Hyperhomocysteinemia Alters Cardiac Substrate Metabolism by Impairing Nitric Oxide Bioavailability Through Oxidative Stress

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Background—Hyperhomocysteinemia (HHcy) has been considered a vascular disease associated with increased levels of oxidative stress that results in scavenging of NO. However, little is known of the impact of HHcy on cardiac function and especially myocardial metabolism.

Methods and Results—L-Homocysteine was intravenously infused into conscious dogs, and the dogs were fed methionine to increase plasma homocysteine to 10 μmol/L for acute and 24 μmol/L for chronic HHcy. There was no significant change in hemodynamics with HHcy. Veratrine-induced, NO-dependent, coronary vasodilation (Bezold-Jarisch reflex) was reduced by 32% but was restored by simultaneous intravenous infusion of ascorbic acid or apocynin. Acute and chronic HHcy significantly increased uptake of glucose and lactate and decreased uptake of free fatty acid by the heart. HHcy significantly decreased bradykinin- or carbachol-induced reduction of myocardial oxygen consumption in vitro, and this effect was completely restored by coinubcation with ascorbic acid, Tempol, or apocynin. Western blot analysis indicated an increase in Nox2 (82%) and a reduction in endothelial nitric oxide synthase (39%), phospho-endothelial nitric oxide synthase (39%), and superoxide dismutase-1 (45%). Microarray analysis of gene expression in heart tissue from chronic HHcy indicated a switch in cardiac phenotype to enzymes that metabolize glucose.

Conclusions—HHcy directly modulates substrate use by the heart independent of changes in hemodynamics or ventricular function by reducing NO bioavailability through the generation of superoxide. The progression of cardiac or coronary heart disease associated with HHcy should be evaluated in light of the impact of alterations in the regulation of cardiac metabolism and substrate use. (Circulation. 2007;115:255-262.)

Key Words: blood flow ■ heart failure ■ metabolism ■ nitric oxide ■ physiology

Homocysteine (Hcy) is a sulfur-containing amino acid that is derived from dietary methionine. An elevation in plasma Hcy, hyperhomocysteinemia (HHcy), has been shown to be an independent risk factor for atherosclerosis, which includes peripheral vascular disease,1 venous thrombosis,2 coronary artery disease,3 and cerebrovascular disease.4 Hcy concentrations are elevated in up to 30% of patients with atherosclerosis,3 and levels only 12% above the upper limit of normal are associated with a 3-fold increase in the risk of acute myocardial infarction.5 In healthy adult humans, Bellamy et al6 have shown that oral methionine loading raises plasma Hcy and impairs flow-mediated endothelium-dependent vasodilation. Chambers et al7 also have shown that this effect is mediated through increased oxidative stress. Upchurch et al8 have demonstrated with animal models that HHcy reduces glutathione peroxidase activity and decreases the bioavailability of NO in cultured bovine aortic endothelial cells. Ungvari et al9 have also shown that HHcy increases tumor necrosis factor α expression, which induces a proinflammatory vascular phenotype through oxidative stress in coronary arteries of Wistar rats. Although the precise mechanisms are still not well understood, a number of in vitro and in vivo studies in humans and in animals have indicated that HHcy causes vascular endothelial dysfunction that leads to atherosclerosis mainly by increasing oxidative stress and attenuating NO bioavailability.

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On the other hand, little is known about the effects of HHcy on cardiac function and myocardial metabolism. In recent years, several investigators, including those participating in the present study, have proposed that NO may play an
important role in the regulation of cardiac metabolism. NO, which is a potent vasodilator, modulates mitochondrial respiration in vivo\textsuperscript{10} and in vitro.\textsuperscript{11} NO attenuates mitochondrial respiration by inhibition of complexes I and II of the electron transport chain\textsuperscript{12–14} and by interactions with cytochrome oxidase.\textsuperscript{13} Through a cGMP-mediated mechanism, NO also regulates glucose uptake\textsuperscript{15} by the heart.

