Phagocytic NADPH Oxidase Overactivity Underlies Oxidative Stress in Metabolic Syndrome

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Original Article

Oxidative stress plays a critical role in the pathogenesis of atherosclerosis in patients with metabolic syndrome. This study aimed to investigate whether a relationship exists between phagocytic NADPH oxidase activity and oxidative stress and atherosclerosis in metabolic syndrome patients.

The past few years have also increased the recognition of its frequent association with cardiovascular disease, especially atherosclerosis, which constitutes one of the major causes of morbidity and mortality in metabolic syndrome.

Oxidative stress, and mainly superoxide anion \( (\cdot O_2^-) \), plays a critical role in the pathogenesis of hypertension, hypertriglyceridemia, diabetes, and obesity—risk factors defining metabolic syndrome (4–9). In fact, available evidence substantiates that metabolic syndrome associates with elevated systemic oxidative stress (10,11). Among other effects, an excess of \( \cdot O_2^- \) may inactivate nitric oxide (NO), thus leading to endothelial dysfunction and, in turn, facilitating vascular abnormalities (12). In addition, an increased production of \( \cdot O_2^- \) may facilitate oxidative modification of proteins (9), by rendering nitrotyrosine, which constitutes a strong and independent predictor of cardiovascular disease (13). \( \cdot O_2^- \) is also involved in LDL oxidation, a key step in the initiation and progression of atherosclerosis (14).

The NADPH oxidase systems, which constitute the most important sources of \( \cdot O_2^- \) in the vessel wall, are present in endothelial cells, smooth muscle cells, fibroblasts, and infiltrated monocytes/macrophages (15,16). A number of findings suggest that vascular NADPH oxidase overactivity may be involved in atherosclerosis (17–21). The phagocytic NADPH oxidase is similar, although it differs structurally and biochemically from the vascular oxidase. It consists of a membrane-associated cytochrome, \( b_{558} \), and three cytosolic components, \( p47^{phox} \), \( p67^{phox} \), and \( rac1/2 \). Cytochrome \( b_{558} \) comprises a large subunit, \( gp91^{phox} \), and a smaller, \( p22^{phox} \) (16). It has been shown that phagocytic NADPH oxidase is crucial also in the development of the atherosclerotic lesion (18–21). Moreover, enhanced phagocytic NADPH oxidase activity has been shown recently to be associated with subclinical atherosclerosis in asymptomatic subjects (22).

We have hypothesized that phagocytic NADPH oxidase is enhanced in metabolic syndrome and that NADPH oxidase overactivity favors the establishment of systemic oxidative stress in metabolic syndrome patients. In addition, and because hyperinsulinemia plays a central role in the development of metabolic syndrome, we hypothesize also that insulin is involved in the activation of NADPH oxidase in this syndrome.

RESEARCH DESIGN AND METHODS

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. The study was performed in 183 unrelated individuals consecutively referred to our institution for routine medical work-up after a 12-h overnight fast. Fifty-six patients were classified as having metabolic syndrome (metabolic syndrome group) by the criteria of the National Cholesterol Education Program Adult Treatment Panel
TABLE 1
Demographical and clinical characteristics of the subjects included in the study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cardiovascular risk factor</th>
<th>Metabolic syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (men/women)</td>
<td>20/8</td>
<td>82/17</td>
<td>44/12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49 ± 2</td>
<td>55 ± 1*</td>
<td>57 ± 2†</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 ± 0.6</td>
<td>28.0 ± 0.4*</td>
<td>31.2 ± 0.6†</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>90 ± 2.3</td>
<td>92 ± 3.7</td>
<td>104 ± 2†</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.0 ± 0.1</td>
<td>5.4 ± 0.1*</td>
<td>6.4 ± 0.2†</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>42.0 ± 1.8</td>
<td>75.6 ± 3.6*</td>
<td>105 ± 6.6†</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.5 ± 0.1</td>
<td>2.9 ± 0.2*</td>
<td>5.1 ± 0.4†</td>
</tr>
<tr>
<td>sBP (mmHg)</td>
<td>111 ± 2</td>
<td>133 ± 2*</td>
<td>139 ± 2†</td>
</tr>
<tr>
<td>dBP (mmHg)</td>
<td>72 ± 1</td>
<td>84 ± 1*</td>
<td>85 ± 1†</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.69 ± 0.23</td>
<td>5.87 ± 0.10</td>
<td>5.74 ± 0.16</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.85 ± 0.21</td>
<td>4.01 ± 0.08</td>
<td>3.88 ± 0.13</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.40 ± 0.01</td>
<td>1.27 ± 0.01*</td>
<td>1.03 ± 0.01†</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.93 ± 0.04</td>
<td>1.18 ± 0.04*</td>
<td>1.74 ± 0.08†</td>
</tr>
<tr>
<td>von Willebrand factor (%)</td>
<td>100 ± 9</td>
<td>114 ± 4</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>275 ± 11</td>
<td>285 ± 6</td>
<td>280 ± 7</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>0.18 ± 0.02</td>
<td>0.27 ± 0.02*</td>
<td>0.27 ± 0.02*</td>
</tr>
<tr>
<td>Carotid IMT (mm)</td>
<td>0.61 ± 0.02</td>
<td>0.64 ± 0.01</td>
<td>0.77 ± 0.03†</td>
</tr>
</tbody>
</table>

