Potential Mechanisms of Low Sodium Diet–Induced Cardiac Disease
Superoxide-NO in the Heart

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Rationale: Patients on a low salt (LS) diet have increased mortality.

Objective: To determine whether reduction in NO bioactivity may contribute to the LS-induced cardiac dysfunction and mortality.

Methods and Results: Adult male mongrel dogs were placed on LS (0.05% sodium chloride) for 2 weeks. Body weight (25.4±0.4 to 23.6±0.4 kg), left ventricular systolic pressure (137.0±3.4 to 124.0±6.7 mm Hg), and mean aortic pressure (111±3.1 to 98±4.3 mm Hg) decreased. Plasma angiotensin II concentration increased (4.4±0.7 to 14.8±3.7 pg/mL). Veratrine-induced (5 μg/kg) NO-mediated vasodilation was inhibited by 44% in LS; however, the simultaneous intravenous infusion of ascorbic acid or apocynin acutely and completely reversed this inhibition. In LS heart tissues, lucigenin chemiluminescence was increased 2.3-fold to angiotensin II (10⁻⁸ mol/L), and bradykinin (10⁻⁴ mol/L) induced reduction of myocardial oxygen consumption in vitro was decreased (40±1.3% to 16±6.3%) and completely restored by coinoculation with tiron, tempol or apocynin. Switching of substrate uptake from free fatty acid to glucose by the heart was observed (free fatty acid: 8.97±1.39 to 4.53±1.12 μmol/min; glucose: 1.31±0.52 to 6.86±1.78 μmol/min). Western blotting indicated an increase in both p47phox (121%) and gp91phox (44%) as did RNA microarray analysis (433 genes changed) showed an increase in p47phox (1.6-fold) and gp91phox (2.0 fold) in the LS heart tissue.

Conclusions: LS diet induces the activation of the renin–angiotensin system, which increases oxidative stress via the NADPH oxidase and attenuates NO bioavailability in the heart. (Circ Res. 2010;106:593-600.)

Key Words: low salt diet ■ nitric oxide ■ free radicals ■ angiotensin II ■ heart diseases

The role of angiotensin in the control of plasma volume and especially sodium homeostasis is well known.1,2 Historically, restriction of sodium intake has resulted in an increase in plasma renin and angiotensin I and II, an increase in plasma aldosterone,3 and enhanced sodium reabsorption. In lower species, including fishes and amphibians, it is the action of angiotensin II that conserves sodium.4 In addition, it has recently become clear that angiotensin II through its interaction with the angiotensin II type 1 (AT₁) receptor activates the NADPH oxidase to increase superoxide production.5 In fact, a portion, perhaps 50%, of the increase in arterial pressure during chronic angiotensin infusion in the rat, is dependent on increased superoxide production,6 as evidenced by the fact that the hypertension is reduced by scavenging superoxide. In tissues from dogs, angiotensin II increases superoxide as measured by lucigenin chemiluminescence in vitro and reduces NO bioactivity both of which are restored to control by addition of agents that scavenge superoxide or by apocynin.7 In hearts from human, primate, dog, hamster, mouse, rat, and frog, in vitro, bradykinin (BK) reduces cardiac oxygen consumption and this is blocked by an NO synthase inhibitor.7–9 In hearts from endothelial NO synthase (eNOS) knockout mice, NO-dependent (BK) signaling to mitochondria does not occur, suggesting the importance of eNOS.8 Low salt (LS)/angiotensin II may regulate the development of atherosclerosis and renal hormone production.9,10 Thus, there is an NO-dependent regulation of mitochondrial function that is important in the control of tissue oxygen metabolism. Most importantly, the ability of NO to reduce oxygen consumption in vitro in the normal mouse heart is almost abolished during incubation with angiotensin II via an AT₁ receptor–dependent mechanism.9 This does not occur in heart from gp91phox knockout mice, suggesting the essential role of assembly of the NADPH oxidase. Thus, during restriction in salt intake, it is already established that there is an increase in plasma angiotensin II.10 Through the actions on the kidney and the adrenal cortex, this results in an increase in sodium reabsorption and a reduction in urine and sodium excretion.
Our studies may shed light on the controversial clinical finding that reduced sodium intake or reduced urine output inversely correlates with cardiac mortality in patients.\textsuperscript{11–14} With regard to the effect of a LS diet in patients, there is some controversy. Many physicians still recommend LS intake in the treatment of both hypertension and heart failure.\textsuperscript{11–14} However, an interesting series of clinical studies by Alderman over the past 20 years call into question the wisdom of this approach, lacking a strong mechanistic scientific basis such as reduced NO bioavailability in the heart.\textsuperscript{15–18} For instance, patients on a LS diet have an increase in coronary events compared to those on normal salt (NS) intake,\textsuperscript{11,13} leading to the claim that mortality is inversely proportional to salt intake or urine output.\textsuperscript{12} Patients on a LS diet who increase their salt intake have a reduction in cardiac events,\textsuperscript{12,13} but not peripheral or cerebral vascular events. Using an almost circular reasoning, it has been shown the AT\textsubscript{1} receptor blockers have little effect on arterial pressure in some patients\textsuperscript{19}; however, placing a patient on a LS diet (which increases plasma angiotensin II and perhaps the local renin angiotensin system) increases the "efficacy of angiotensin II activation of the AT\textsubscript{1} receptor leads to uncoupling of the control of cardiac cell mitochondrial function by NO, potentially causing myocardial metabolic dysfunction.