Recent studies have also detected elevated levels of superoxide in HHcy, which results in impaired NO-dependent vasodilatation.\textsuperscript{9,16–18} Such dysfunction of coronary endothelial-um-mediated relaxation is a strong predictor of atherosclerotic disease and future cardiovascular events.\textsuperscript{19} We have also shown previously that HHcy dose-dependently inhibits the NO-dependent regulation of cardiac O\textsubscript{2} consumption in vitro through the generation of superoxide by NADPH oxidase in rats and mice that were fed methionine for 5 to 9 weeks.\textsuperscript{20} However, the mechanism by which superoxide levels are increased in HHcy has not yet been fully elucidated. Because the hypertension of HHcy is modest at best and certainly less than that after complete NO synthesis inhibition, it is plausible that only certain aspects of the function of NO are altered; i.e., the threshold for effects on cardiac metabolism are less than those for vascular dysfunction. Therefore, the causes, extent, and consequences of NO inactivation in HHcy on cardiac function, oxygen consumption, and substrate use all require further investigation. Thus the purpose of our present study was to evaluate the potential impact of HHcy on cardiac metabolism with special reference to the production of superoxide and scavenging of NO.

Methods

Surgical Preparation

Twenty male mongrel dogs (25 to 27 kg) were sedated with acepromazine maleate (1 mg/kg IM), anesthetized with sodium pentobarbital (25 mg/kg IV), and ventilated with room air. A thoracotomy was performed and catheters (Tygon) were placed in the aorta, left atrium, and coronary sinus. A left ventricular (LV) pressure gauge (P 6.5, Konigsberg Instruments, Inc, Pasadena, Calif), a Doppler flow transducer (Craig Hartley), and a pair of pacing electrodes were implanted as described previously.\textsuperscript{21} All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College.

Hemodynamic Recordings

Arterial pressure, LV pressure, and left circumflex coronary blood flow (CBF) were measured, heart rate was monitored, and mean arterial pressure and mean CBF were derived, as we described previously.\textsuperscript{22}

Cardiac Metabolites

Blood samples from aorta and coronary sinus were collected in plastic syringes treated with heparin or EDTA. Blood gases, lactate, and glucose were measured with a blood gas analyzer (IL-682 CO-Oximeter). Free fatty acid (FFA) analysis was performed on plasma from EDTA-treated samples with a colorimetric assay (NEFA C kit from Wako Diagnostics, Richmond Va) as described previously.\textsuperscript{23} Triglyceride content in the myocardium was measured as previously reported.\textsuperscript{24}

Measurement of Plasma Hcy

Plasma Hcy was quantified with a Hcy microplate enzyme immunoassay (Bio-Rad Laboratories, Hercules, Calif) as we have done previously.\textsuperscript{20} Activity was assessed with a Power Wave 200 spectrophotometer (Bio-Tek Instruments, Winooski, Vt) at 450 nm after addition of the substrate tetramethylbenzidine.

Protocols

Acute Hyperhomocysteinemia

To examine the effects of acute HHcy on the regulation of cardiac function and substrate use by NO, L-Homocysteine (Sigma-Aldrich) was intravenously infused into conscious dogs at 0.8 mg/min for 120 minutes.

Effects of Hcy on Activation of Bezold-Jarisch Reflex by Veratrine

We measured the NO-dependent response of CBF to veratrine at baseline, 40 minutes, 80 minutes, and 120 minutes during intravenous infusion of L-Hcy as described previously.\textsuperscript{25} After 120 minutes of L-Hcy infusion, ascorbic acid was administered intravenously at an initial dose of 2000 mg and followed by a constant infusion of 25 mg/min for 120 minutes (simultaneous with L-Hcy infusion) to scavenge superoxide as previously reported.\textsuperscript{25}

Chronic Hyperhomocysteinemia

L-methionine (Sigma-Aldrich) was added to the drinking water (10 g/L) of dogs for 2 weeks. We performed the veratrine-induced activation of the Bezold-Jarisch reflex, and we also collected blood samples for the measurement of glucose, lactate, FFA, and Hcy concentration. Apocynin, which was administered intravenously at a dose of 10 mg/kg for 120 minutes, was used as an inhibitor of NADPH oxidase activation. Ascorbic acid was given on another day to scavenge superoxide. After these experiments, we euthanized the dogs and took hearts for the measurement of oxygen consumption, Western blotting, and gene expression. Control hearts were harvested from 7 similarly instrumented dogs.

