Role of NADPH Oxidase in Endothelial Ischemia/Reperfusion Injury in Humans

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Background—Reactive oxygen species have been implicated in the pathogenesis of ischemia/reperfusion (IR) injury. Recent studies suggest that NADPH oxidase may be a source of ROS during IR. Using an in vivo model of endothelial IR injury in the arm, we compared the response to IR in healthy volunteers with that in patients with chronic granulomatous disease. These patients have a molecular lesion in a subunit of NADPH oxidase that renders the enzyme inactive.

Methods and Results—Flow-mediated dilatation was used to assess endothelial function in patients with X-linked (NOX2) or autosomal (p47) chronic granulomatous disease. IR injury was induced by 20 minutes of upper limb ischemia followed by reperfusion. Flow-mediated dilatation was determined before IR and after 20 minutes of reperfusion. The response to IR in chronic granulomatous disease patients was compared with that in age- and sex-matched healthy control subjects. Flow-mediated dilatation was expressed as mean and compared statistically with mixed linear models. IR caused a significant reduction in flow-mediated dilatation in control subjects (–5.1%; 95% confidence interval, 6.3 to 3.9%; P<0.001; n=11). A significant reduction in flow-mediated dilatation was not observed in p47-chronic granulomatous disease patients (–1.5%; 95% confidence interval, –3.1 to 0.2; P=0.08; n=6) in contrast to healthy control subjects (–6.5%; 95% confidence interval, –8.2 to –4.9%; P<0.001; n=6).

Conclusions—These data indicate, for the first time in humans in vivo, that reactive oxygen species produced by NADPH oxidase are determinants of endothelial function after IR injury in humans. These findings have implications for the design of strategies to limit clinical IR injury. (Circulation. 2010;121:2310-2316.)

Key Words: granulomatous disease, chronicendothelium ischemia NADPH oxidase reactive oxygen species reperfusion

Reactive oxygen species (ROS) have a central role in the pathogenesis of cardiovascular disease. A common feature of proatherosclerotic states, including hypercholesterolemia, hypertension, and diabetes mellitus, is the ROS-mediated loss of homeostatic functions of the vascular endothelium, which normally acts to promote vasodilatation and to inhibit inflammation and thrombosis. Endothelial dysfunction has been associated with risk factors for the development of atherosclerosis and identified as a predictor of adverse cardiovascular events.

Clinical Perspective on p 2316

Despite the large body of experimental evidence associating diverse cardiovascular risk factors with elevated indexes of oxidative stress, the results of several large clinical trials of ROS scavengers in cardiovascular disease have been disappointing. An alternative therapeutic approach would be to inhibit ROS production directly by targeting specific sources. Recent experimental evidence suggests that there may be a “hierarchy” in ROS-producing enzymes, with a neutrophil-type NADPH oxidase being central to the regulation of superoxide generation from other sources. Unfortunately, this observation has yet to be confirmed in human in vivo studies, mainly because specific inhibitors of NADPH oxidase are not available for use in humans.

One way to overcome this limitation is to study vascular injury in patients with chronic granulomatous disease (CGD). These patients have mutations in genes coding for specific...
NADPH oxidase subunits that cause almost complete disruption in oxidase activity in neutrophils, resulting in recurrent, severe infections. Although the limited number of CGD patients and the clinical course of the disease preclude large cohort studies to compare the incidence of atherosclerosis and adverse cardiovascular events with the general population, these human NADPH oxidase "knockouts" provide the opportunity to investigate the role of the enzyme in acute ROS-mediated cardiovascular injury.

One such form of acute injury is ischemia/reperfusion (IR) injury, which is the major clinical manifestation of atherosclerosis. ROS, generated during reperfusion, have been shown to be pathogenic in animal models of IR injury and have been associated with post-IR endothelial dysfunction. We have developed an in vivo model of endothelial IR that results in transient endothelial dysfunction in conduit and resistance vessels of the arm. In the present study, we used this model to determine the role of NADPH oxidase in IR injury in humans by comparing the endothelial response to IR between healthy control subjects and patients with CGD.