Medication

Antihypertensives (%) — 42 39
Statins (%) — 19 21
Oral hypoglycemics (%) — 6 21†

Data are means ± SE. *P < 0.05 compared with control group; †P < 0.05 compared with cardiovascular risk factor group. dBP, diastolic blood pressure; sBP, systolic blood pressure.

III (23). In accordance with that, the diagnosis of metabolic syndrome was established when three or more of the following alterations were present: central obesity defined as waist circumference >102 cm in men and >88 cm in women; hypertriglyceridemia defined as triglycerides ≥1.695 mmol/l; low HDL cholesterol defined as HDL cholesterol <1.036 mmol/l in men and <1.295 mmol/l in women; high blood pressure defined as systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg, or use of antihypertensive medication; or high fasting glucose defined as glucose ≥6.1 mmol/l. Furthermore, we identified a group of 90 patients without clinical evidence of metabolic syndrome that presented one or two conventional cardiovascular risk factors (cardiovascular risk factor group), including hypertension, obesity, dyslipidemia, or diabetes. Finally, 28 healthy subjects with no cardiovascular risk factors constituted the control group. Clinical screenings were based on medical history, physical examination, and routine analytical tests. The homeostasis model assessment (HOMA) index (fasting glucose [mmol/l] × fasting insulin [µU/ml])/22.5 was used to assess insulin resistance.

Determination of NADPH oxidase activity. We measured O₂⁻ 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 µmol/l), and using 5 µmol/l lucigenin by a chemiluminescent method previously described (5,22). In addition, the effects of 5 µmol/l diphenylene iodonium (DPI), a flavoprotein inhibitor, and 2.5 mmol/l apocynin, a specific intracellular inhibitor of NADPH oxidase assembly, were studied. To verify the specificity of the lucigenin assay for O₂⁻, the effect of superoxide dismutase (SOD) 10,000 units/ml, an enzymatic scavenger of O₂⁻, was examined. Although lucigenin concentration was low enough to avoid autodissociation, the measurements were validated against an independent measurement of O₂⁻ production using SOD-inhibitable ferricytochrome c reduction as previously reported (5,22). The measurement of O₂⁻ production using SOD-inhibitable ferricytochrome c reduction closely correlated with lucigenin measurements. In some subjects, phagocytic O₂⁻ production was evaluated in response to human recombiant insulin in presence and absence of apocynin, DPI, and bisindolylmaleide (BIS) I, an inhibitor of protein kinase C (PKC). Lucigenin, PMA, DPI, SOD, and ferricytochrome c were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), and apocynin and BIS I were from Calbiochem (Darmstadt, Germany).

Study of the expression of NADPH oxidase subunits. Protein levels of NADPH oxidase components were determined by Western blot. Phagocytic mononuclear cells isolated from peripheral blood were homogenized in lysis buffer (5 mmol/l EDTA, 150 mmol/l KCl, 0.5% NP-40, and 10 mmol/l Tris-HCl, pH 7.4) containing a protease inhibitor cocktail Complete (Roche). Protein abundance of NADPH oxidase components was determined with polyclonal antibodies directed against p22(phox), gp91(phox), p47(phox), and p67(phox). The expression of the housekeeping protein β-actin was evaluated using a monoclonal anti-β-actin antibody. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Determination of circulating markers of oxidative stress. Markers of oxidative stress were evaluated in a subgroup of 90 subjects within the overall population. Serum and plasma samples collected after overnight fasting were prepared after centrifugation of blood samples. An enzyme-linked immunosorbent assay was performed to determine levels of protein-associated 3-nitrotyrosine in human plasma (Hyctyl Biotechnology, Uden, the Netherlands). Levels of oxidized LDL (oxLDL) were measured in plasma by an enzyme-linked immunosorbent assay procedure using the murine monoclonal antibody, mAb-4E6 (Mercodia AB, Uppsala, Sweden). Nitrate and nitrite (NO₃⁻/NO₂⁻, NOx) concentration was measured in serum samples after ultracentrifugation. NOx concentration was evaluated directly from the supernatant using a colorimetric assay based on Griess reaction (Cayman Chemical, Ann Arbor, MI).

