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# Increased phagocytic nicotinamide adenine dinucleotide phosphate oxidase–dependent superoxide production in patients with early chronic kidney disease

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

*Division of Cardiovascular Sciences, Centre for Applied Medical Research, and Departments of Internal Medicine, and Cardiology and Cardiovascular Surgery, University Clinic, School of Medicine, University of Navarra, Pamplona, Spain*

**Increased phagocytic nicotinamide adenine dinucleotide phosphate oxidase–dependent superoxide production in patients with early chronic kidney disease.**

*Background.* Oxidative stress has been implicated in the pathogenesis of atherosclerosis that develops in patients with advanced chronic kidney disease (CKD). This study was designed to investigate whether a relationship exists between phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–dependent superoxide anion ($\bullet O_2^-$) production and subclinical atherosclerosis in patients with early CKD.

*Methods.* Superoxide production was assayed by chemiluminescence under baseline and stimulated conditions on mononuclear cells obtained from asymptomatic patients with stage 1 to 2 CKD ($N = 22$) and healthy controls ($N = 21$). Ultrasonographic determination of carotid intima-media thickness (IMT) was used to assess the presence of atherosclerosis.

*Results.* Although there were no differences in baseline $\bullet O_2^-$ production between controls and patients, the $\bullet O_2^-$ production in phorbol myristate acetate–stimulated mononuclear cells was increased ($P < 0.05$) in patients compared with controls. The phorbol myristate acetate–induced $\bullet O_2^-$ production was completely abolished by apocynin, a specific inhibitor of NADPH oxidase. A direct correlation ($r = 0.441$, $P < 0.05$) was found between plasma insulin levels and NADPH oxidase–mediated $\bullet O_2^-$ production in patients. Carotid IMT was higher ($P < 0.005$) in patients than in controls. Carotid IMT values above the upper normal limit in controls were found in 70% and 40% of patients with increased or normal NADPH oxidase–mediated $\bullet O_2^-$ production, respectively.

*Conclusion.* Generation of $\bullet O_2^-$ that is mainly dependent on NADPH oxidase is abnormally enhanced in patients with early CKD. It is suggested that this alteration could be related to the development of subclinical atherosclerosis in these patients.

Patients with stage 3 to 5 chronic kidney disease (CKD), according to the National Kidney Foundation Practice Guidelines for Chronic Kidney Disease [1], appear to be at greater risk of developing atherosclerosis and its associated morbidity and mortality [2]. Thus, most patients with stage 3 to 5 CKD will die of cardiovascular complications before developing end-stage renal disease [3]. Oxidative stress has been proposed to play a major role in the development of atherosclerosis in these patients [4]. Clinical assessment of oxidative stress in these patients is based on the finding of increased blood concentrations of reactants resulting from oxidation by reactive oxygen species [5].

A number of findings supports the notion that superoxide anion ($\bullet O_2^-$) plays an important role in the pathophysiology of atherosclerosis [6]. For instance, enhanced generation of $\bullet O_2^-$ may facilitate oxidative modification of low-density lipoproteins (LDL), which play a key role in the formation of atherosclerotic lesions [7]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is considered the most important source of $\bullet O_2^-$ in vascular cells [8], and a number of studies have demonstrated the involvement of this oxidase in experimental and clinical atherosclerosis [9]. NADPH oxidase is also the major inducible source of $\bullet O_2^-$ in phagocytic cells, including lymphocytes, monocytes, and neutrophils [9]. Interestingly, enhanced phagocytic NADPH oxidase activity has been recently shown to be associated with carotid atherosclerosis in asymptomatic patients [10].

A recent review of the literature has demonstrated that the association between kidney dysfunction and atherosclerosis-related cardiovascular risk appears early during the evolution of kidney disease [11]. Thus, we have hypothesized that phagocytic NADPH oxidase overactivity may be present in patients with early CKD. To test this hypothesis, NADPH oxidase–mediated $\bullet O_2^-$ production by mononuclear cells was assessed in healthy controls and patients with stage 1 to 2 CKD according to the National Kidney Foundation Practice Guidelines for Chronic Kidney Disease [1].

