Basic and clinical research has established that an increase in plasma homocysteine (Hcy) is an independent and graded risk factor for cardiovascular disease. McCully was the first to propose such a relationship in 1969 after finding postmortem evidence of atherosclerosis in patients with hyperhomocysteinemia (HHcy). Subsequent studies have linked even mildly or moderately elevated plasma concentrations of Hcy, a sulfur-containing amino acid produced via methionine metabolism, to coronary artery disease, peripheral vascular disease, venous thrombosis, peripheral vascular disease, venous thrombosis, myocardial infarction, and stroke, but the mechanism by which HHcy increases one’s risk for heart disease remains unclear.

Nitric oxide (NO) has been established as an important molecule in the regulation of cardiovascular function. In addition to its prominent roles in controlling vascular tone and platelet aggregation, NO regulates tissue O2 consumption by reversibly inhibiting cytochrome c oxidase in the mitochondrial respiratory chain. In the cardiovascular system, NO is synthesized in the thin endothelial lining of blood vessels by endothelial NO synthase (eNOS), which acts on the substrate L-arginine; however, NO reacts rapidly with reactive oxygen species, and the degradation of NO by superoxide (O2−) has been shown in studies on atherosclerosis, hypertension, hypercholesterolemia, heart failure, cardiac hypertrophy, and aging. These articles implicate NADPH oxidase—a large enzyme composed primarily of 2 membrane-bound subunits (p22phox and gp91phox), 3 cytosolic subunits (p40phox, p47phox, and p67phox), and GTP-binding proteins (such as rac-1)—as a primary enzymatic source of O2− in cardiovascular disease states.

Recent studies have also detected elevated levels of O2− in HHcy, which results in impaired NO-dependent vasodilation. Such dysfunction of coronary endothelium-mediated relaxation is a strong predictor of atherosclerotic disease and future cardiovascular events. However, the means by which O2− levels are increased in HHcy have not yet been elucidated, and because the hypertension of HHcy is less profound than that after complete NO synthesis inhibition, it is plausible that only certain functions of NO activity are altered. Therefore, the causes, extent, and consequences of NO inactivation in HHcy all require further investigation.

We hypothesized that the ability of NO to regulate myocardial O2 consumption is impaired in rats and mice with...
methionine diet–induced HHCy and that this is \textit{O}_2^- dependent. To investigate this contention, we included studies directed toward (1) the effect of antioxidant treatment on NO bioactivity, (2) the quantification of various proteins involved in the synthesis and degradation of \textit{O}_2^-, (3) the assessment of hemodynamic function, and (4) the effect of HHCy in genetically modified mice that lack a functional gp91\textsuperscript{phox} subunit of NADPH oxidase.

**Methods**

Male Wistar-Kyoto rats were obtained from Jackson Laboratories (Bar Harbor, Me) at an age of 3 months and fed for 5 to 8 weeks. To raise the plasma Hcy levels of the experimental group, the amino acid methionine was added to the drinking water of half the animals (9 g/L). Additional experiments were performed in genetically modified B6.129S6-Cybb\textsuperscript{tm1Din/J} mice (Jackson), which exhibit the X-linked chronic granulomatous disease because they lack a functional gp91\textsuperscript{phox} component to assemble NADPH oxidase.

**Cardiac Tissue Preparation and Measurement of \textit{O}_2 Consumption**

Rats and mice were anesthetized by injection of sodium pentobarbital (50 mg/kg). From the parasternal long-axis view, images by the 2D-guided M-mode cursor were used to measure left ventricular (LV) dimensions in addition to interventricular septum and posterior wall thickness. All M-mode data were collected from an average of 3 consecutive cardiac cycles. Additionally, images from the bidimensional parasternal long-axis view were used to measure the LV diastolic (LVDA) and systolic (LVSA) areas. Ejection fraction was then calculated as (LVDA – LVSA)/LVDA and expressed as a percent.