Measurement of carotid intima-media thickness. Ultrasonography of the common carotid arteries was performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI) as previously reported (22). The measurement of intima-media thickness (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 carotid artery. Subjects were examined by the same two certified sonographers.

![FIG. 1. NADPH oxidase–dependent O₂⁻ production was determined by lucigenin chemiluminescence after PMA stimulation in 4 × 10⁸ phagocytes from control subjects (C) and from cardiovascular risk factor (CRF) and metabolic syndrome (MetS) patients. *P < 0.05 compared with control group; †P < 0.05 compared with cardiovascular risk factor group.](image-url)
phases blinded to all clinical information. The intraobserver and interobserver coefficients of variation were 5 and 10%, respectively.

Statistical analysis. Data are expressed as means ± SE. Differences in the demographic and clinical characteristics among subgroups of subjects were assessed by one-way ANOVA once normality was demonstrated (Shapiro-Wilks test), and a Scheffé post hoc test was used to examine differences between groups when significance was achieved; otherwise a Kruskal-Wallis followed by a Mann-Whitney U test was used. The χ² analysis was used to search for differences in qualitative variables. Pearson’s correlation test was used to assess correlations between continuously distributed variables. Relationships between Ô₂⁻ production and cardiovascular risk factors were analyzed using a Mann-Whitney U test. Statistical significance was established as P < 0.05.

RESULTS

Population characteristics. The demographic and clinical characteristics of the studied subjects are summarized in Table 1. No significant difference in sex distribution was found among the three groups. The metabolic syndrome and cardiovascular risk factor patients were significantly older than control subjects. As expected, metabolic syndrome patients displayed significantly higher waist circumference, BMI, systolic blood pressure, plasma levels of glucose, triglycerides, insulin, and HOMA index, and a lower level of HDL cholesterol compared with cardiovascular risk factor and control groups. In addition, metabolic syndrome patients exhibited increased carotid IMT compared with cardiovascular risk factor and control groups. Diastolic blood pressure values and C-reactive protein levels, which were significantly higher in metabolic syndrome and cardiovascular risk factor groups than in the control group, were similar between cardiovascular risk factor and metabolic syndrome groups. No significant differences were found in the remaining parameters among the three groups of subjects. Finally, no differences were found in the frequency of cardiovascular medications between the cardiovascular risk factor and metabolic syndrome groups, except for oral hypoglycemic therapy.

Phagocytic NADPH oxidase activity. In agreement with previous studies of our group (5,22), PMA-induced Ô₂⁻ production was inhibited by DPI and apocynin, and SOD completely abolished the chemiluminescent signal (data not shown), thus demonstrating that the enzymatic source of Ô₂⁻ in phagocytic cells is the NADPH oxidase system.

NADPH oxidase activity was higher (P < 0.05) in the metabolic syndrome group (24.3 ± 2.6 counts/s) than in the other two groups (cardiovascular risk factor, 13.5 ± 0.9; control, 7.6 ± 0.3) (Fig. 1). The upper normal limit of the NADPH oxidase activity was 22.8 counts/s, calculated with 99% confidence in the control group. We found that the percentage of subjects presenting values of NADPH oxidase activity above this limit was higher (P < 0.05) in metabolic syndrome group (39%) than in cardiovascular risk factor (15%) and control (7%) groups.

Expression of NADPH oxidase components. p22phox protein abundance was higher (P < 0.05) in metabolic syndrome patients (2.1 ± 0.4 arbitrary units) than in cardiovascular risk factor patients (0.7 ± 0.2) and control subjects (1.0 ± 0.2) (Fig. 2). No differences were found among the three groups of subjects in protein abundance for the other subunits of the phagocytic NADPH oxidase (Fig. 2).