**Key words:** chronic kidney disease, mononuclear cells, NADPH oxidase.

## METHODS

The study population consisted of two groups of patients who attended for routine medical examination at the University Clinic of Navarra. The CKD group consisted of 22 stage 1 to 2 CKD patients with no clinical manifestations of atherosclerotic disease. All patients exhibited a urine albumin-to-urine creatinine ratio >17 mg/g (men) or >25 mg/g (women) on two measurements. Glomerular filtration rate (GFR) estimated from serum creatinine by abbreviated Modification of Diet in Renal Disease Study equation was ≥90 mL/min/1.73 $m^2$ in patients with stage 1 CKD and <90 and ≥60 mL/min/1.73 $m^2$ in patients with stage 2 CKD. The control group consisted of 21 apparently healthy patients with no pathologic abnormalities or markers of kidney damage. All patients gave written informed consent to participate in the study, and the Institutional Review Committee of the University Clinic approved the protocol. The study conformed to the principles of the Helsinki Declaration.

Blood and urine samples were collected after an overnight (12-14 hour) fast, and biochemical and hormonal parameters were determined by standard laboratory protocols. All patients underwent ultrasonography of the common carotid arteries, and carotid artery intima-media thickness (IMT) was determined as previously reported [12].

Phagocytic cells were isolated from blood samples using a Ficoll-Hypaque gradient. Luminescence assays with lucigenin (10 μmol/L; Sigma, St. Louis, MO, USA) as the electron acceptor were used to measure $\bullet O_2^-$ production in $4 \times 10^5$ mononuclear cells that were incubated at 37°C for 30 minutes alone or in the presence of stimuli or inhibitors, as previously reported [13]. The reaction was started by the addition of lucigenin to cell samples. Luminescence was measured every 11 seconds for 5 minutes in a tube luminometer (Berthold Detection System, Sirius, Pforzheim, Germany). A buffer blank was subtracted from each reading. The determinations were performed under baseline conditions and also after maximum stimulation by the protein kinase C activator, phorbol myristate acetate (PMA, 2 mg/L, Sigma) at 37°C. A kinetic study of baseline and stimulated mononuclear cell response was carried out, and the value of the area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units produced per second (RLU/s). In some experiments, the effect of diphenylene iodonium (5 μmol/L; Sigma), a flavoprotein inhibitor, and apocynin ($2.5 \times 10^{-3}$ mol/L; Calbiochem, Darmstadt, Germany), a specific intracellular inhibitor of NADPH oxidase assembly, were studied. To verify the specificity of the lucigenin assay for $\bullet O_2^-$ production, the effect of superoxide dismutase (SOD, 10000 U/mL, Sigma), an enzymatic scavenger of $\bullet O_2^-$, was examined.

Although lucigenin concentration was low enough to avoid auto-oxidation, the measurements were validated against an independent measurement of $\bullet O_2^-$ production using SOD-inhibitable ferricytochrome c reduction. Mononuclear cells ($4 \times 10^5$) were incubated in 500 μL of buffer containing ferricytochrome c (80 μmol/L, Sigma) at 37°C for 60 minutes in the presence or absence of SOD (10,000 U/mL, Sigma), and finally the absorbance was measured at 550 nm. The PMA-stimulated production measured by lucigenin-enhanced chemiluminescence was closely related with measurements determined in parallel by ferricytochrome c reduction.

Oxidized LDL (oxLDL) concentrations in plasma were measured by a sandwich enzyme-linked immunosorbent assay procedure using the murine monoclonal antibody, mAb-4E6, as the capture antibody bound to microtitration wells, and a peroxidase-conjugated antibody recognizing oxLDL bound to the solid phase (oxLDL; Mercodia AB, Uppsala, Sweden).

