Unstable forms of oxygen are highly reactive and have been widely implicated in disease pathogenesis. Indeed, interest in detecting their formation extends beyond this planet.1 Given their intrinsic evanescence, the generation of reactive oxygen species (ROS) in vivo has been inferred by the quantitation of products of their reaction with lipids,2 proteins,3 and DNA.4 Such data have implicated ROS in cardiovascular diseases such as atherosclerosis5–8 and ischemia-reperfusion syndromes.9,10 Evidence consistent with increased ROS generation based on such methodology has emerged in individuals with cardiovascular risk factors such as hypercholesterolemia,11,12 cigarette smoking,13,14 and alcoholism.15 Despite this, the outcome of trials of antioxidants as protective agents in cardiovascular diseases have been contradictory.16–18 However, a limitation of these trials is that none of them have included an assessment of ROS generation, either as an entry criterion or as a basis for dose selection. As in model systems in vitro,19 the response of humans to exogenous antioxidants is highly conditioned by the extent to which endogenous antioxidant defenses are depleted.20,21 Thus, inclusion of phenotypically inappropriate patients in such trials may have undermined sample size calculations and, consequently, the basis of statistical inference.

If the functional competence of the diverse enzymes that comprise our defense against ROS is a critical determinant of our capacity to respond to exogenous antioxidants, genetic variations in the activity or expression of such enzymes may contribute to interindividual differences in susceptibility to ROS-mediated diseases. Reduced activity of the superoxide dismutase expressed in endothelial cells has been reported in patients with coronary artery disease (CAD) and correlates with their degree of endothelial dysfunction.22 Similarly, serum levels of paraoxonase (PON) 1, an enzyme that protects low density lipoprotein from oxidative modification, are low in patients with CAD relative to controls.23 In this instance, a PON promoter polymorphism that defines a low-expression phenotype mandates the low levels of PON activity in patients with CAD. However, the story becomes more complicated. The promoter polymorphism interacts significantly with a second polymorphism, this time in the coding sequence, which itself is an independent risk factor for CAD. The presence of the risk-conferring promoter variant seems to neutralize partly the risk associated with the coding sequence polymorphism.24 Environmental factors usually add to the mix by enhancing the impact of “at risk” genotypes. Thus, the relationship between polymorphisms in hemostatic proteins and phenotypic changes that contribute to the pathogenesis of cardiovascular disease is often strengthened in smokers.25,26 Although such a relationship has not been established for antioxidant enzymes, smoking increases ROS generation in vivo13,14 and is an independent risk factor for lower PON activity and concentration in patients with CAD.27

The article by Guzik et al28 in a recent issue of Circulation touches on an aspect of a similarly complex scenario: that genetic variability in enzymes which generate ROS might contribute to interindividual susceptibility to CAD. NAD(P)H oxidases are membrane-associated enzymes that catalyze the 1-electron reduction of oxygen using either NADH or NADPH as the electron donor, and they are the major oxidases in vascular tissue.29,30 NAD(P)H oxidase comprises several distinct subunits; gp91phox and p22phox are electron-transfer proteins, and both are expressed in endothelial cells.31 Two additional cytosolic proteins, p47phox and p67phox, together with the low-molecular-weight G protein rac2, are also present in vascular cells32–34 and associate with and modulate the function of the aforementioned 2 phox subunits. Although these proteins are homologous with those expressed in phagocytic neutrophils, cell-specific isoforms exist. The electron-transfer proteins are predominantly intracellular in endothelial cells, whereas those in neutrophils are extracellular. This may explain cell-specific differences in the function of the 2 enzymes. For example, the capacity to generate $O_2^-$ is lower in vascular cells,35 in which the enzyme activity seems to be constitutively expressed.36 Humoral factors, such as angiotensin 11, thrombin, and platelet-derived growth factor, regulate the expression of NAD(P)H oxidases in the vasculature, as was reviewed nicely by Griendling et al.37 Given its importance in generating singlet oxygen in vascular tissues in vitro, one might anticipate that polymorphisms which disrupt the function of NAD(P)H oxidase might reduce the likelihood of developing ROS-associated diseases such as atherosclerosis.