Markers of oxidative stress. Plasma levels of oxLDL were higher (P < 0.05) in metabolic syndrome group (78 ± 0.2 units/l) than in the cardiovascular risk factor group (64 ± 0.1) and the control group (62 ± 0.1) (Fig. 3A).

FIG. 2. Expression of NADPH oxidase components. A: Representative Western blots for p22phox, p47phox, p67phox, gp91phox, and β-actin. B: Densitometric analysis, normalized for β-actin, revealed increased p22phox in the metabolic syndrome (MetS) group compared with control (C) and cardiovascular risk factor (CRF) groups. *P < 0.05 compared with control and cardiovascular risk factor groups.

FIG. 3. Levels of oxLDL were determined in plasma samples from 90 subjects. A: Bars show means ± SE of oxLDL levels in control subjects (C, n = 19), cardiovascular risk factor patients (CRF, n = 41), and metabolic syndrome patients (MetS, n = 30). *P < 0.05 compared with control and cardiovascular risk factor groups. B: Correlation (γ = 62.2 ± 0.37x) between NADPH oxidase–mediated Ô₂⁻ production and oxLDL levels in all subjects.
Plasma levels of nitrotyrosine were enhanced \((P < 0.05)\) in the metabolic syndrome group \((5.8 \pm 0.9 \text{ nmol/l})\) compared with the cardiovascular risk factor group \((2.6 \pm 0.2)\) and the control group \((2.8 \pm 0.3)\) (Fig. 4A). In addition, serum values of NOx were similar in the three groups of subjects \((\text{control}, 4.1 \pm 0.6 \mu \text{mol/l}; \text{cardiovascular risk factor}, 4.5 \pm 0.3; \text{metabolic syndrome}, 4.7 \pm 0.3)\).

**Analysis of associations.** An analysis of risk factors defining metabolic syndrome that could affect phagocytic \(-\text{O}_2^-\) production revealed no major effect of a single risk factor on NADPH oxidase activity in metabolic syndrome patients (Table 2). This suggests that a synergistic effect of risk factors may be more important than the individual effect of each one on the activation of phagocytic NADPH oxidase as observed in these patients.

There was a significant positive bivariate correlation between insulin levels and phagocytic NADPH oxidase–dependent \(-\text{O}_2^-\) production in all subjects (Fig. 5). Besides, the values of HOMA also correlated with phagocytic 

\[ r = \frac{y - \bar{y}}{s_y} \times \frac{x - \bar{x}}{s_x} \]

\[ r = 0.393; P = 0.03 \]

A positive correlation between insulin levels and oxLDL values \((r = 0.393; P = 0.03)\) was also found in the overall population. In addition, a positive correlation between NADPH oxidase–dependent \(-\text{O}_2^-\) production and oxLDL levels was found in the overall population (Fig. 3B). A positive correlation between insulin levels and oxLDL values \((y = 55.1 + 0.152x; r = 0.393; P = 0.001)\) was also found in the overall population. In addition, a positive correlation between NADPH oxidase–dependent \(-\text{O}_2^-\) production and nitrotyrosine levels was found in the overall population (Fig. 4B). Finally, a positive correlation between NADPH oxidase–dependent \(-\text{O}_2^-\) production and carotid IMT \((y = 0.6 + 0.023x; r = 0.232; P = 0.002)\) was found in the overall population.

**Effects of insulin on phagocytic NADPH oxidase activity.** The effect of different concentrations of human recombinant insulin on \(-\text{O}_2^-\) production in mononuclear phagocytic cells isolated from five volunteers is shown in Fig. 6A. Insulin increased \(-\text{O}_2^-\) production in a sigmoidal fashion. Compared with control, insulin \((10^3 \text{ pmol/l})\) significantly increased \(-\text{O}_2^-\) production.

Insulin \((10^3 \text{ pmol/l})\) stimulated \(-\text{O}_2^-\) production (basal, \(0.92 \pm 0.03 \text{ counts/s}; \text{insulin}, 1.66 \pm 0.12; P < 0.05)\) in mononuclear phagocytic cells from five volunteers (Fig. 6B). This insulin-stimulated \(-\text{O}_2^-\) production was completely inhibited by apocynin \((1.01 \pm 0.09 \text{ counts/s})\) and DPI \((1.11 \pm 0.15)\), thus demonstrating a specific role of NADPH oxidase on insulin-mediated \(-\text{O}_2^-\) production. BIS I \((20 \mu \text{mol/l})\) also abolished the insulin-induced phagocytic NADPH oxidase activity \((1.12 \pm 0.11 \text{ counts/s})\), suggesting that this effect of insulin is mediated by PKC.