**Effect of NO on Myocardial \textit{O}_2 Consumption**

In rat hearts, myocardial \textit{O}_2 consumption was measured at baseline and in response to cumulative doses (10\textsuperscript{-5} to 10\textsuperscript{-7} mol/L) of 1 of 3 drugs that release NO: the \textit{B}\textsubscript{2} kinin receptor agonist bradykinin (Sigma-Aldrich; n=12), the muscarinic 2 receptor agonist carbachol (Sigma-Aldrich; n=7), or the exogenous NO donor \textit{S}-nitroso-\textit{N}-acetyl-penicillamine (SNAP; Sigma-Aldrich; n=5). These responses were also examined in the presence of the NO synthase inhibitor \textit{N}-nitro-l-arginine methyl ester (L-NAME; Sigma-Aldrich; n=7 for bradykinin and carbachol, n=5 for SNAP), which was added in a concentration of 10\textsuperscript{-4} mol/L to the tissue bath.

In hearts from wild-type and gp91\textsuperscript{phox}-knockout mice, myocardial \textit{O}_2 consumption was again measured at baseline and in response to cumulative doses (10\textsuperscript{-5} to 10\textsuperscript{-7} mol/L) of bradykinin. Responses were also examined in the presence of L-NAME.

**Effect of Antioxidants on NO-Regulated Myocardial \textit{O}_2 Consumption**

Plasma Hcy was quantified in rats (n=16) and mice (n=9) with a homocysteine microplate enzyme immunoassay (Bio-Rad Laboratories). All mixed disulphide and protein-bound forms of Hcy in plasma samples were first reduced by dithiothreitol to form free Hcy and then converted enzymatically to \textit{S}-adenosyl-l-homocysteine (SAH) by SAH hydrolase and excess adenosine. Once placed inside the wells of a microtiter plate, the SAH in the sample competed with SAH bound to the walls of each well for a monoclonal anti-SAH antibody. The plate was then washed, thereby removing any antibody not bound to the wall, and a secondary rabbit anti-mouse antibody labeled with horseradish peroxidase (HRP) was added. HRP activity was assessed with a Power Wave 200 spectrophotometer (Bio-Tek Instruments) at 450 nm after addition of the substrate tetramethylbenzidine. Because absorption by HRP was directly proportional to the quantity of anti-SAH antibody bound to the walls of the microtiter plate, it was inversely related to the level of Hcy in the original sample. Five control samples (with Hcy concentrations of 2, 4, 8, 15, 30, and 50 μmol/L) were run in this immunoassay to relate Hcy concentration to absorption by HRP, and a dose-response logistic curve was generated (SliWrite Plus Version 6.00) to calculate the Hcy concentration of the experimental samples.

**Total Protein Western Blotting**

After being immersed in liquid nitrogen at ~80°C, myocardial tissues (n=12 for methionine-fed rats; n=9 for control rats) were manually ground and subsequently homogenized in lysis buffer (0.05 mol/L Tris-HCl at pH 7.2, 1 mmol/L EDTA, 10 mmol/L dithiothreitol, 1 mg/mL PMSF, 100 μg/mL leupeptin, 100 μg/mL soybean trypsin inhibitor, and 20 μg/mL aprotinin) 3 times for 15 seconds. After 1 minute of sonication, samples were centrifuged for 10 minutes at 13,000 rpm, and the protein concentrations of the resulting supernatants were assessed with the Bio-Rad Protein Assay. Solutions of supernatant and loading dye were prepared to contain 75 μg of protein and then loaded into separate lanes of a 10% polyacrylamide gel that contained the detergent SDS. Standard techniques for electrophoresis (150 V) and Western blotting were used to separate proteins by molecular weight and transfer them to

**Plasma Hcy**

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polyvinylidene difluoride membranes. After 1 hour of incubation with 5% milk/PBS to block all remaining protein-binding sites, the membrane was allowed to incubate overnight in 1% milk/PBS with antibodies to eNOS (BD Transduction Laboratories; 1:1000 dilution), Cu/Zn SOD (SOD-1; BD Transduction; 1:5000 dilution), Mn SOD (SOD-2; CalBioChem-NovaBioChem Corporation; 1:10000 dilution), or 1 of the following subunits of NADPH oxidase: p22phox (Santa Cruz Biotechnology; 1:2500 dilution), gp91phox (BD Transduction; 1:1000 dilution), p40phox (Santa Cruz; 1:1500 dilution), p47phox (Santa Cruz; 1:1500 dilution), p67phox (Upstate, Inc; 1:2000 dilution), or rac-1 (Santa Cruz; 1:5000 dilution). Finally, a secondary antibody labeled with HRP was added (Upstate, Inc; 1:2000 dilution), or rac-1 (Santa Cruz; 1:5000 dilution). Images were scanned and absorption was calculated with the Alphamager 2000 program (Alpha Innotech Corporation).