Two potentially interesting polymorphisms in the p22phox component of the NAD(P)H oxidase have been studied. The first, and the subject of the article by Guzik et al,28 is a C242T nucleotide transition that results in the substitution of histidine-72 with tyrosine, thereby modifying 1 of the 2 heme-binding sites that is thought to be essential for the
stability of the protein.\textsuperscript{37} This might be expected to reduce the generation of $O_2^-$ and be associated with a reduced incidence of atherosclerosis. Indeed, Inoue et al.\textsuperscript{40} reported that the risk of CAD was lower in individuals carrying the T allele (TT plus CT individuals) in a Japanese case-control study of 201 individuals in each group. However, a similarly sized case-control study by Ito et al.\textsuperscript{49} reported an increased risk of stroke in association with the T allele. Although ethnic and geographic factors are unlikely to be relevant to the disparity of these results obtained in Tokyo and Kobe, they may contribute to differences across continents. Indeed, the T allele frequency in Japanese seems much lower than that in whites.\textsuperscript{39} Thus, there seemed to be no difference in T allele frequency in a US study of 149 mostly (83\%) white patients with CAD and 103 controls, and no relationship between genotype and endothelial function.\textsuperscript{40} Similarly, Gardemann and colleagues\textsuperscript{41} found no difference in the frequency of CAD or myocardial infarction as a function of the C242T polymorphism in a study of $>$2000 German subjects. They did, however, find that the second polymorphism, A640G, which occurs in 3'UTR, was associated with CAD, especially in young individuals in whom genetic contributions to risk are usually more prominent. More recently, a study of 689 Australians found an increase in the frequency of the T allele in patients <45 years of age who have CAD,\textsuperscript{42} and an assessment of 368 Americans randomized to placebo therapy indicated that those with the T allele had more progression and less regression of angiographically visualized CAD over the 2.5-year period of the study.\textsuperscript{43} A surprising and paradoxical feature of the latter study was that there was no baseline inequality in disease severity as a function of C242T genotype status, at which time a pathogenic influence of the T allele might have been expected to have influenced the clinical phenotype.

Many factors might explain the discrepancies described above. All of the studies were small for investigations of this kind. Perhaps their outcomes merely represent random variation about the mean. Similar disarray was apparent in the results of small studies seeking an association of polymorphisms in the ACE gene with CAD. The oxidase studies differ not only across ethnic groups, but also by definition of cases and controls and in methods of statistical analysis. In the present study, Guzik and colleagues\textsuperscript{28} address a more fundamental issue relating to the biological significance of the C242T polymorphism: does the T variant actually result in diminished generation of $O_2^-$, as might be anticipated from the role of heme binding in maintaining the stability of the enzyme complex? They addressed this question by measuring $O_2^-$ using a lucigenin-enhanced chemiluminescence assay and observed that basal activity was reduced by $\approx$40\% and that induced activity was reduced by $\approx$20\% when the NADH substrate was added to activate the enzyme. However, the accuracy of this assay method has been criticized: among other limitations, lucigenin can actually generate $O_2^-$. Although it lacks the specificity of other approaches that are based on electron paramagnetic resonance,\textsuperscript{44,45} the results do accord with the a priori hypothesis—based loss of the heme binding site. Previous work showing that p21phox is expressed in vascular cells was confirmed in the study by Guzik et al.\textsuperscript{28} Thus, established risk factors for CAD, such as hypercholesterolemia, smoking, and diabetes, are associated with increased vascular NAD(P)H oxidase activity\textsuperscript{37} and ROS generation in vivo; however, the authors suggest that a mild functional variant of the same enzyme independently increases risk for CAD. Something seems awry with this contention.

There are obvious limitations to the study by Guzik et al.\textsuperscript{28} The numbers of samples analyzed are small, the data are fragmentary, the assay method is imprecise, and the measurements were performed ex vivo. Data were obtained from both the mammary artery and the saphenous vein. Although the trends were similar, $O_2^-$ generation, irrespective of genotype, was considerably lower in samples from the artery than in those from the vein. Extrapolation of such reduced measurements to an association of the T allele with progression (which the authors favor, but do not address in the present study) of arterial disease may be tricky and unwarranted. It is possible that such “counterintuitive” results might be elucidated by indirect measurements of ROS generation in vivo. For example, the capacity of atherosclerotic vasculature to generate prostacyclin is reduced ex vivo but, given the imbalance between capacity and actual biosynthetic rates in vivo, it is unsurprising that actual in vivo biosynthesis is increased in individuals with severe atherosclerosis due to accelerated platelet-vessel wall interactions.\textsuperscript{46}

Clearly, we still need clarity on 2 issues. First, are either of the NAD(P)H oxidase polymorphisms associated with increased or decreased cardiovascular risk? This answer will emerge only from studies that are sufficiently large to address the question in distinct ethnic subgroups. Second, what is the effect of these polymorphisms on ROS generation in arterial tissue ex vivo and in vivo in the same individuals? Technologies have emerged that will enable investigators to address these issues with increased specificity and quantitative precision. Genetic variation in pro-oxidant and antioxidant enzymes may contribute substantially to the impact of environmental variables on the individual risk of disease. Ultimately, the screening of “at risk” populations such as those discussed here may be of value. However, the reduction of this hypothesis to clinical practice is not yet ready for general release.

References

7. Rong JX, Rangaswamy S, Shen L, et al. Arterial injury by cholesterol oxidation products causes endothelial dysfunction and arterial wall cho-

Downloaded from http://circ.ahajournals.org by guest on April 16, 2017


25. Humphries SE, Whitehead and FitzGerald Screening for Phox 9


**KEY WORDS:** Editorials ■ genetics ■ oxygen
Twenty-First Century Phox: Not Yet Ready for Widespread Screening
Alexander Steven Whitehead and Garret A. FitzGerald

Circulation. 2001;103:7-9
doi: 10.1161/01.CIR.103.1.7

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/103/1/7

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/