Calculation of Myocardial Oxygen Consumption, ATP Equivalent, and Triple Product

Myocardial oxygen consumption (MVO\textsubscript{2}) was calculated by multiplying the arterial-coronary sinus oxygen content difference in oxygen content by CBF. Assumed total ATP synthesis levels in both control and HHcy were calculated by the theoretical ATP yield from each substrate as follows: glucose 36, lactate 18, and palmitate 129 (mol ATP/mol substrate). Triple product, an index of mechanically related oxygen consumption, was calculated as LV systolic pressure multiplied by dP/dt\textsubscript{max} and heart rate and expressed in units.

Measurement of O\textsubscript{2} Consumption in Cardiac Muscle Segments

Myocardial tissue was isolated from the LV free wall of hearts. MVO\textsubscript{2} was measured polarographically in vitro with a Clark-type oxygen electrode (YSI-5331, Yellow Springs Instruments, Yellow Springs, Ohio). Tissue respiration was calculated as the rate of decrease in oxygen concentration after the addition of muscle slices with the assumption of an initial oxygen concentration of 224 nmol/mL; tissue respiration was expressed as nanomoles of oxygen consumed per minute per gram of tissue. Cumulative doses (10\textsuperscript{–7} to 10\textsuperscript{–4} mol/L) of 1 of 3 drugs: the B\textsubscript{2} kinin receptor agonist BK, the muscarinic-2 receptor agonist carbachol, or the exogenous NO donor S-nitroso-N-acetyl-penicillamine were used. The O\textsubscript{2} scavenger ascorbic acid (10\textsuperscript{–3} mol/L), the superoxide dismutase (SOD) mimetic Tempol (10\textsuperscript{–3} mol/L), or the NADPH oxidase inhibitor apocynin (10\textsuperscript{–4} mol/L) were used as antioxidants.\textsuperscript{20}

Western Blot Analysis

The preparation of protein samples from myocardial tissues was performed as described previously.\textsuperscript{20} Standard techniques for electrophoresis (150 V) and Western blotting were used to separate proteins by molecular weight and transfer them to a polyvinylidene difluoride membrane (Amersham Pharmaica Biotech, Piscataway, NJ). Antibodies to endothelial NO synthase (eNOS; Affinity Bioreagents, Golden, Colo; 1:1000 dilution), phospho-eNOS (serine-1177; Cell Signaling Technology, Danvers, Mass; 1:1000 dilution), Cu/Zn SOD (SOD-1; Calbiochern, EMD Biosciences, Darmstadt, Germany;
TABLE 1. Hemodynamic Data

<table>
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<th></th>
<th>Control Dogs (n=10)</th>
<th>120 Minutes (n=10)</th>
<th>1 Week (n=10)</th>
<th>2 Weeks (n=10)</th>
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<tr>
<td>Body weight, kg</td>
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<td>LVSP, mm Hg</td>
<td>136±4.8</td>
<td>128±4.1</td>
<td>136±8.2</td>
<td>132±6.7</td>
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<td>SBP, mm Hg</td>
<td>136±4.8</td>
<td>127±4.7</td>
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<td>DBP, mm Hg</td>
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<td>88±6.5</td>
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<td>mAoP, mm Hg</td>
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<td>CBF, mL/min</td>
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<td>dP/dtmax</td>
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<td>3349±319</td>
<td>3064±220</td>
<td>3295±222</td>
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<tr>
<td>Heart rate, bpm</td>
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<td>93±6.1</td>
<td>96±6.3</td>
<td>102±7.0</td>
</tr>
</tbody>
</table>

Summary data for the body weight, plasma Hcy concentration, and hemodynamic parameters of both control dogs and HHcy dogs (120 minutes, 1 week, and 2 weeks). LVSP indicates LV systolic pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; mAoP, mean arterial pressure.