Data are reported as means ± standard error. Statistical differences between mean values were tested by the Student *t test* for unpaired data once normality was demonstrated (Shapiro-Wilk test); otherwise, a Mann-Whitney *U* test was used. Categorical variables were analyzed with the $\chi^2$ Fisher's exact test when necessary. Correlation between continuously distributed variables was analyzed by calculation of linear regression and correlation coefficients. The significant level for all tests was taken as $P < 0.05$.

## RESULTS

Arterial hypertension and dyslipidemia were present in 82% and 38% of patients with stage 1 to 2 CKD, respectively. Obesity and diabetes mellitus were diagnosed in 36% and 32% of patients with stage 1 to 2 CKD, respectively. None of these conditions was present in patients from the control group. Smoking was identified in 32% of patients in each group. Thirteen patients with stage 1 to 2 CKD were under treatment with antihypertensive and/or hypolipidemic and/or antidiabetic drugs when included in the study. The remaining nine patients were free of these medications.

The clinical characteristics of the studied patients are summarized in Table 1. Insulin levels were higher ($P = 0.001$) in patients with stage 1 to 2 CKD than in controls. In addition, patients with stage 1 to 2 CKD exhibited increased carotid IMT compared with controls (0.74 ± 0.03 mm vs. 0.62 ± 0.03 mm, $P < 0.005$) (Fig. 1).

The PMA-stimulated $\bullet O_2^-$ production was inhibited by diphenylene iodonium and apocynin. SOD and the protein kinase C (PKC) inhibitor bisindolinmalenide I (10 μmol/L) also abolished the cellular-induced increase

**Table 1.** Clinical and biochemical parameters assessed in control subjects and patients with stage 1 to 2 chronic kidney disease

| | Controls | Patients | *P* |
|---|---|---|---|
| Age *years* | 48 ± 3 | 57 ± 2 | 0.013 |
| Gender *m/f* | 14/7 | 19/3 | 0.151 |
| BMI $kg/m^2$ | 25.5 ± 0.6 | 29.5 ± 0.9 | 0.001 |
| SBP *mm Hg* | 110 ± 2 | 141 ± 4 | 0.001 |
| DBP *mm Hg* | 72 ± 2 | 89 ± 3 | 0.001 |
| Glucose *mg/dL* | 91 ± 2 | 99 ± 2 | 0.014 |
| Total cholesterol *mg/dL* | 220 ± 12 | 230 ± 11 | 0.538 |
| HDL-cholesterol *mg/dL* | 52 ± 2 | 45 ± 10 | 0.019 |
| LDL-cholesterol *mg/dL* | 151 ± 11 | 154 ± 9 | 0.774 |
| Triglycerides *mg/d*L | 83 ± 4 | 130 ± 10 | 0.001 |
| Insulin μ*U/mL* | 7.0 ± 0.4 | 15.8 ± 1.6 | 0.001 |
| Glomerular filtration rate $mL/min/1.73\ m^2$ | 90 ± 4 | 91 ± 4 | 0.875 |
| Urine albumin: urine creatinine *mg/g* | 3.6 ± 0.4 | 33.8 ± 2.4 | 0.001 |

Abbreviations are: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoproteins; LDL, low-density lipoproteins. Values are mean ± standard error or number of subjects.

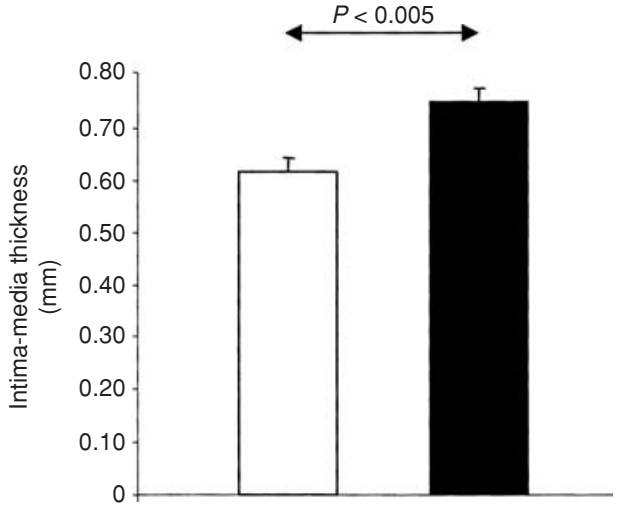


**Fig. 1. Carotid IMT in healthy controls (open bar) and patients with stage 1 to 2 chronic kidney disease (closed bar).** Data are presented as mean + standard error.