**Statistical Analysis**

Differences from control were calculated with an unpaired *t* test (Microsoft Excel), and significance was determined at *P* < 0.05. Regression analyses, including calculation of the *r* value, were performed with the least-squares test (SlideWrite).

**Results**

**Hemodynamic Measurements in Rats**

All hemodynamic data are shown in the Table. Blood pressure and heart rate in conscious resting rats did not differ significantly between the control and methionine-fed groups. The ratio of heart weight to body weight was similar between the 2 groups, and echocardiography revealed that methionine feeding did not cause any significant changes in LV dimensions, wall thickness, ejection fraction, or fractional shortening.

**Effect of Bradykinin or Carbachol on O2 Consumption**

LV tissues from both control and methionine-fed rats consumed O2 from a HEPES-containing Krebs bicarbonate solution at similar rates (92 ± 6.9 mmol O2 · min⁻¹ · g⁻¹ for control, 87 ± 5.8 for methionine-fed rats). In control hearts, bradykinin or carbachol (10⁻⁷ to 10⁻⁴ mol/L) caused concentration-dependent decreases in tissue O2 consumption. At the highest dose of each drug, the reductions were 22 ± 1.2% and 23 ± 2.3%, respectively. These responses were significantly inhibited in the presence of L-NAME: O2 consumption decreased by only 14 ± 2.5% after the addition of 10⁻⁴ mol/L bradykinin and 8.4 ± 2.4% after 10⁻⁴ mol/L carbachol. However, the effect of NO agonists was significantly less at each concentration in hearts from methionine-fed rats, and the highest concentrations of bradykinin or carbachol reduced O2 consumption by just 13 ± 1.6% and 14 ± 1.7% in these animals (Figures 1A and 1B). L-NAME did not significantly alter responses in the methionine-fed group.

**Effect of SNAP on O2 Consumption**

The exogenous NO donor SNAP (10⁻⁷ to 10⁻⁴ mol/L) caused concentration-dependent reductions in cardiac O2 consumption that were not significantly different between the 2 groups of rats (at highest dose, 32 ± 1.7% for control and 28 ± 1.7% for methionine-fed rats). These responses were not altered significantly by the presence of L-NAME.

**Measurement of Plasma Hcy**

The concentration of Hcy in rat and mouse plasma samples was assessed by immunoassay and differed significantly between the 2 groups. Hcy levels were fairly homogeneous among control rats (a mean of 6.7 ± 0.6 µmol/L), whereas measurements for the methionine-fed group varied greatly (from ~10 to 43 µmol/L) and reached a mean of 20 ± 2.6 µmol/L. A dose-response logistic curve (SlideWrite) was used to compare the Hcy concentration of each animal and the corresponding reduction in myocardial O2 consumption after the highest concentration of bradykinin or carbachol (Figure 2A). This indicated that as plasma Hcy level increased, NO bioactivity decreased proportionally. Plasma homocysteine was also measured in wild-type and knockout mice. Plasma Hcy was 3.84 ± 0.39 and 3.40 ± 0.49 µmol/L in wild-type and knockout mice, respectively.
knockout mice, respectively (n = 6). In wild-type and knockout mice fed methionine, plasma Hcy was 10.85 ± 0.92 and 10.09 ± 1.41 μmol/L, respectively. There was no difference in plasma Hcy between wild-type and knockout mice fed methionine.

Effect of Antioxidants on NO-Dependent Regulation of O2 Consumption
LV tissues from rats were treated with ascorbic acid (an O2 scavenger), tempol (an SOD mimetic), or apocynin (an inhibitor of NADPH oxidase assembly), and O2 consumption by these tissues was assessed in response to increasing concentrations of bradykinin (10^-7 to 10^-4 mol/L). In control rats, the presence of antioxidants did not significantly alter NO bioactivity. Meanwhile, addition of these chemicals restored the depressed NO-dependent regulation of O2 metabolism in the methionine-fed group to approximately normal levels (Figure 3).