**Methods**

**Subjects**

**Patients**

Seventeen patients with confirmed CGD were recruited from CGD clinics at the Academic Medical Centre, University of Amsterdam (the Netherlands) and University College London Hospital NHS Foundation Trust (London, UK). Eleven CGD patients (all male; mean age, 22±6 years) had mutations in the CYBB gene on the X chromosome that resulted in disruption of the NOX2 (gp91phox) subunit of neutrophil NADPH oxidase. Ten of these patients suffered from the "classic" type of X-linked CGD (NOX20), with no detectable NOX2 protein expression and complete lack of NADPH oxidase activity. One NOX2-CGD patient had the "variant" CGD phenotype (normal protein expression [NOX2+] but almost complete lack of NADPH oxidase activity [2% of normal]; Table 1). These 11 patients formed NOX2 CGD study group 1. The remaining 6 CGD patients (3 male and 3 female patients; mean age, 33±16 years) suffered from the autosomal form of the disease and had mutations in the NCF1 gene on chromosome 7, with resulting molecular lesions in the p47phox subunit of NADPH oxidase. All patients in the p47-CGD group had no p47phox protein expression and <5% of neutrophil NADPH oxidase activity (Table 2). This group of 6 individuals formed study group 2, the p47 CGD group.

In 3 patients in the NOX2-CGD group, the exact CYBB gene mutation was not known. For all other patients, mutation and NADPH oxidase activity data were provided by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam.

All NOX2-CGD patients were on long-term antibacterial (cotrimoxazole) regimen at the time of study. Five NOX2-CGD patients received antifungal medication (itraconazole), and 5 received interferon-γ-1b as part of standard treatment. In the p47-CGD group, the majority of patients took no medication, and only 2 were on cotrimoxazole at the time of study. None of the CGD patients had a history of hypertension, diabetes mellitus, or hypercholesterolemia.

**Table 1. Summary of X-Linked CGD Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>CGD Type</th>
<th>CYBB Mutation</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Activity, % of Normal</th>
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<td>C793→T</td>
<td>Glu261→Stop</td>
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<td>M</td>
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<tr>
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<td>Glu377→Stop</td>
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<td>Missense</td>
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<td>Pro339→His</td>
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<td>M</td>
<td>26</td>
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<td>Splice</td>
<td>gtaag→gtaaa</td>
<td>Deletion exon 3</td>
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<td>NOX20</td>
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<td>...</td>
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</tbody>
</table>

Nucleotide and amino acid changes are represented with respect to normal (wild-type) sequence.

**Table 2. Summary of Autosomal CGD Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, y</th>
<th>CGD Type</th>
<th>NCF1 Mutation</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Activity, % of Normal</th>
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</thead>
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<td>p47/H/Z</td>
<td>Deletion</td>
<td>(GT 75,76)</td>
<td>Frameshift</td>
<td>&lt;5</td>
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<td>55</td>
<td>p47/H/Z</td>
<td>Deletion</td>
<td>(GT 75,76)</td>
<td>Frameshift</td>
<td>&lt;5</td>
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<tr>
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<td>51</td>
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<td>Deletion</td>
<td>(GT 75,76)</td>
<td>Frameshift</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>17</td>
<td>p47/H/Z</td>
<td>Deletion</td>
<td>(GT 75,76)</td>
<td>Frameshift</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>35</td>
<td>p47/H/Z</td>
<td>Deletion</td>
<td>(GT 75,76)</td>
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<tr>
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<td>M</td>
<td>25</td>
<td>p47/H/Z</td>
<td>Deletion</td>
<td>(GT 75,76)</td>
<td>Frameshift</td>
<td>&lt;5</td>
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</table>

p47 indicates autosomal recessive, no p47phox protein expression; H/Z, homozygous. Data were provided by the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam.
and all were life-long nonsmokers. Their body mass index was 22.9±2.1, and none were on vasoactive medication.