**DISCUSSION**

The main finding of this study is that phagocytic NADPH oxidase–dependent \(-\text{O}_2^-\) production is significantly enhanced in patients with metabolic syndrome. In addition, patients with metabolic syndrome present features of oxidative stress (i.e., increased levels of oxLDL and nitrotyrosine) and subclinical atherosclerosis (i.e., enhanced carotid IMT) that are associated with phagocytic NADPH oxidase activity.

Previous studies have shown that hypertriglyceridemia, hypertension, hyperglycemia, and obesity are independently associated with systemic oxidative stress (4–9). Furthermore, it has been reported that diabetes, hypertension, and obesity are each associated with increased production of \(-\text{O}_2^-\) via the NADPH oxidase pathway (5,24–26). Because obesity associates with increased reactive oxygen species generation by mononuclear phagocytic cells, probably in relation to increased macronutrient intake (27), and a majority (64%) of the metabolic syn-

**TABLE 2**

Risk factors associated with phagocytic NADPH oxidase activity in metabolic syndrome

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Patients</th>
<th>NADPH oxidase activity (counts/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With RF</td>
<td>Without RF</td>
</tr>
<tr>
<td>Hypertension</td>
<td>38</td>
<td>18</td>
</tr>
<tr>
<td>Obesity</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>Diabetes</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td>High triglycerides</td>
<td>33</td>
<td>23</td>
</tr>
<tr>
<td>Low HDL cholesterol</td>
<td>39</td>
<td>17</td>
</tr>
</tbody>
</table>

Data are \(n\) or means ± SE. RF, risk factor.
drome patients of our study were obese, the NADPH oxidase overactivity in metabolic syndrome group might be related to obesity. Nevertheless, we did not find differences in NADPH oxidase activity between obese and nonobese within the group of metabolic syndrome patients. Furthermore, the analysis presented in Table 2 suggests that phagocytic NADPH oxidase overactivity in metabolic syndrome may be a consequence of the synergistic effects of the different risk factors, including in metabolic syndrome.

A number of factors may be related to increased phagocytic NADPH oxidase–mediated \(-\text{O}_2^-\) production in metabolic syndrome. Among these, growing evidence suggests a role for insulin. In fact, recent evidence indicates a role for this hormone in the upregulation of NADPH oxidase activity in vascular smooth muscle cells (28), fibroblasts (29), and adipocytes (30). In addition, NADPH oxidase overactivity is involved in the development of cardiovascular complications in models of insulin resistance (31,32). In this regard, our finding showing a correlation of hyperinsulinemia with NADPH oxidase overactivity suggests a role for insulin in the regulation of NADPH oxidase activity in phagocytic cells. This possibility is supported by our observation that high doses of insulin activate NADPH oxidase in human phagocytic cells. It has been shown that infusion of low doses of insulin induces a diminished reactive oxygen species generation in the mononuclear phagocytic cells from obese (33). Our findings showing that only high concentrations of insulin significantly increase NADPH oxidase activity suggest that insulin may possess a dual effect on reactive oxygen species generation in phagocytic cells by a dose-dependent mechanism. The potential pathophysiological relevance of this finding is given by the fact that such concentrations may be reached in the postprandial situation (34). Among the underlying mechanisms for activation of phagocytic NADPH oxidase, it has been suggested a critical role for PKC-dependent \(p47^{\text{phox}}\) and \(p67^{\text{phox}}\) phosphorylation (35). In accordance with this possibility, we found that enhanced insulin-stimulated NADPH oxidase activity was abolished by the PKC inhibitor BIS I.

Alternatively, it is also likely that the enhanced \(p22^{\text{phox}}\) subunit expression found in metabolic syndrome patients plays a role in NADPH oxidase overactivity. Enhanced NADPH oxidase activity in lymphoblasts from hypertensive patients has been found to be associated with greater \(p22^{\text{phox}}\) subunit abundance but not with changes in the other NADPH oxidase subunits (36). Furthermore, a functional polymorphism of \(CYBA\), the human gene that encodes the \(p22^{\text{phox}}\) subunit, has been described that regulates NADPH oxidase activity by modulating the \(p22^{\text{phox}}\) expression in hypertensive patients (37). It is likely that \(p22^{\text{phox}}\) levels constitute a limiting factor capable of regulating NADPH oxidase activity. Accordingly, a genetic study shows that overexpression of \(p22^{\text{phox}}\) increases the NADPH oxidase activity in mice aortas (38). The clinical relevance of these findings is underlined by a recent study showing that thiazolidinediones, which can beneficially influence insulin resistance (39), reduce the expression of the NADPH oxidase \(p22^{\text{phox}}\) subunit (40).