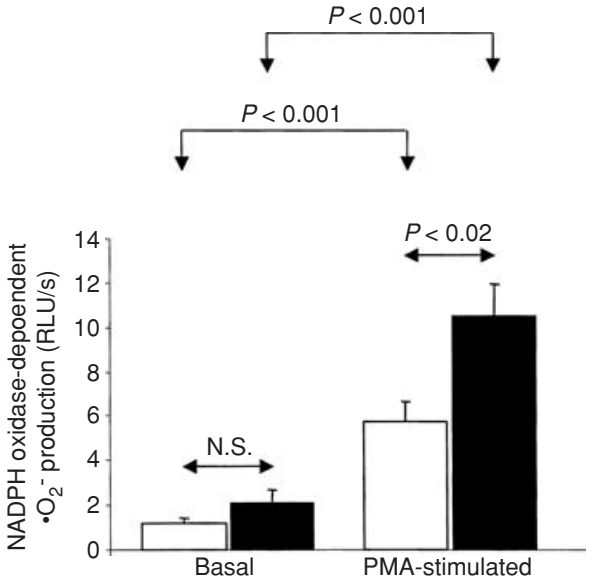


**Fig. 2. Baseline and PMA-stimulated NADPH oxidase–dependent superoxide anion ($\cdot O_2^-$) generation in healthy controls (open bars) and patients with stage 1 to 2 chronic kidney disease (closed bars).** Data are presented as mean + standard error. RLU/s, relative light units per second.

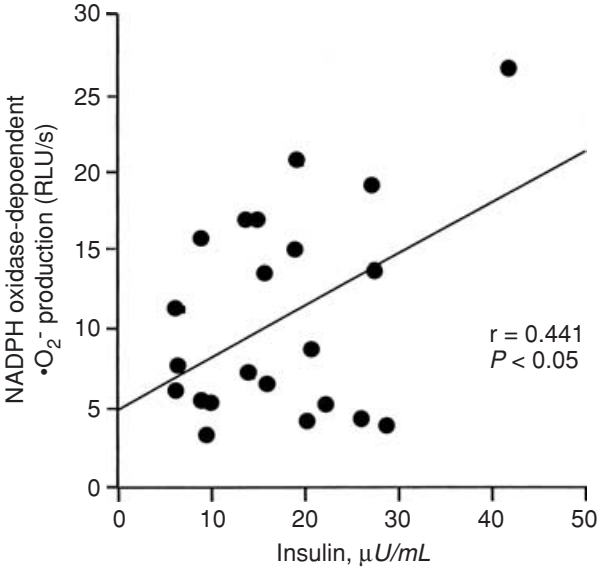


**Fig. 3. Direct correlation (y = 4.9 + 0.3x) between plasma insulin and phorbol-myristate acetate-stimulated NADPH oxidase–dependent superoxide anion ($\bullet O_2^-$) generation in patients with stage 1 to 2 chronic kidney disease.**

in chemiluminescence under PMA-stimulated conditions. No differences in basal levels of chemiluminescence derived from unstimulated cells were observed. Thus, these results suggest that the enzymatic complex of NADPH oxidase may be the enzymatic source of $\bullet O_2^-$ from mononuclear cells under the conditions of the present study.