**Healthy Volunteers**
Seventeen healthy volunteers in total were recruited as controls and matched 1 to 1 with patients for age and sex. Eleven healthy subjects matched to the NOX2-CGD group (study group 1) had a mean age of 22±5 years, and 6 volunteers matched to the p47-CGD group (study group 2) had a mean age of 33±15 years. None of the healthy volunteers had a history of hypertension, diabetes mellitus, or hypercholesterolemia, and all were life-long nonsmokers. Their average body mass index was 23.2±2.5, and none were on vasoactive medication. NADPH oxidase activity was measured at the Centre for Molecular Medicine, University College London as described previously.20

Studies were approved by local research ethics committees, and all individuals gave informed consent. Studies were performed in temperature-controlled laboratories (24°C to 26°C) at the Department of Vascular Medicine, Academic Medical Centre, University of Amsterdam and the Vascular Physiology Unit, Institute of Child Health, University College London.

**Assessment of Conduit Vessel Function**
Conduit vessel endothelial function was assessed by measuring flow-mediated dilatation (FMD) of the brachial artery in the non-dominant arm as previously described.31

**Assessment of the Effect of IR on Conduit Vessel Endothelial Function**
The nondominant arm was rendered ischemic by inflating a 9-cm-wide blood pressure cuff placed around the upper part of the arm to a pressure of 200 mm Hg for 20 minutes, after which the cuff was deflated, allowing the arm to reperfuse. To determine the effect of IR on endothelial function, FMD was assessed before ischemia (baseline) and at 20 minutes after reperfusion. We have previously demonstrated that this protocol results in brachial artery endothelial dysfunction but does not have an effect on vascular smooth muscle function because it does not alter the vascular dilator response to glyceryl trinitrate.22

**Statistical Analysis**
Data analysis was performed at the Vascular Physiology Unit, Institute of Child Health, University College London by a single operator blinded to subject group allocation. Brachial artery diameter was measured in millimeters, and dilatation was expressed as percentage increase from baseline diameter. The FMD flow stimulus during reactive hyperemia was expressed as the ratio of peak to baseline volume flow per minute. Mixed linear models were used to compare baseline characteristics, pre- and post-IR FMD, and hemodynamic parameters in the study groups, taking into account repeated measures, individual matching, and data being nested in each individual. The model assessed outcomes (Yijv) measured at visit V (baseline, V=0; follow-up visit, V=1), for person i in matched pair j, using random effects for pair j and person i and a fixed effect for the time of visit, and group (G, with CGD coded as 1 and others as 0) with an interaction term to model the difference in effect over time within matched pairs:

\[ Y_{ijv} = \mu + G_{ij} + \epsilon_{ijv} \]

\[ \beta_{ij} = \beta_{0} + \beta_{1} \times G_{ij} + \epsilon_{ijv} \]

\[ V_{ijv} = \frac{\max{V_{ijv}}}{\min{V_{ijv}}} \]

For both data sets, random-intercept and random-slopes models were compared with the nested random-intercept models using likelihood ratio tests to select the best model to fit the data. There was no evidence that the effect over time was dependent on person or group. Normality of residuals was checked at each stage. In all cases, values of P<0.05 were considered statistically significant. All data are expressed as mean and 95% confidence interval (CI) unless otherwise stated.

**Results**
All subjects tolerated the procedures without any complications.

**Baseline Characteristics**
There were no differences between the CGD and healthy control groups in baseline systolic blood pressure, diastolic blood pressure, heart rate, and brachial artery diameter (data summarized in Tables 3 and 4). NADPH oxidase activity in healthy volunteers was 7.2±0.3 nmol O₂/10⁶ cells per minute (n=10).

**Effect of IR on Brachial Artery Endothelial Function**
Overall, a random-intercept model was the most parsimonious fit to the data in both data sets, using random effects for individuals and further individual random effects to account for 1-to-1 matching of patients and healthy control subjects. There was no evidence of person-specific variation in changes in FMD or any of the other measured traits over time for both data sets. The likelihood ratio test suggested that a random-intercept model was most parsimonious (P=0.46 for the NOX2-CGD group, P=0.83 for the p47-CGD group). All residuals satisfied normality. For both data sets, residual variation in FMD is accounted for by differences between individuals and between time points. Within matched pairs, there was very strong evidence of a difference in pre- and post-IR FMD (P<0.001), indicating evidence of a difference in vascular response between individuals with CGD and healthy control subjects.

**NOX2-CGD Group**
IR did not affect blood pressure, heart rate, arterial diameter, and flow stimulus in both CGD patients and matched control subjects.