In the present study, we show for the first time that metabolic syndrome patients exhibit enhanced levels of nitrotyrosine. The reaction of \(-\text{O}_2^-\) with NO leads to generation of peroxynitrite, a powerful oxidant of macromolecules, including proteins (9). Increased nitrotyrosine levels have been reported in diabetic patients (41) and in healthy subjects during a hyperglycemic clamp (42) and associate with endothelial dysfunction (43). In addition, it has been shown that increased levels of nitrotyrosine are strong and independent predictors of cardiovascular disease (13). Thus, our finding showing an association of NADPH oxidase overactivity with high nitrotyrosine levels supports the possibility that phagocytic NADPH oxidase may be involved in endothelial dysfunction in metabolic syndrome.

Plasma levels of oxLDL were enhanced in patients with metabolic syndrome, confirming previous data reported by others (10,11). LDL is oxidized by myeloperoxidase in the presence of \(\text{H}_2\text{O}_2\) (44). Because increased NADPH oxidase–dependent \(-\text{O}_2^-\) production associates with in-

![Diagram](image.png)

**FIG. 5.** Correlation \((y = 2.1 + 0.0233x)\) between plasma levels of insulin and NADPH oxidase–dependent \(-\text{O}_2^-\) production in all subjects.

**FIG. 6.** Effect of insulin on phagocytic \(-\text{O}_2^-\) production. A: \(8 \times 10^4\) phagocytic cells were stimulated with increasing concentrations of insulin (10 pmol/l to 1 pmol/l). Data are means \(\pm\) SE of five independent experiments. \(*P < 0.05\) compared with the control experiment (cells without insulin). B: Basal and insulin (10 pmol/l)-stimulated NADPH oxidase activity was measured in phagocytic cells. We analyzed the effect of 5 pmol/l DPI, 2.5 \(\times\) \(10^{-5}\) pmol/l apocynin (Apo), and 20 pmol/l BIS I on insulin-stimulated \(-\text{O}_2^-\) generation. Bars show means \(\pm\) SE of five independent experiments. \(*P < 0.05\) compared with the other conditions.
creased levels of $\text{H}_2\text{O}_2$ (45), it may be suggested that phagocytic NADPH oxidase overactivity facilitates the oxidation of LDL. Our finding of a positive correlation between NADPH oxidase–dependent $\text{O}_2^-$ production and oxLDL levels supports this possibility. High levels of oxLDL have been found to be associated with hyperinsulinemia in vivo (46). Thus, our results showing an association of high levels of insulin with increased levels of oxLDL suggest that insulin-stimulated NADPH oxidase overactivity can promote LDL oxidation in metabolic syndrome patients.

We have found that phagocytic NADPH oxidase overactivity associates with enhanced carotid IMT (22), an independent risk factor for coronary heart disease events, stroke, and transient cerebral ischemia that provides a useful surrogate marker for atherosclerotic disease (47). The association found here of NADPH oxidase activity with carotid IMT suggests a relationship between phagocytic NADPH oxidase–mediated oxidative stress and the development of atherosclerosis in metabolic syndrome patients. This is supported by previous reports showing that increased NADPH oxidase activity impairs endothelial function in atherosclerotic patients (17) and that the severity of the atherosclerotic lesion correlates with overexpression of gp91$^{\text{phox}}$ and p22$^{\text{phox}}$ subunits (18–20).

In conclusion, this study provides evidence for the first time that phagocytic NADPH oxidase–dependent $\text{O}_2^-$ production is increased in patients with metabolic syndrome. Although we cannot exclude the role of other enzymatic sources of oxidant molecules, our results point to an association between phagocytic NADPH oxidase overactivity and oxidative stress and atherosclerosis in these patients. In addition, our findings also suggest that hyperinsulinemia, in association with other proinflammatory and pro-oxidant factors (i.e., tumor necrosis factor-$\alpha$, interleukin-6, MCP-1, etc.), may be implied in the phagocytic NADPH oxidase overactivity.

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