrotyrosine, carotid intima media thickness, and an increased presence of carotid plaques.

Conclusions—Enhanced NADPH oxidase-dependent O$_2^-$ production stimulates MMP-9 in monocytes and this relationship may be relevant in the atherosclerotic process. Moreover, MMP-9 emerges as an important mediator of the phagocytic NADPH oxidase-dependent oxidative stress in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:587-593.)

Key Words: atherosclerosis • NADPH oxidase • superoxide • MMP

The NADPH oxidase systems, which constitute the most important sources of superoxide (O$_2^-$) in the vessel wall, are present in endothelial cells, smooth muscle cells (SMCs), fibroblasts, and infiltrated monocytes/macrophages.1,2 The phagocytic NADPH oxidase consists of a membrane-associated cytochrome b$_{558}$, which comprises gp91phox and p22phox subunits, and cytosolic components p47 phox, p67 phox, and rac.2 Several studies have shown a key role for vascular NADPH oxidase isoforms in the development of human atherosclerosis.3–7 Interestingly, phagocytic NADPH oxidase seems to play also a key role in the development and progression of atherosclerotic lesion.5–7 Recently, enhanced phagocytic NADPH oxidase-dependent O$_2^-$ production has been correlated positively with carotid intima-media thickness (IMT), a surrogate marker of atherosclerosis.8 Metalloproteinasises (MMPs) are a family of zinc-dependent endopeptidases capable as a class of degrading extracellular matrix components, which participate in the atherosclerotic process by remodeling the extracellular matrix.9 Available evidence substantiates that plasma MMP-9 levels correlate with the presence of atherosclerosis10 and represent an independent risk factor for atherothrombotic events (ie, coronary heart disease events and cerebrovascular disease).11,12 Thus, plasma MMP-9 levels can provide a useful emerging plasma biomarker in the prediction of atherothrombotic events.13 Whereas NADPH oxidase-mediated O$_2^-$ generation participates in MMP-2 and MMP-9 activation in cardiomyocytes, endothelial cells, and SMCs,14–18 no data are available on its ability to regulate MMPs in blood phagocytic cells. We therefore hypothesized that an association may exist between phagocytic NADPH oxidase and MMP-9 in human atherosclerosis. To test this hypothesis, we performed the study at 3 levels; (1) we analyzed in vitro the ability of NADPH oxidase to regulate MMP-9 in human monocytes; (2) we studied the association between NADPH oxidase and MMP-9 in athero-
sclerotic plaques from endarterectomy specimens; and (3) we explored the relationship of NADPH oxidase with plasma MMP-9 and carotid atherosclerosis in asymptomatic subjects.

**Patients and Methods**

**Cell Culture Experiments**

Human monocytes were isolated from peripheral blood mononuclear cells with the MACS Column Technology (Miltenyi Biotec) and maintained in RPMI 1640 supplemented with 0.2% fetal calf serum. Human THP-1 macrophagic cell line was obtained from ATCC, and was maintained in RPMI 1640 supplemented with 10% fetal calf serum. For experiments, cells (500000 cells/mL) were maintained in RPMI 1640 supplemented with 0.2% fetal calf serum for 24 hours. After this period of time, cells were incubated with phorbol myristate acetate (PMA, 6.4×10⁻⁶ mol/L) in the presence and the absence of apocynin, a specific intracellular inhibitor of NADPH oxidase assembly.

**Detection In Vitro of -O₂⁻ Generation**

We measured -O₂⁻ production in human monocytes in the presence of 5 μmol/L lucigenin. Luminescence was measured for 30 minutes in a plaque luminometer. A buffer blank was subtracted from each reading. Data were expressed as relative light units produced per second. In some experiments, the effects of apocynin, a NADPH oxidase inhibitor, was studied.

**Determination In Vitro of MMP-9 Expression and Activity**

A sandwich enzyme-linked immunosorbent assay (ELISA) (Amer sham Biosciences), an assay that quantified both the precursor form and the active form complexed with the tissue inhibitor of metalloproteinase-1, was used to determine MMP-9 levels in cultured cell supernatants. MMP-9 gelatinolytic activity was assessed by zymography in supernatants from cells. Samples were prepared in nonreducing sample buffer (0.625 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 2% bromphenol blue), and run through 10% zymogram gelatin gels.

**Subjects**

Two studies were performed. The first study was performed in 5 male patients (mean age, 67 years) undergoing carotid endarterectomy. Surgical intervention criteria were internal carotid stenosis >75%. The second study was performed in 188 consecutive apparently healthy subjects (80% men; mean age, 53 years). The criteria for defining the absence of atherosclerosis have been recently described (please see http://atvb.ahajournals.org). A sandwich ELISA (Amersham Biosciences) was used to determine MMP-9 levels in plasma samples.