![Table 3. Summary of Pre-IR and Post-IR Data From Studies in X-Linked CGD Patients (NOX2-CGD) and Healthy Control Subjects (n=11)](image_url)
subjects (Table 3). There was no evidence for a difference in pre-IR FMD between patients and healthy control subjects (\(\Delta\text{FMD [pre-IR NOX2-CGD−pre-IR control]}=1.1\%; 95\% \text{ CI, } -1.1 \text{ to } 3.4; \ P=0.32\)). IR resulted in FMD reduction in controls (\(\Delta\text{FMD [post-IR−pre-IR]}=-5.1\%; 95\% \text{ CI, } -6.3 \text{ to } -3.9; \ P<0.001; \text{ Figure 1A}) but had no effect on endothelial function in patients with NOX2-CGD (\(\Delta\text{FMD [pre-IR−post-IR]}=-0.9\%; 95\% \text{ CI, } -2.1 \text{ to } 0.3; \ P=0.12; \text{ Figure 1B}) \). The mean difference of \(\Delta\text{FMD (post-IR minus pre-IR) between the 2 groups was 4.2\% (95\% CI, 2.5 \text{ to } 5.6; \ P<0.001). There was strong evidence for interaction between CGD status and change in FMD over time (\(P<0.001\)), with a significant difference in post-IR FMD between patients and healthy individuals (\(\Delta\text{FMD [post-IR NOX2−post-IR control]}=4.4\%; 95\% \text{ CI, } 2.5 \text{ to } 5.9; \ P<0.001\)).

### p47-CGD Patient Group
IR did not affect blood pressure, heart rate, arterial diameter, and flow stimulus in both CGD patients and matched control subjects (Table 4). There was no evidence for a difference in baseline (pre-IR) FMD between patients and healthy control subjects (\(\Delta\text{FMD [pre-IR p47-CGD−pre-IR control]}=0.5\%; 95\% \text{ CI, } -3.7 \text{ to } 2.7; \ P=0.74\)). IR resulted in FMD reduction in the control group (\(\Delta\text{FMD [pre-IR−post-IR]}=-6.5\%; 95\% \text{ CI, } -8.1 \text{ to } 4.9; \ P<0.001; \text{ Figure 2A}) but had no effect on endothelial function in p47-CGD patients (\(\Delta\text{FMD [pre-IR−post-IR]}=-1.5\%; 95\% \text{ CI, } -3.1 \text{ to } 0.12; \ P=0.08; \text{ Figure 2B}) \). The mean difference of \(\Delta\text{FMD (post-IR minus pre-IR) between the 2 groups was 5.1\% (95\% CI, 2.7 \text{ to } 7.4; \ P<0.001). As for the NOX2-CGD group, there was strong evidence for a statistical interaction between time and group (\(P<0.001\)), with a significant difference in post-IR FMD between patients and healthy individuals (\(\Delta\text{FMD [post-IR p47-CGD−post-IR control]}=5.1\%; 95\% \text{ CI, } 2.7 \text{ to } 7.34; \ P<0.001\)).

### Discussion

This study demonstrates, for the first time, that disruption of NADPH oxidase prevents endothelial IR injury in humans in vivo. Brachial artery endothelial function was preserved after IR in patients with CGD, which was in contrast to what was observed in healthy control subjects. These observations suggest that ROS generated by NADPH oxidase are pathogenic in human IR injury.

ROS play an important role in the pathophysiology of IR injury.\textsuperscript{23,24} The rapid reintroduction of molecular oxygen in postischemic tissues results in a “burst” of ROS during the first minutes of reperfusion.\textsuperscript{25,26} Evidence consistent with ROS-mediated injury during IR is indirect and based on a reduction of injury by ROS scavengers\textsuperscript{27,28} or genetic overexpression of antioxidant enzymes.\textsuperscript{29} However, attempts to extend experimental concepts to clinical IR injury have been largely unsuccessful.\textsuperscript{30,31} Although this has added to the skepticism about the significance of ROS in cardiovascular disease, the lack of specificity of chemical antioxidants, the limited tissue penetration of enzymatic antioxidants, and uncertainties about the dose or timing of these agents limit the
conclusions that can be drawn from negative clinical studies. Understanding the complex mechanisms of oxidative stress in humans is fundamental to overcome these limitations.

