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stable 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,11–13 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 patho-
genesis 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 intra-
cellular 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. This might be expected to reduce the generation of $O_2^-$ and be associated with a reduced incidence of atherosclerosis. Indeed, Inoue et al. 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. 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. 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. Similarly, Gardemann and colleagues 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, 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. 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 oxidative 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 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 ≈40% and that induced activity was reduced by ≈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. 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. Thus, established risk factors for CAD, such as hypercholesterolemia, smoking, and diabetes, are associated with increased vascular NAD(P)H oxidase activity 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. 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.

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.

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