Results

Plasma Homocysteine

Hcy levels of control were 4.4±0.2 μmol/L; infusion of L-Hcy for 120 minutes significantly increased Hcy levels to 8.9±0.5 μmol/L, and Hcy levels finally reached 10.1±1.8 μmol/L after 90 more minutes during the simultaneous infusion of ascorbic acid (P<0.01, n=10). Addition of methionine to diet for 2 weeks increased Hcy concentration to 24.3±9.6 μmol/L (P<0.01, n=10).

Hemodynamic Measurements in Dogs

All hemodynamic data and body weight are shown in Table 1. LV systolic pressure, systolic arterial blood pressure, diastolic arterial blood pressure, mean arterial pressure, mean CBF, dP/dtmax, and heart rate in resting conscious dogs did not differ significantly between control and HHcy.

Effects of Veratrine on the Coronary Circulation

Figure 1 shows the representative recordings of veratrine-induced, NO-dependent, coronary vasodilation (Bezold-Jarisch reflex) during acute HHcy. Veratrine administration at a dose of 5 μg/kg caused significant increases in CBF (76±19%, n=7). Infusion of L-Hcy significantly decreased this response by 42%, and this effect was completely reversed by the simultaneous infusion of ascorbic acid (P<0.05, n=7). Similar to acute HHcy, 2 weeks of methionine-enhanced diet significantly attenuated this response by 32% (P<0.05, n=8) (Figure 2), which was reversed by the simultaneous infusion of ascorbic acid (n=5) or apocynin (n=6).

Effects of Hcy on Energy Metabolism

The short-term infusion of Hcy significantly increased glucose uptake (1.47±0.72 to 7.48±1.58 μmol/min, P<0.01, n=7) and also decreased FFA uptake (7.38±1.09 to 3.88±1.08 μmol/min, P<0.05, n=6). Lactate uptake tended to increase (3.76±2.94 to 12.15±6.21 μmol/min, P=0.25, n=6), but this did not reach the statistical significance. All of this substrate switching occurred in the absence of hypertension or any change in CBF, which indicates a lack of effect of HHcy on resistance vessels. Figure 3 shows the relationship...
There was significant reduction in the coronary vasodilation caused by veratrine, which was restored by infusion of either ascorbic acid or apocynin. Values are mean ± SEM, *P < 0.05 for difference from control (n = 7).

Effects of Hcy on MVO₂ in Tissue
Cumulative doses of BK caused concentration-dependent decreases in MVO₂ in control heart (−39.7 ± 1.4%, n = 6). BK-induced reduction in MVO₂ was significantly attenuated by HHcy (−19.6 ± 2.5%, P < 0.01, n = 6). The inhibitory effects of HHcy on MVO₂ in response to BK were completely restored with co-incubation with ascorbic acid (−29.5 ± 2.3%), tempol (−29.3 ± 1.7%), or apocynin (−31.6 ± 3.0%), respectively (P < 0.05, n = 6). In the same manner, carbachol-induced reduction in MVO₂ was also significantly decreased by HHcy (−19.7 ± 2.1%, P < 0.01, n = 6) and was reversed by ascorbic acid (−33.0 ± 1.3%), tempol (−32.9 ± 2.1%) or apocynin (−29.0 ± 3.6%), respectively (P < 0.05, n = 6). These data are summarized in Figure 4. In contrast to reduction induced by BK and carbachol, S-nitroso-N-acetyl-penicillamine–induced reduction in MVO₂ (−37.1 ± 1.6%) was not affected by HHcy (−33.2 ± 3.7%) (n = 6).