\textbf{Methods}

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

\textbf{Surgical Preparation}

Male mongrel dogs (n=10, 25 to 28kg) were anesthetized with sodium pentobarbital (25 mg/kg IV). A thoracotomy was performed, and instruments were implanted for measurement of pressure, flow, and blood sampling as described previously.\textsuperscript{24–28} All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College.

\textbf{Hemodynamic Recordings}

Hemodynamics were recorded in conscious dogs as we described previously.\textsuperscript{24–29}

\textbf{Measurement of Plasma Angiotensin II}

Plasma concentration of Angiotensin II was measured with peptide enzyme immunoassay (Peninsula Laboratories Inc).

\textbf{Furosemide and LS Diet}

After performing a control experiment, we gave an intramuscular injection of 100 mg furosemide to dogs to acutely reduce blood volume, and then we put them on a LS diet (0.05% of sodium chloride, approximately 150 mg/d) for 2 weeks to further reduce blood volume.

\textbf{Cardiac Metabolites}

Blood samples from aorta and coronary sinus were collected for the measurements of free fatty acid (FFA), glucose, and lactate as we described previously.\textsuperscript{26,27}

\textbf{Effects of LS Diet on Activation of Bezold–Jarisch Reflex by Veratrine}

We measured the NO-dependent response of coronary blood flow to veratrine as previously described.\textsuperscript{24–26}

\textbf{Nitrite Production of Coronary Microvessels}

To measure the capacity of the heart to produce NO, nitrite production of coronary microvessels was measured as described previously.\textsuperscript{30,31}

\textbf{Measurement of O\textsubscript{2}}\textsuperscript{- Consumption in Cardiac Muscle}

Myocardial tissue was isolated from the left ventricular (LV) free wall of hearts. Myocardial oxygen consumption (MVO\textsubscript{2}) was measured polarographically in vitro using a Clark-type oxygen electrode (YSI-5331, Yellow Springs Instruments, Yellow Springs, Ohio). Cumulative doses (10\textsuperscript{-7} to 10\textsuperscript{-4} mol/L) of BK or carbachol were used. The superoxide scavenger tiron (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.\textsuperscript{24,26}

\textbf{Lucigenin Chemiluminescence}

The chemiluminescence elicited by O\textsuperscript{2}\textsuperscript{-} in the presence of lucigenin (5 μmol/L) was measured in cardiac muscle segments from the same dog that was used for measurements of MVO\textsubscript{2} as described previously.\textsuperscript{24,26}

\textbf{Western Blotting Analysis}

The preparation of protein samples from myocardial tissues was as described previously.\textsuperscript{26}

\textbf{RNA Isolation and Microarray Analysis}

Total cardiac RNA was extracted from the left ventricle from the control and LS groups (n=4) as described previously.\textsuperscript{32} All the hybridization data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO) (http://www.ncbi.nlm.nih.gov/geo) with GEO accession numbers for series GSE17149.