The values of basal and PMA-stimulated $\bullet O_2^-$ production in mononuclear cells from controls and patients with stage 1 to 2 CKD are shown in Figure 2. Although the levels of $\bullet O_2^-$ production from nonstimulated mononuclear cells were higher in patients with stage 1 to 2 CKD than in controls, the difference did not reach statistical significance. The addition of PMA stimulated $\bullet O_2^-$ production both in controls (1.2 ± 0.2 RLU/s vs. 5.7 ± 0.9 RLU/s, $P < 0.001$) and patients with stage 1 to 2 CKD (2.1 ± 0.6 RLU/s vs. 10.5 ± 1.4 RLU/s, $P < 0.001$). The magnitude of this stimulation was higher ($P < 0.05$) in cells from patients with stage 1 to 2 CKD than in cells from controls. As a consequence, PMA-stimulated $\bullet O_2^-$ production was higher ($P < 0.02$) in patients with stage 1 to 2 CKD than in controls. Values of PMA-stimulated $\bullet O_2^-$ production were above the upper normal limit (13.5 RLU/s)

in 45% of patients with stage 1 to 2 CKD. There was no difference in the values of basal and PMA-stimulated NADPH oxidase–dependent $\bullet O_2^-$ production between treated and untreated patients.

No differences in oxLDL were found between controls and patients with stage 1 to 2 CKD (71.6 ± 6.6 U/L vs. 75.0 ± 5.5 U/L). The oxLDL values were not influenced by treatment in patients with stage 1 to 2 CKD.

A direct correlation ($r = 0.441$, $P < 0.05$) was found between insulin levels and NADPH oxidase activity in patients with stage 1 to 2 CKD but not in controls (Fig. 3). The frequency of patients with carotid IMT values were above the upper normal limit in our laboratory of vascular radiology (0.69 mm) [12] was higher in patients with elevated NADPH oxidase activity than in patients with normal activity (70.5% vs. 40%), respectively, but the difference did not reach statistical significance.

## DISCUSSION

The two major findings of this study are as follows: (1) $\bullet O_2^-$ production is abnormally enhanced in PMA-stimulated mononuclear cells from patients with stage 1 to 2 CKD, and (2) patients with stage 1 to 2 CKD exhibit features of preclinical atherosclerosis (i.e., abnormally increased carotid IMT).

The NADPH oxidase is a multicomponent enzyme that has a membrane portion collectively known as cytochrome $b_{558}$, which is inactive until it is associated with the cytosolic components (see [10] for review). Apocynin is a potent intracellular inhibitor of the assembly of the NADPH oxidase on stimulation, because it impedes the assembly of the $p47^{phox}$ and $p67^{phox}$ subunits within the membrane NADPH oxidase complex [14]. This agent has no known inhibitory effects on the other potential sources of reactive oxygen species [14]. Thus, our findings showing complete inhibition of PMA-stimulated $\bullet O_2^-$ production observed in the presence of apocynin strongly suggest that the enzymatic complex of NADPH oxidase is the source of $\bullet O_2^-$. In accordance with this, results presented here indicate that NADPH oxidase activity is increased in patients with stage 1 to 2 CKD. This observation expands on the previous data by Galli et al [15] who reported on abnormally enhanced NADPH oxidase–mediated $\bullet O_2^-$ production in neutrophils from hemodialysis patients. Collectively, these findings suggest that phagocytic NADPH oxidase may represent an early and maintained alteration in the course of kidney disease.

The exaggerated response to PMA of cells from patients with stage 1 to 2 CKD might be the result of a state of preactivation. In fact, it has been shown that preactivated monocytes from patients with CKD exhibit enhanced reactive oxygen species production and increased release of cytokines on stimulation [16]. Underlying mechanisms for the increased $\bullet O_2^-$ production are unclear, although some data suggest a critical role for PKC-dependent $p47^{phox}$ and $p67^{phox}$ phosphorylation in the regulation of NADPH oxidase activity in mononuclear cells [17]. In accordance with this possibility, we found that increased PMA-stimulated $\bullet O_2^-$ production was blocked by the PKC inhibitor, bisindolinmalenide I.