Western Blot Analysis
There was an upregulation of Nox2 (gp91phox) during chronic HHcy accompanied by a reduction in eNOS and phospho-
eNOS (39%), SOD-1 and phospho-p47 (73%, Figure 5) protein expression. There was no change in p22phox (9%), p67phox (19%), SOD-2 (4%), or p47phox (6%). Thus these may also serve as loading controls.

Comparison of Gene Expression During HHcy

The microarray data revealed that 459 genes were differentially expressed (P≤0.05; fold change of at least 2). One hundred twenty-seven genes were expressed at significantly greater levels in HHcy, and 332 were significantly reduced in HHcy. Examples of these genes are shown in Figure 6 and Table 2. As expected, a number of cytokines and immune response genes were regulated. These included serum amyloid A protein (12.0-fold), interleukin-8 (9.4-fold), selectin (5.0-fold), tissue inhibitor of metalloproteinases (3.6-fold), CD48 (2.4-fold), and macrophage inflammatory protein 3 (2.3-fold). Unexpectedly, a substantial number of cardiac and metabolic genes were differentially regulated. Examples of these are shown in Figure 6 and Table 2. All genes that are significantly increased or decreased can be found in the online-only Data Supplement, Table I.

Discussion

The major findings of the present study are as follows. (1) Both acute and chronic HHcy caused a shift of cardiac substrate uptake from FFA to carbohydrate. These alterations were not accompanied by any hemodynamic changes (work) or by alterations in calculated ATP availability or oxygen consumption. (2) Both acute and chronic HHcy reduced veratrine-induced, NO-dependent, coronary vasodilation (Bezold-Jarisch reflex). This effect was restored by infusion of ascorbic acid or apocynin. (3) The ability of BK or carbachol to reduce oxygen consumption in vitro was abrogated in chronic HHcy. This effect was restored by antioxidants. (4) Protein expression of Nox2 (gp91phox) was upregulated, whereas it was downregulated for eNOS, phospho-eNOS, and SOD-1, which indicates potential sources of superoxide anion. (5) There was a switch in cardiac gene expression toward a new cardiac phenotype. In the present study, the serum concentration of Hcy was elevated to 24.3±9.6 μmol/L, which is considered mild clinical HHcy. Under these experimental conditions, we did not see any changes in hemodynamics or LV function. Therefore, all significant changes we observed were caused by the direct effects of HHcy rather than by the altered cardiac work associated with hypertension or heart failure. This may be the result of the limited exposure to HHcy over 2 weeks and may be evidence of the beginning of a process that leads to altered cardiac dynamics. It should be noted that there was an alteration in control of the coronary circulation at this time because the response to veratrine was significantly reduced. Our data with the gene chips also indicated what is perhaps an early step that contributes to cardiac dysfunction. Be that as it may, the present is the first study to indicate altered cardiac substrate uptake in HHcy, until this time indicative of cardiac disease.
TABLE 2. Gene Expressions in Dogs With HHcy

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<td>5-lipoxygenase activating protein</td>
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<td>ubiquinol-cytochrome-c reductase complex core protein 2</td>
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List of genes that are upregulated or downregulated in chronic HHcy. Affy probe set IDs refers to the Affymetrix probe set identifier.

In 1867, von Bezold and Hirt observed that an intravenous injection of veratrum alkaloids caused a decrease in blood pressure and heart rate, which was then named the Bezold-Jarisch reflex. Activation of this reflex also causes coronary vasodilation. Our recent studies have indicated that the coronary vasodilation induced by activation of the Bezold-Jarisch reflex is entirely NO-dependent and is selectively impaired after pacing-induced heart failure and enhanced after short-term exercise training in proportion to the altered release of NO and upregulation or downregulation of eNOS. The present results indicate that veratrine administration at a dose of 5 μg/kg significantly increased CBF (76%). Infusion of L-Hcy decreased this response by 42%, and the response was completely reversed by infusion of ascorbic acid. Similarly, after 2 weeks of a diet high in methionine, there was a 32% reduction of this response, which was completely restored by the infusion of either ascorbic acid or apocynin. Thus, there appear to be both short-term and longer-term effects of HHcy mediated in part by changes in protein and gene expression. Recent studies in animals and humans have demonstrated that mild HHcy induces endothelial dysfunction. Flow-mediated vasodilation as well as vasodilation induced by acetylcholine is impaired in patients with HHcy, which indicates alterations in NO-mediated responses. Hcy may decrease NO bioavailability through the formation of disulfides and the generation of hydrogen peroxide and superoxide anion. Therefore, the decrease in the coronary vasodilation induced by the activation of the Bezold-Jarisch reflex in HHcy is thought to be caused by impaired NO bioavailability through oxidative stress. Indeed, this reduction of the reflex recovered completely with simultaneous infusion of ascorbic acid or apocynin in both acute and chronic HHcy.