\textbf{Data Analysis}

All data are presented as means and SEM. Statistical significance of differences was determined with Student t test for each peak response, and differences between groups were determined with
Table. Effects of LS Diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>2 Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>25.4±0.4</td>
<td>23.6±0.4†</td>
</tr>
<tr>
<td>LVSP (mm Hg)</td>
<td>137.0±3.4</td>
<td>124.0±6.7*</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>110.8±3.1</td>
<td>97.6±4.3†</td>
</tr>
<tr>
<td>CBF (mL/min)</td>
<td>29.3±4.6</td>
<td>29.6±4.4</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>99.6±6.3</td>
<td>106.1±6.3</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>46.0±1.0</td>
<td>40.5±1.4†</td>
</tr>
<tr>
<td>LVPWD (cm)</td>
<td>1.04±0.05</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>IVSWD (cm)</td>
<td>0.97±0.05</td>
<td>0.87±0.01</td>
</tr>
<tr>
<td>Cardiac output (L/min)</td>
<td>3.44±0.19</td>
<td>2.10±0.28†</td>
</tr>
<tr>
<td>LV dp/dt (mm Hg/sec)</td>
<td>3848.0±384.6</td>
<td>3915.3±669.2</td>
</tr>
<tr>
<td>TPR (dyn · sec · cm⁻²)</td>
<td>19.1±2.6</td>
<td>26.9±4.4 (P&lt;0.12)</td>
</tr>
<tr>
<td>EF (%)</td>
<td>64.8±2.9</td>
<td>60.3±4.3</td>
</tr>
</tbody>
</table>

Data are means±SEM. CBF indicates coronary blood flow; HR, heart rate; IVSWD, interventricular septal wall diameter; LVEDD, LV end-diastolic diameter; LVPWD, left ventricular end-diastolic posterior wall dimension; LVSP, LV systolic pressure; MAP, mean arterial pressure; TPR, total peripheral vascular resistance. *P<0.05, †P<0.01.

Results

Plasma Angiotensin II
Plasma concentration of angiotensin II of control dogs was 4.3±0.7 µmol/L (n=10), and 2 weeks of LS diet significantly increased by 340±84% (14.8±3.7 µmol/L).

Hemodynamic Measurements in Dogs
All hemodynamic data and body weight are shown in Table. Body weight significantly decreased (P<0.01, n=10), and both LV systolic pressure (P<0.05, n=10) and mean arterial pressure (P<0.01, n=10) were also significantly decreased. However, LV dp/dt, coronary blood flow, and heart rate were maintained after 2 weeks of LS. There was a tendency for total peripheral resistance (P<0.12) to increase, but this did not reach statistical significance.

Echocardiographic Measurement
The Table also shows the summary data of echocardiographic analysis of LV function in both baseline and LS. LV end-diastolic diameter was decreased (P<0.01, n=6), which was accompanied by the simultaneous decrease of CO (P<0.01, n=6). However, ejection fraction was unchanged. There was no change in LV posterior wall or interventricular septum wall thickness in diastole, indicating a lack of hypertrophy after 2 weeks on LS.

Effects of Veratrine on the Coronary Circulation
Figure 1 is the summary of veratrine-induced NO dependent coronary vasodilation (Bezold–Jarisch reflex). Veratrine administration at a dose of 5 µg/kg caused significant increases in coronary blood flow (65±8%, n=10). Two weeks of LS inhibited this response by 44% (P<0.05); the simultaneous infusion of either ascorbic acid or apocynin completely reversed this inhibition (P<0.05, n=6 respectively).

Nitrite Production of Coronary Microvessels
There was no significant difference in the baseline production of nitrite in coronary microvessels between NS and LS (P=NS, n=10). BK (10⁻⁵ mol/L) increased nitrite production in a dose-dependent fashion similarly in both groups (normal: 133±6.7%; versus LS: 138±24.6%; P=NS, n=10), ie, the endogenous NO production was preserved in LS. This effect of BK was completely inhibited by coincubation of NO synthesis inhibitor N⁵,G-1-nitro-arginine methyl ester (L-NAME) (10⁻⁴ mol/L) in both groups. Similar effects were found using carbachol (not shown).