**Measurement of Carotid IMT and Assessment of Carotid Plaques**

Ultrasonography of the common carotid arteries was performed with a linear-array transducer (ATL 500 HDI) as previously reported. Measurement of IMT was made 1 cm proximal to the carotid bulb of each common carotid artery at plaque-free sites. Atheroma plaques were defined as echogenic structures protruding into the lumen with a distinct area 50% greater than the intimal plus media thickness of neighboring sites.

**Statistical Analysis**

Data are expressed as mean±SEM. The χ² analysis was used to search for differences for qualitative variables. Pearson correlation test was used to assess correlations between -O₂⁻ production and all continuous variables. Multivariate linear regression analysis was performed to evaluate factors related to MMP-9 levels and the possibility of interactions. For association studies, subjects were stratified according to quartiles of -O₂⁻ production (quartile 1, -O₂⁻ production ≤8 counts/second; quartile 2, -O₂⁻ production ≥8 and <16 counts/second; quartile 3, -O₂⁻ production ≥16 and <30 counts/second; quartile 4, -O₂⁻ production ≥30 counts/second). The cardiovascular medication intake (dichotomous: yes versus no) was tested, but revealed P=0.05. Likewise, if each class of cardiovascular medication were tested separately in the models, they were not significant covariates at the 5% test level. The linear trend of variables, according to -O₂⁻ production quartiles, was compared by use of ANOVA for continuous variables and the linear test of the χ² analysis for categorical variables.

**Results**

**Study in Cultured Cells**

To explore the ability of NADPH oxidase to regulate MMP-9, the effects of the NADPH oxidase-dependent -O₂⁻ production on the activity and secretion of MMP-9 were examined in fresh human monocytes isolated from five patients. First, incubation of monocytes with PMA immediately provoked a threefold enhanced -O₂⁻ generation (P<0.01) (Figure 1A). This effect was prevented in the presence of apocynin, in a dose-dependent way, thus supporting the notion that the
expression was intense in the macrophage rich area and colocalized with MMP-9 staining (supplemental Figure III). 

\( O_2^- \) production in endarterectomies was detected by using DHE staining. \( O_2^- \) was evident in all layers, although an intense area of \( O_2^- \) production was observed throughout the macrophage-rich area (Figure 2). DHE fluorescence was abolished by preincubation with CuZn-SOD, demonstrating the specificity of the assay for \( O_2^- \) (Figure 2). Interestingly, \( O_2^- \) staining was similar to phagocytic NADPH oxidase staining.

The fibrous cap of the lesion was intensely stained for collagen fibers (Figure 2). The collagen staining was practically absent throughout the macrophage rich area. Besides, the diminished collagen staining coincided with enhanced MMP-9 expression.

These data suggest that infiltrated macrophages may favor the remodeling of atherosclerotic plaque by inducing the expression of activated MMP-9 through NADPH oxidase-dependent \( O_2^- \) production.

**Study in Asymptomatic Subjects**

The clinical characteristics of the asymptomatic subjects are shown in Table 1. Whereas the cholesterol values were above the upper normal limit, the values of the remaining parameters tested were normal.

The values of phagocytic \( O_2^- \) production and plasma MMP-9 in the whole population were 19.1 ± 1.2 counts/second and 14.8 ± 0.6 ng/mL, respectively. There was a significant positive bivariate correlation between phagocytic \( O_2^- \) production and plasma levels of MMP-9 (Figure 3), which remained highly significant after controlling for age and sex (supplemental Table I). \( O_2^- \) production was also significantly associated with triglycerides and body mass index, with the latter also remaining statistically significant after controlling for age and sex (supplemental Table I). When we analyzed only those subjects that were not receiving cardiovascular treatment, \( O_2^- \) production was also associated with systolic blood pressure values, which remained significant after controlling for age and sex \((r=0.160, P=0.032)\). However, plasma MMP-9 did not correlate with other analyzed variables.

In a multivariate analysis, the association between phagocytic \( O_2^- \) production and MMP-9 remaining statistically significant after adjusting for some potentially confounding cardiovascular risk factors (Table 2), with the \( O_2^- \) production explaining up to 16% of the MMP-9 variance.

The classification of the subjects by quartiles of \( O_2^- \) production showed a linear trend in MMP-9 levels \((P<0.05)\) (Table 3). Furthermore, values of oxidized low-density lipoprotein and NT, 2 oxidative stress markers that have been associated with atherosclerosis, were enhanced in subjects in the fourth quartile compared with subjects in the other quartiles \((P<0.05)\). Likewise, subjects in the fourth quartile exhibited higher frequency of carotid plaques and increased carotid IMT compared with subjects in the other quartiles \((P<0.05)\). Interestingly, carotid IMT exhibited a positive bivariate correlation with \( O_2^- \) production \((r=0.262, P=0.001)\) and MMP-9 \((r=0.181, P=0.017)\), after controlling for age and sex. Finally, subjects in the fourth quartile
exhibited a higher prevalence of metabolic syndrome and obesity than subjects in the other quartiles ($P<0.05$).