Western Blot of Total Protein
Overall protein concentrations, assessed by the Western blot of β-actin, did not vary significantly between control (229±15 U of density) and methionine-fed (220±13 U of density) rats. Average protein densities between the 2 groups were also not different for eNOS, iNOS, SOD-1, SOD-2 (control versus methionine-fed: 120±5 versus 117±4 U for eNOS; 333±1 versus 333±1 U for iNOS; 130±1 versus 131±1 U for SOD-1; and 73±16 versus 66±14 U for SOD-2), and the following components of NADPH oxidase: p40phox, p47phox, p67phox, and rac-1 (Figure 4B). Although there

Figure 1. Inhibition of myocardial O2 consumption after addition of bradykinin (A) or carbachol (B). In control rat hearts (○), O2 consumption was significantly reduced at each dose, decreasing by >20% at 10^-4 mol/L for both drugs; however, bradykinin or carbachol reduced O2 consumption to a significantly smaller degree in hearts from methionine-fed rats (●). *Significant difference vs control, P<0.05.

Figure 2. A, Dose-response logistic curve illustrates graded relationship between plasma Hcy concentration and reduced NO bioactivity for control (○) and methionine-fed (●) rats. Each point relates individual rat’s plasma Hcy level to percent reduction in myocardial O2 consumption after highest dose of bradykinin or carbachol for same animal. Similar dose-response logistic curve in B illustrates relationship between plasma Hcy level and increased p22phox protein concentration (assessed by Western blot).

Figure 3. Effect of various antioxidants on regulation of myocardial O2 consumption by highest dose of bradykinin (BK). Hearts from methionine-fed rats exhibited low NO bioactivity; however, presence of ascorbic acid (AA), NADPH oxidase inhibitor apocynin (Apo), or SOD mimetic tempol (Temp) raised response to bradykinin to level observed in hearts from control rats, which indicates that NO bioactivity was restored. *Significant difference vs control, P<0.05.
was a statistically significant difference between the 2 groups for gp91phox (130 ± 3 U for control and 113 ± 3 U for methionine-fed), these differences may be too small to account for any meaningful physiological effect; however, the concentration of the p22phox subunit of NADPH oxidase (Figure 4A) was more than twice as great in methionine-fed rats (97 ± 22 U) than in the control group (43 ± 13 U, P < 0.05). There was also a sigmoidal relationship between plasma Hcy and p22phox protein concentration (Figure 2B).

Western Blot of Plasma Membrane Fraction
Western blotting showed that the isolated PMF was enriched 6-fold in Na\(^+\)-K\(^+\)-ATPase compared with the cytosolic fraction. As shown in Figure 5, the concentration of p22phox in the PMF was significantly higher in hearts from methionine-fed rats than in control hearts, whereas gp91phox was significantly lower. There was a trend toward increased p67phox and rac-1 in the PMF of methionine-fed rats compared with control rats.

Studies in Wild-Type and gp91phox-Knockout Mice
There was no significant difference in blood pressure in any group of mice. For instance, systolic blood pressure was 129 ± 2.5 mm Hg in wild-type mice, 120 ± 3.8 mm Hg in wild-type mice fed methionine, 125 ± 4.2 mm Hg in gp91phox-knockout mice, and 118 ± 5.0 mm Hg in gp91phox-knockout mice fed methionine. As shown in Figure 6, bradykinin caused dose-dependent reductions in myocardial O\(_2\) consumption in normally fed wild-type mice and significantly greater reductions in normally fed gp91phox-knockout mice. Whereas methionine feeding in wild-type mice significantly impaired the response to bradykinin (at doses 10\(^{-9}\) through 10\(^{-5}\)), methionine feeding had no effect on the response to bradykinin in mice lacking gp91phox. L-NAME significantly inhibited the effects of bradykinin in the normally fed wild-type mice and in both feeding groups of the knockout mice at each dose (data not shown).

Discussion
The major findings of this study are as follows: (1) elevated plasma concentrations of Hcy significantly inhibit the NO-dependent regulation of cardiac O\(_2\) consumption; (2) the inactivation of NO has a marked and graded relation to plasma Hcy level; (3) antioxidant treatment can restore normal regulation of cardiac O\(_2\) consumption by NO in rats with increased plasma Hcy; (4) the inactivation of NO by O\(_2\) is due to the increased assembly of NADPH oxidase after p22phox upregulation; and (5) the absence of functional gp91phox in mice prevents the impairment of NO bioactivity.