Effects of LS Diet on MVO₂ in Tissue
Cumulative doses of BK caused concentration-dependent decreases in MVO₂ in control hearts (40±1.3%). BK-induced reduction in MVO₂ was significantly attenuated by LS (16±6.3%, P<0.01). The inhibitory effects of LS on MVO₂ in response to BK was restored with coincubation with tiron (28±2.5%, P<0.05), tempol (35±3.9%), or apocynin (34±2.5%) (P<0.05). Coincubation with L-NAME in normal tissue also significantly inhibited BK-induced reduction in MVO₂ (17±7.1%, P<0.01; Figure 2).

O₂⁻ Production by Angiotensin II
Angiotensin II resulted in significant increase in production of O₂⁻ only in LS. O₂⁻ production was inhibited by coincubation with Tiron or Tiron plus SOD (Figure 3).

Effects of LS Diet on Energy Metabolism
The LS significantly increased glucose uptake (P<0.05) and also decreased FFA uptake (P<0.05). Lactate uptake tended to increase (P=0.11; Figure 4).
Western Blotting Analysis
The protein expression of p47phox (121%) and gp91phox (44%) were upregulated in the LV tissues after 2 weeks of LS (Figure 5). There was no change in inducible NOS, neuronal NOS, SOD1, SOD2, p67, phospho-p47, p22, Rac-1, nitrotyrosine, or \( \beta \)-actin. For instance, by scanning, there was no change in eNOS or phospho-eNOS (802 ± 16 and 774 ± 26 in normal and 767 ± 94 and 767 ± 26 in LS, respectively). In NS and LS, \( \beta \)-actin was 721 ± 135 and 651 ± 111 (\( P = 0.50 \)), respectively.

RNA Microarray Analysis
Affymetrix Canine Gene Array 2.0 was used to determine differential gene expression in heart tissues from four LS and four control dogs. There were 443 genes that changed, 110 decreasing and 333 increasing. There was an increase in mRNA for p47\( ^{\text{phox}} \) (1.6-fold), gp91\( ^{\text{phox}} \) (2.0-fold) and in SOD1 (1.7-fold, all \( P < 0.05 \); Figure 6). In addition to the metabolic genes there was no difference in RNA for p67, p22, p40, rac-1, SOD2, eNOS, inducible NOS, or neuronal NOS. All the genes are listed in Online Table I. eNOS was 16.8 ± 0.6 pixels in normal and 17.4 ± 0.5 pixels in LS (\( P = \text{NS} \)).

Discussion
All of our data point toward an important role of NO and the renin–angiotensin–NADPH system as potential mediators of the LS-induced cardiac dysfunction and potentially the mortality associated with salt restriction in patients.\(^{11-14,23} \) Firstly, there was a 340% increase in plasma angiotensin during LS feeding of dogs. There was the expected reduction in body weight, arterial pressure, diastolic dimensions in the heart, along with a reduction in cardiac output with LS. This was accompanied by a reduction in NO-mediated vasodilation in vivo with no change in nitrite production from coronary microvessels in vitro. There was no change in eNOS protein pointing toward a reduction in NO bioactivity during LS, most likely through the scavenging of NO by superoxide. The obtunded NO-mediated coronary vasodilation in vivo was restored by ascorbic acid. The suppression of the ability of BK to regulate MVO\(_2\) in cardiac tissue in vitro was restored by tiron and tempol. Most directly, there was an increase in lucigenin-induced chemiluminescence, a direct measure of superoxide, in response to angiotensin, in cardiac tissues from dogs fed LS. The source of superoxide is most likely the NADPH oxidase because: (1) the reduced NO-mediated vasodilation was restored by apocynin in vivo; (2) the ability of BK to control MVO\(_2\) was restored by apocynin; (3) there was an increased gene expression of p47\( ^{\text{phox}} \) and gp91\( ^{\text{phox}} \) using microarrays; and (4) there was an increase in p47\( ^{\text{phox}} \) and gp91\( ^{\text{phox}} \) protein by Western blotting. Interestingly and previously unreported, there was a switching of substrate uptake by the heart from fatty acids to glucose, a signature in our previous studies for the involvement of NO. A reduction in NO bioactivity causes the heart to switch from fatty acid uptake and oxidation to oxidation of glucose.\(^{33,34} \) There is also a change in gene expression by microarray analysis toward genes which encode for enzymes of intermediary metabolism, supporting our finding of altered substrate uptake, as yet a new feature of LS consumption. Thus, there are potentially defects in coronary...
vascular control, in mitochondrial function, in oxygen use, in intermediate metabolism, and in protein and gene expression as a consequence of a LS diet, and all of these may contribute to the development of cardiac disease.