We have previously reported the potential role of NO in the control of myocardial metabolism with both in vivo and in vitro models. Recchia et al. found a significant increase in myocardial glucose uptake and decrease in FFA uptake associated with a fall in cardiac NO production in conscious dogs with pacing-induced heart failure. Moreover, this shift in substrate use was also observed in normal dogs with short-term pharmacological inhibition of NO synthase with nitro-L-arginine, which was completely reversed by a NO donor. Tada et al. have shown that glucose uptake is elevated in Langendorff-perfused hearts from eNOS knockout mice, a condition that was mimicked by NOS inhibition in hearts from wild-type mice. Furthermore, this increase in glucose uptake was normalized by 8-Br-cGMP or S-nitroso-N-acetyl-penicillamine, which indicates that cardiac NO regulates myocardial glucose uptake via a cGMP-dependent mechanism. These previous reports suggest that NO plays an important role in the regulation of myocardial substrate use. Our current study shows that HHcy causes a change in cardiac energy substrate utilization in the absence of any hemodynamic changes. Taking these findings into account, we conclude that HHcy directly alters cardiac metabolism by impairing NO bioavailability at least partly through oxidative stress.

In a normal metabolic state, FFA is the major substrate for the heart and provides the highest yield of ATP compared with glucose or lactate. On the other hand, downregulation of myocardial FFA oxidization and accelerated glucose oxidization has been reported in a number of models of heart failure, such as pacing-induced heart failure in dogs, myocardial infarction in rats, and also in idiopathic dilated cardiomyopathy in humans. This substrate switching may be considered either to be a marker of heart disease or to be a contributor to the development and progression of heart
HHcy on hearts independent of the secondary effects such as atherosclerosis or hypertension have not been investigated. Our results indicate, however, that the progression of cardiac or coronary disease associated with HHcy should be viewed in light of alterations in the regulation of cardiac metabolism, substrate use, and gene expression. Indeed, a recent clinical study\(^7\) has also shown that the treatment of HHcy by the combination of folic acid and vitamins B\(_6\) and B\(_{12}\) did not reduce the risk of major cardiovascular events in patients with vascular disease, even though it significantly lowered Hcy levels. Even though the plasma level of Hcy is low or the duration of HHcy is short, the potential that HHcy has direct effects on the heart and in that way may increase the risk of future cardiovascular events should be taken into account during the assessment of patients with HHcy.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Our studies indicate that hyperhomocysteinemia, which was once considered a vascular disease, should now also be considered a cardiac disease. As such, it is important to recognize the potential involvement of alterations in the control of cardiac metabolism to this disease process. The present study supports the conclusion that the scavenging of NO by superoxide anion in hyperhomocysteinemia results in a switch of cardiac substrate use from fatty acids to lactate and glucose and dysregulation of myocardial oxygen consumption. Furthermore, after just 2 weeks of methionine-enhanced diet, gene chip analysis indicates changes in cardiac gene expression, some of which are quite surprising. Thus, our present study draws attention to the fact that clinicians should consider hyperhomocysteinemia a cardiac metabolic disease as well as a vascular disease.
Hyperhomocysteinemia Alters Cardiac Substrate Metabolism by Impairing Nitric Oxide Bioavailability Through Oxidative Stress
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