The ability of NO to regulate myocardial O\(_2\) consumption through the inhibition of cytochrome c oxidase in mitochondria has been well established by our laboratory and others.\(^9\)\(^{-11}\) Although the release of NO is small under basal conditions because of the lack of agonists and flow, the presence of an agonist causes dose-dependent reductions in
tissue O₂ consumption, an effect that can be inhibited by L-NAME. The present results demonstrate that this important regulatory mechanism is impaired in mice and rats with elevated plasma Hcy concentrations. Whereas baseline O₂ consumption was not significantly different between control and methionine-fed groups, responses to bradykinin or carbachol were greatly diminished in LVs from methionine-fed rats (by ≈40% at the highest dose of each drug), which suggests reduced NO bioavailability. These data are in accordance with studies showing decreased NO-dependent vasodilation in HHcy.²⁴ Moreover, a recent study by Zylberstein et al.²⁴ found that elevated plasma Hcy correlated with an increased risk of myocardial infarction and consequent mortality in women over a 24-year period. By focusing on myocardial metabolism rather than vessel dysfunction, the present study offers a link between the previously reported inactivation of NO in HHcy and the cardiac tissue damage suggested by Zylberstein et al.²⁴

All rats in the experimental group were fed methionine similarly, but the subsequent increase in plasma Hcy level varied greatly. This variability allowed us to study, over a wide range of values, the correlation between Hcy concentration and the ability of bradykinin or carbachol to reduce myocardial O₂ consumption. The relationship between increased plasma Hcy and decreased NO bioactivity was graded and formed a sigmoidal curve (Figure 2A). For the most part, responses to the NO agonists in rats began to decrease at Hcy concentrations between 10 and 15 μmol/L, a range that is quite similar to what is considered a risk factor in humans (ie, the American Heart Association recommends a therapeutic goal of <10 μmol/L). Plasma Hcy increased almost 3-fold during methionine feeding in both the wild-type and knockout mouse, reaching 10 μmol/L, and these values were not statistically different.

Despite altered NO bioactivity in rats and wild-type mice with high levels of plasma Hcy, hemodynamic measurements (including blood pressure, heart rate, LV dimensions, wall thickness, ejection fraction, and fractional shortening) revealed little difference between control and methionine-fed animals. This strongly suggests that the changes observed in the NO-mediated regulation of O₂ consumption were due to the direct effects of HHcy rather than to the onset of hypertension or heart failure. However, the striking similarity in blood pressure between the 2 groups is somewhat surprising given the low bioavailability of NO in HHcy. Whereas comparable NO inhibition by L-NAME would be sufficient to make rats hypertensive, this was not observed in rats fed methionine. One possible explanation is that the rats and mice were fed methionine for only 5 to 9 weeks and thus experienced HHcy for a very short period of time. At the same time, even some large-scale clinical trials have suggested that high plasma Hcy, although a risk factor for many cardiovascular diseases, is not a strong predictor of hypertension in humans.²⁵ Clearly, this is a subject that requires further investigation.

Bradykinin (a B₂ kinin receptor agonist) or carbachol (a muscarinic 2 receptor agonist) both stimulate the endogenous release of NO by activating eNOS through different mechanisms. Thus, L-NAME significantly impaired the regulation of O₂ consumption by these agonists in hearts from control rats. However, the present data suggest that the reduced NO bioactivity observed in HHcy is not caused by a decrease in eNOS activity or a loss of sensitivity to NO in mitochondria. Western blotting revealed that eNOS protein is preserved, not depressed, in rats with high plasma Hcy. Moreover, the exogenous NO donor SNAP reduced myocardial O₂ consumption to an extent that was not different between control and methionine-fed rats. It is therefore unlikely that the differences between the 2 groups observed with bradykinin or carbachol were due to decreased binding sites for NO in mitochondria.