There was, as expected, a reduction in cardiac end-diastolic dimensions and cardiac output along with a reduction in body weight, all characteristic of a reduction in blood volume similar to that in patients with LS diet.11–14 There was also a reduction in LV systolic pressure and phasic and mean arterial pressure as a consequence of LS consumption. Our data did not reach statistical significance (P<0.12); however, they suggest that there is actually an increase in total peripheral resistance, as reported in patients;35 another of our studies in mice (Y Skayian, TH Hintze, unpublished data, 2009) indicate a statistically significant increase in total peripheral resistance with LS feeding, which can be interpreted as peripheral vasoconstriction. There was no sign of hypertrophy using measures of anterior wall and septum wall thickness. Caution has to be used when creating ratios of LV/body weight to assess hypertrophy because LS reduced body weight by almost 10% in 2 weeks. Furthermore, the measurement of heart weight by echo is model-dependent and not specific for the canine heart.

The Bezold–Jarisch induced increase in coronary blood flow following injection of veratrine is entirely NO-dependent, because it is abolished by L-NAME.24–26 It is also reduced during the development of dilated cardiomyopathy36 or insulin dependent diabetes mellitus,24 conditions in which NO production is reduced and during hyperhomocysteinemia29 and endothelial stunning7 in which NO bioactivity is reduced because of superoxide anion. In contrast, with pregnancy or exercise training, the reflex dilation is largely attributable to upregulation of eNOS.25,26 During LS feeding, the reflex dilation is also reduced but it is restored by infusion of ascorbic acid, indicating a reduction in NO bioavailability. Previous studies by us showed that vagal stimulation in the anesthetized dog, to directly control efferent activity, resulted in a entirely NO dependent coronary vasodilation.28 Furthermore, the bradycardia following veratrine injection was unaffected by NO synthesis inhibition.36 Thus, it is unlikely that the reduction in the reflex dilation was caused by an antioxidant effect on the ganglion or nuclei in the brain stem.

We have shown previously that BK through an NO-dependent mechanism regulates cardiac oxygen consumption in vitro.15–18 These data support the concept that NO, through a competition with oxygen for cytochrome oxidase, regulates oxygen consumption in all types of cells from cardiac muscle to endothelial cells.15–17,37–40 This effect occurs in the wild-type mouse heart but not in the eNOS−/− heart and not in the presence of L-NAME. In our studies, BK reduced cardiac tissue oxygen consumption; this was blocked by L-NAME, indicating that it was NO dependent and was reduced to a similar degree in hearts from dogs fed a LS diet. Although this was not...
attributable to a reduction in NO production, because there was no decrease in nitrite production by coronary microvessels, it was restored by superoxide scavengers. We have previously used these techniques in a model of “endothelial stunning” in the dog heart,7 in which there was no change in NO or nitrite production but a reduction in NO ability to regulate oxygen consumption caused by a reduction in the half life of NO. Similar results were found in both the rat and dog heart after methionine feeding, leading to hyperhomocysteinemia.28 Thus, the measurement of cardiac tissue oxygen consumption is a sensitive index of NO bioactivity and also addresses a mechanism perhaps contributing to cardiac dysfunction.