**Discussion**
The main findings of this study are as follows: (1) enhanced phagocytic NADPH oxidase-dependent $\cdot O_2^-$ production stimulates MMP-9 in human monocytes; (2) phagocytic NADPH oxidase is associated with MMP-9 in plaques from atherosclerotic patients; and (3) enhanced phagocytic NADPH oxidase-dependent $\cdot O_2^-$ production is related to both plasma levels of MMP-9 and carotid atherosclerosis in asymptomatic subjects. This relationship is independent of confounding variables, including some conventional cardiovascular risk factors.
In recent years, it has been demonstrated that reactive oxygen species (ROS) production may participate in MMP-9 activation. In addition, results from several approaches confirm that the in vitro relationship between NADPH oxidase-dependent ROS production and plasma levels of MMP-9 is positive.

**Table 1. Baseline Characteristics of the Studied Population**

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>n=188</th>
<th>Age, y</th>
<th>53±1</th>
<th>Sex, M/F</th>
<th>150/38</th>
<th>Body mass index, kg/m²</th>
<th>28±1</th>
<th>Systolic blood pressure, mm Hg</th>
<th>131±1</th>
<th>Diastolic blood pressure, mm Hg</th>
<th>83±1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>5.77±0.11</td>
<td>Total cholesterol, mmol/L</td>
<td>5.84±0.08</td>
<td>HDL cholesterol, mmol/L</td>
<td>1.22±0.03</td>
<td>LDL cholesterol, mmol/L</td>
<td>3.98±0.08</td>
<td>Triglycerides, mmol/L</td>
<td>1.41±0.06</td>
<td>Smokers, %</td>
<td>33</td>
</tr>
<tr>
<td>Hypertensive, %</td>
<td>55</td>
<td>Diabetic, %</td>
<td>15</td>
<td>Obese, %</td>
<td>37</td>
<td>Metabolic syndrome, %</td>
<td>35</td>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensives, %</td>
<td>31</td>
<td>Statins, %</td>
<td>18</td>
<td>Oral hypoglycemics, %</td>
<td>10†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In this context, our finding showing that apocynin attenuated both MMP-9 secretion and activation in human blood monocytes, demonstrates for the first time that NADPH oxidase-mediated ROS effects on other MMPs in human blood monocytes, in fact, NADPH oxidase-dependent ROS production may participate in MMP-2 activation in other cell types.

**Table 2. Association of the MMP-9 with NADPH Oxidase-Dependent \( \mathrm{O}_2^\cdot \) Production in Multiple Linear Regression Analysis**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>( \beta )</th>
<th>( P^* )</th>
<th>Partial ( r^2 )%</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mathrm{O}_2^\cdot ) production, counts/sec</td>
<td>0.386</td>
<td>0.001</td>
<td>16.0</td>
</tr>
<tr>
<td>Age, y</td>
<td>0.013</td>
<td>0.866</td>
<td>0.1</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>0.036</td>
<td>0.628</td>
<td>0.1</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>0.025</td>
<td>0.745</td>
<td>0.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>-0.072</td>
<td>0.365</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>0.005</td>
<td>0.258</td>
<td>0.5</td>
</tr>
<tr>
<td>Smokers, no/yes</td>
<td>0.033</td>
<td>0.659</td>
<td>0.2</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.001</td>
<td>0.577</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, body mass index, systolic blood pressure, glucose, smoking, and triglycerides; \( r^2 \) for the total population was 17.4%.

MMP-9 activity in these cells. We cannot discard other NADPH oxidase-dependent ROS effects on other MMPs in human monocytes. In fact, NADPH oxidase-dependent \( \mathrm{O}_2^\cdot \) production may participate in MMP-2 activation in other cell types.