Major cellular sources of ROS in the cardiovascular system include NADPH oxidase, xanthine oxidoreductase, endothelial nitric oxide synthase, and myeloperoxidase. Although their relative contribution in cardiovascular injury (acute or chronic) is not clear at present, emerging evidence supports a crucial role for NADPH oxidase. Superoxide generated by NADPH oxidase has been shown to modulate ROS production from xanthine oxidoreductase (by promoting conversion to the oxidase isoform) and endothelial nitric oxide synthase (by oxidation of tetrahydrobiopterin and uncoupling). This makes NADPH oxidase a potentially important target for therapeutic intervention.

NADPH oxidase is a superoxide-producing enzyme that was first identified in neutrophils, where it is involved in nonspecific host defense against microbes. Five isoforms of NADPH oxidase (called NOX) have been identified in a variety of tissues. All isoforms consist of an NOX subunit (NOX1 through NOX5) and a smaller p22phox subunit. NOX2 (gp91phox) is found in the phagocytic NADPH oxidase but is also present in endothelial cells and cardiomyocytes. NOX1 and NOX4 may also be important in the cardiovascular system because they are expressed in endothelial cells, vascular smooth muscle cells, and cardiomyocytes. Activation of NOX1 and NOX2 requires association of other cytoplasmic regulatory subunits, including p47phox (NOX2 and possibly NOX1), the p47phox homolog NOXO1 (NOX1), and Rac (NOX1 and NOX2). Little is known about the regulatory mechanisms of NOX4, although it appears to be constitutively active and does not require any regulatory subunits for activation.

NADPH oxidases have been shown to be a major source of superoxide in the majority of cardiovascular disease models, including IR. NOX2 is upregulated in human cardiomyocytes after myocardial infarction, and studies in isolated endothelial and vascular smooth muscle cells subjected to IR have demonstrated significant ROS production that could be blocked by inhibition of NADPH oxidase activity. Studies in NADPH oxidase “knockout” mice demonstrated attenuated IR-induced injury in the myocardium, lung, liver, and brain of NOX2−/− mice and in the myocardium, liver, and brain of p47phox−/− mice. In addition, studies in transgenic mice with hepatic overexpression of dominant-negative Rac demonstrated a reduction in liver necrosis after IR.

To investigate the role of NADPH oxidase in human endothelial IR injury, we used our well-established in vivo model in a cohort of patients with CGD. These patients have mutations in genes coding for the NOX2 and p47phox subunits, resulting in almost complete loss of oxidase activity in neutrophils. In contrast to the substantial IR-induced reduction in FMD observed in healthy volunteers, post-IR FMD in CGD patients was not significantly different from baseline. The endothelial response to IR was similar in all CGD patients regardless of whether vascular or neutrophil NADPH oxidase activity was impaired, suggesting that the endothelial response to IR in CGD patients cannot be attributed to a lack of oxidase activity. However, some of the NOX2 CGD patients were treated with itraconazole at the time of the study. Antifungal imidazoles have been shown to inhibit endothelial nitric oxide synthase and to affect FMD in vitro. Despite this finding, there were no differences in baseline FMD between treated/untreated CGD patients and healthy volunteers, and the endothelial response to IR was similar in all CGD patients regardless of treatment. This makes it unlikely that baseline differences in drug therapy have influenced our results.

Uncertainties remain about the precise role of other NOX enzymes (lack of NOX2 or p47phox may not result in full...
disruption of NADPH oxidase activity) and other sources of ROS (xanthine oxidoreductase, endothelial nitric oxide synthase, myeloperoxidase) in endothelial IR injury because we did observe a small (although nonsignificant) reduction in FMD after IR in CGD patients. Moreover, there are insufficient clinical data in CGD patients to determine whether they are protected from more substantial IR injury such as that caused by acute myocardial infarction or stroke. However, our study indicates a key role for ROS derived from NADPH oxidase in endothelial IR injury in humans. Replication of our findings in patients at higher cardiovascular risk would further support the development of antioxidant strategies targeting NADPH oxidase to reduce IR injury in the clinical setting.

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Disclosures
None.

References


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