In addition, potential extracellular stimulating factors of the NADPH oxidase enzymatic system deserve to be considered. On the one hand, there is evidence that hyperinsulinemia may be involved in the generation of oxidative stress [18]. Insulin stimulates NADPH oxidase in human cells, including adipocytes [19] and skin fibroblasts [20]. Furthermore, insulin has been shown to enhance respiratory burst activity induced by PMA in human leukocytes [21]. This is probably because insulin also activates PKC [22]. The potential clinical relevance of these data is remarked by our findings that insulin levels were abnormally enhanced in patients with stage 1 to 2 CKD, and that insulin was associated with NADPH oxidase activity in these patients. On the other hand, in vitro experiments show that advanced oxidation protein products (AOPP) activate NADPH oxidase in human mononuclear cells [23]. Interestingly, it has been reported that in vivo AOPP levels are elevated early in the course of CKD (i.e., GFR >80 mL/min/1.73 $m^2$), increase with the progression of the disease, and are closely related to monocyte activation state [24]. Clearly, further studies are necessary to ascertain whether AOPP contribute to the overactivity of NADPH oxidase seen in patients with stage 1 to 2 CKD.

Evidence substantiates that carotid IMT correlates with the presence of coronary atherosclerosis and that enhanced carotid IMT represents an independent risk factor for coronary heart disease events, stroke, and transient cerebral ischemia [25]. Thus, our finding that carotid IMT is abnormally increased in patients with stage 1 to 2 CKD reinforces the notion that the cardiovascular system becomes affected by atherosclerosis in kidney disease well before the stage of kidney failure is reached [2], and adds further support to the recent observation that the smallest impairment in renal function associated with a significant increase in cardiovascular risk is a GFR of 90 mL/min/1.73 $m^2$ [11].

Upregulated expression of some subunits of the NADPH oxidase and increased activity of the enzyme have been reported in human atherosclerosis [26]. In addition, enhanced phagocytic NADPH oxidase overactivity has been shown recently to be associated with increased carotid IMT in a large sample of 184 asymptomatic patients [10]. Therefore, because NADPH oxidase overactivity and increased carotid IMT did tend to be associated in patients with stage 1 to 2 CKD, it is tempting to speculate that phagocytic NADPH oxidase–derived $\bullet O_2^-$ can be involved in subclinical atherosclerosis in these patients. $\bullet O_2^-$ may contribute to the pathogenesis of atherosclerosis mediating the formation

of oxLDL [27]. However, circulating levels of oxLDL were not increased in patients with stage 1 to 2 CKD compared with controls. Thus, alternative pathways must be considered for the proatherogenic biologic actions of $\bullet O_2^-$ in these patients [17]. Among these, the production of reactive oxygen species induces stress responses that alter phagocyte cell function, including adhesion, proliferation, and motility. $\bullet O_2^-$ is also a very effective scavenger of nitric oxide and can thereby regulate endothelial relaxation and, in the process, also generate highly reactive peroxynitrite. In this regard, some available evidence has led to the proposal that oxidative stress may induce microinflammation and endothelial dysfunction in patients with minor renal dysfunction [28].

In conclusion, this study demonstrates for the first time that the production of $\bullet O_2^-$ by NADPH oxidase is increased in PMA-stimulated mononuclear cells from patients with stage 1 to 2 CKD. Furthermore, our results point to an association between hyperinsulinemia and exaggerated phagocytic NADPH oxidase–dependent $\bullet O_2^-$ production in these patients. Because this study was performed in a small population of patients, further studies in larger samples are required to confirm whether phagocytic NADPH oxidase–mediated oxidant stress contributes to the development of atherosclerosis in the initial stages of CKD.

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*Reprint requests to Dr. Javier Díez, Área de Ciencias Cardiovasculares, Edificio CIMA, Avda. Pío XII, 55 31008 Pamplona, Spain.*
*E-mail: jadimar@unav.es*

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