The phagocytic NADPH oxidase plays a major role in human atherosclerosis. It has been reported that the severity of atherosclerotic lesion correlates with p22\textsuperscript{phox} overexpression in coronary arteries. In addition, gp91\textsuperscript{phox} and p22\textsuperscript{phox} increase considerably along the progression of human atherosclerotic plaques, thus suggesting a causal link between the phagocytic NADPH oxidase and the development of lesions. In fact, contribution of gp91\textsuperscript{phox} to lesion progression is caused almost entirely by infiltrated monocytes. Finally, ROS production, mainly generated by infiltrated inflammatory cells, has been associated with p22\textsuperscript{phox} in atherosclerotic human coronary arteries. Likewise, MMP-9 is involved in several stages of atherosclerosis through remodeling of the extracellular matrix. In cell studies, degradation of the matrix by MMP-9 at the endothelial layer promotes recruitment of monocyte-derived cells into the subendothelial space. In experimental models, degradation of the matrix surrounding SMCs promotes SMC migration, whereas macrophage expression of active MMP-9 induces acute plaque disruption in apolipoprotein E\textsuperscript{−/−} mice. In this context, our finding showing that \( \mathrm{O}_2^\cdot \) generation and NADPH oxidase components localized with MMP-9 in macrophage-rich areas in endarterectomy specimens from atherosclerotic patients confirms that the in vitro relationship between NADPH oxidase and MMP-9 in human monocytes is also present in plaques, and allows us to suggest that phagocytic NADPH oxidase-dependent ROS may promote MMP-9-dependent degradation of extracellular matrix and the development of atherosclerotic disease.

Phagocytic NADPH oxidase has been associated with subclinical atherosclerosis (ie, carotid IMT) in asymptomatic subjects, thus suggesting that NADPH oxidase-mediated ROS production plays a crucial role in the initiation and development of atherosclerotic disease. It is thus likely that MMP-9 might be a potential mediator of the NADPH oxidase-dependent ROS production in the atherosclerotic process. In support of this possibility, we found a positive correlation between NADPH oxidase-dependent ROS production and plasma levels of MMP-9.

![Figure 3](image-url) Positive correlation between NADPH oxidase-dependent \( \mathrm{O}_2^\cdot \) production and plasma levels of MMP-9 (\( y=10.98+0.19x, r=0.411, P<0.001 \)).
correlation between NADPH oxidase-dependent \( \text{O}_2^- \) production and MMP-9 levels in asymptomatic subjects. In addition, findings showing that subjects in the upper quartile of \( \text{O}_2^- \) production exhibited increased values of MMP-9, and were associated with high levels of oxidative stress markers and with enhanced carotid IMT and frequency of plaques, identifies a subgroup of subjects who might be prone to develop plaque rupture and/or ischemic events. In fact, it has been shown that MMP-9 levels provide a useful emerging biomarker in the prediction of atherothrombotic events.13 Thus, epidemiologic studies suggest that MMP-9 expression correlates with lesion stability and clinical manifestations of atherosclerosis.16,26–28 In addition, analysis of coronary atherectomies reveal active synthesis of MMP-9 by macrophages in lesions of patients with unstable versus stable angina.29

During the past decade, a significant number of studies support an essential role of vascular NADPH oxidase isoforms (Nox1 and Nox4) in vascular remodeling30–33 and atherogenesis.11–15 Because the present study does not provide direct evidence of the potential role of vascular NADPH oxidases, it is important to point out that the increased phagocytic NADPH oxidase-dependent \( \text{O}_2^- \) production might not necessarily be the only factor responsible for the increased MMP-9 levels.8 In fact, our findings showing that \( \text{O}_2^- \) production explained up to 16% of the MMP-9 variance after adjusting for common risk factors, suggest that vascular NADPH oxidases may also be participating in the atherosclerotic process.3–7 We should also take into account the fact that several enzymes have been proposed as sources of ROS in atherosclerosis other than NADPH oxidases, such as lipoxygenase, xanthine oxidase, and nitric oxide synthase.34

Cardiovascular treatment, including antihypertensive drugs,35,36 statins,37 and thiazolidinediones,38 may reduce NADPH oxidase activity. In the current study, we found no association between \( \text{O}_2^- \) production and blood pressure values in the whole population, which included 99 subjects using cardiovascular medication, contrary to results of a previous study.39 Nevertheless, our results showed a positive association between \( \text{O}_2^- \) production and systolic blood pressure in subjects who were not receiving cardiovascular medication, thus demonstrating a critical role of cardiovascular drugs on phagocytic NADPH oxidase activity.

In summary, we found that enhanced NADPH oxidase-dependent \( \text{O}_2^- \) production stimulates MMP-9 in monocytes and that this relationship may be relevant in the atherosclerotic process. In fact, MMP-9 emerges as a potential mediator of the pro-atherosclerotic actions of phagocytic NADPH oxidase in both symptomatic and asymptomatic subjects.

**Acknowledgments**

We gratefully acknowledge technical assistance by Raquel Ros and Ana Montoya.

**Sources of Funding**

This project was funded through the agreement between FIMA and “UTE project CIMA,” Foundation MMA, 25/2005 from Department of Health of Government of Navarra, SAF2004-07910 from Ministry of Science and Technology and RECAVA C03/01 from Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Ministry of Health of Government of Navarra, SAF2004-07910 from Ministry of Science and Technology and RECAVA C03/01 from Fondo de Investigaciones Sanitarias, Instituto de Salud Carlos III, Ministry of Health, Spain.

**Disclosures**

None.

**References**