Previous authors have suggested that NO bioactivity is impaired in HHcy because of increased oxidant stress.¹⁸⁻²⁰ In line with these findings, incubation with antioxidants (including ascorbic acid, which directly scavenges O₂⁻, or tempol, an SOD mimic) significantly enhanced the NO-dependent regulation of O₂ consumption in rats with high plasma Hcy, restoring it to control levels at each dose of bradykinin. Such restoration of NO bioactivity also occurred when tissues were pretreated with apocynin, which specifically inhibits NADPH oxidase by blocking the association of p47²⁶⁻ and p67²⁶⁻ with the membrane-bound complex.²⁶ Because the 3 antioxidants had no significant effect on responses in control tissues, these results strongly support the conclusion that elevated O₂⁻ levels occur in rats with increased plasma Hcy concentrations.

To clarify a mechanism behind the increased production of reactive oxygen species, Western blotting was used to assess the effect of HHcy on the expression of various proteins involved in the production and destruction of the O₂⁻ radical. Both SOD-1 and SOD-2 were present at normal levels in hearts from methionine-fed rats, even though tempol was able to improve NO bioactivity in these same animals. This suggests that whereas addition of a chemical that mimics SOD was able to lower O₂⁻ levels, a deficiency in this enzyme was not responsible for the increased O₂⁻ associated with high plasma Hcy. Instead, enhanced free radical generation is more likely a result of upregulated NADPH oxidase, as suggested by previous studies¹⁹,²⁰ and the fact that apocynin improved the regulation of O₂ consumption by bradykinin. This notion was further supported by Western blotting, which revealed that the p22phox subunit of the enzyme was present at a concentration 2-fold greater in hearts from methionine-fed rats than in control hearts. Furthermore, this increase in p22phox correlated with increasing plasma Hcy in a sigmoidal relationship (Figure 2B).

Although these findings point to upregulated NADPH oxidase in HHcy, we were surprised to discover that only 1 subunit (p22phox) was present at higher levels in hearts from methionine-fed rats. We therefore investigated whether the increase in p22phox was sufficient to cause the oxidase to assemble in the plasma membrane (and thus produce O₂⁻) more often. In fact, Western blots with the isolated PMF not only confirmed the increase in p22phox in hearts from methionine-fed rats but also indicated that greater amounts of p67²⁶⁻ (a cytosolic component) and rac-1 (a GTP-binding protein) had translocated to the membrane. This suggests that NADPH oxidase assembly increases in HHcy, even if many of the subunits do not increase in quantity. And although previous authors have suggested that HHcy also upregulates iNOS and uncouples NO synthase in vascular tissues, the complete restoration of NO bioactivity in the presence of apocynin and the lack of upregulation of eNOS and iNOS in hearts from methionine-fed rats makes it unlikely
that NO synthase–derived O$_3^-$ is a major cause of NO inactivation in cardiomyocytes.

The role of NADPH oxidase in HHcy was further supported by studies in genetically modified mice lacking a functional gp91$^{phox}$ subunit. Methionine-feeding for 9 weeks did not alter the ability of bradykinin to reduce myocardial O$_2^-$ consumption in these animals, which suggests that the impaired NO bioactivity in HHcy is due to increased activity of a gp91$^{phox}$-containing NADPH oxidase. This form of the oxidase is expressed in endothelial cells, fibroblasts, and cardiomyocytes but not in vascular smooth muscle cells, in which most NADPH oxidase-derived O$_2^-$ production involves other NOX proteins (gp91$^{phox}$ homologues).27,28 Interestingly, the responses to bradykinin in both groups of the gp91$^{phox}$- knockout mice were greater than responses in normally fed wild-type mice, which indicates that even in a normal mouse, a small amount of NO is still inactivated by O$_3^-$.

To the best of our knowledge, this study is the first to demonstrate that HHcy impairs the regulation of cardiac O$_2^-$ consumption by NO and that this effect is proportional to plasma Hcy level. These findings suggest that one reason why plasma Hcy is a graded risk factor for cardiovascular disease may be the varying extent to which NO is inactivated and unable to fulfill its role in controlling cardiovascular homeostasis. Moreover, this work was supported by the following NIH grants: PO-1 HL 2118 Circulation HHcy as a cause of elevated NADPH oxidase activity, an event that requires the participation of gp91$^{phox}$. These results indicate that the inactivation of NO by NADPH oxidase-derived O$_3^-$ has a major impact on cardiac metabolism beyond that caused by impaired vasodilation.

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Hyperhomocysteinemia, a Cardiac Metabolic Disease: Role of Nitric Oxide and the p22phox Subunit of NADPH Oxidase

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