There was an increase in lucigenin chemiluminescence in vitro to angiotensin II only in the heart of dogs fed a LS diet; this was confirmed using tiron and SOD. The enzymatic source of superoxide was the NADPH oxidase, because the components were upregulated and the biological effects were restored by apocynin both in vivo and in vitro. We have shown previously that apocynin in vivo blocks endothelial stunning7 and restores the NO-dependent coronary vasodilation during hyperhomocysteinemia.28 However, there is some question in the literature regarding whether apocynin not only prevents the assembly of the NADPH oxidase but also has other effects.41 When given chronically in rats, apocynin downregulated components of the NADPH oxidase; however, in our studies, apocynin was given for 1 hour. It is possible that apocynin, particularly the monomeric form, acts as an antioxidant22 and not to inhibit assembly of the oxidase. Myeloperoxidase is needed to form the apocynin dimer and in tissues lacking myeloperoxidase the active dimer does not form. All of our studies in vivo and those using lucigenin or measuring oxygen consumption were performed in mixed tissues containing white cells in which myeloperoxidase allows for dimerization of apocynin, conferring its activity as an assembly inhibitor. If in our studies apocynin only acts as an antioxidant then our studies support finding using SOD, tiron, tempol, and ascorbic acid. If apocynin also acts as an NADPH oxidase inhibitor, it supports our studies using western blotting and the gene array. Thus, experiments using apocynin confirm the role of superoxide and the upregulation of p47phox and gp91phox support the involvement of the NADPH oxidase.

Not to our surprise, hearts from dogs fed a LS diet switched substrate use from predominantly FFAs to primarily glucose. In a number of studies, we have shown that reduction in cardiac NO production or bioactivity resulting from heart failure,43,44 administration of L-NAME,31 during endothelial stunning,7 and hyperhomocysteinemia29 all result in a shift in substrate uptake by the heart. This seems to be in contrast to a healthy or unstressed heart, as for instance in pregnancy and exercise, conditions in which fatty acids take on a larger role as substrate to support increased cardiac mechanical function.26 The first studies to suggest that NO might regulate glucose uptake by the heart by Depre and colleagues45,46 indicated that this was a cGMP-mediated action in contrast to the chemical action of NO through cytochrome oxidase to control MVO2.47,48 Tada et al7 confirmed the studies by Depre et al45 using eNOS−/− mouse heart and a Langendorff heart preparation. In hearts from eNOS−/− mouse BK did not alter glucose uptake whereas in eNOS+/+ heart inhibition of guanylate cyclase with ODQ increases glucose uptake via and 8-Br cGMP, a cGMP mimic, inhibits glucose uptake. The role of NO in the control of cardiac FFA uptake and oxidation is more controversial.44 Firstly, this is best studied using live animals, such as the conscious dog, because fatty acids are carried bound to plasma proteins and are normally consumed by the heart. In the isolated heart, fatty acids are difficult to use as a substrate because they are detergents. However, fatty acid uptake by the heart is dependent on the plasma concentration; at arterial concentrations less than 0.2 to 0.3 mmol/L, there is little uptake.53 In our studies, the arterial plasma FFA concentrations were 0.79±0.08 and 0.71±0.22 mmol/L (P=NS) during control and LS, respectively. Nonetheless, our studies indicate a reduction in fatty acid uptake and increase in glucose uptake, both attributable to a reduction in NO bioactivity.

Although consistent with our previous studies but still surprising, there was a shift in the expression of 433 genes, 110 increasing and 323 decreasing, in dogs fed a LS diet which is also indicative of the dramatic effects of a LS diet on the heart. Although this observation has not been reported previously, it should be noted that the tissues used in this analysis were sections of heart containing many different cell types. There were 6 genes upregulated more than 2-fold and 9 downregulated more than 2-fold. These genes include some related to mitochondrial function such as cytochrome b; some related to fatty acid transport and metabolism such as acetyl-coenzyme A binding protein; and some related to glucose metabolism, such as fructose 1,5-bisphosphatase and glycogen phosphorylase. The exact impact of these changes in gene expression is difficult to evaluate, but the data indicate the profound effect that a LS diet has on the heart. Furthermore, these data support the conclusion that a LS diet has an unexpected impact on the heart when heretofore the major consequences of a LS diet were thought to be vascular, resulting in superoxide generation, removal of NO and vasoconstriction.

There is a remarkable congruence between the gene array and Western blotting. By either method, there was no change in p67phox, p22phox, rac-1, SOD2, eNOS, inducible NOS, or neuronal NOS. By both methods, there was a significant increase in p47phox and gp91phox. There was an increase in SOD1 by gene array but not in the protein. This was not attributable to our inability to measure SOD1 by Western blotting because we have recently done so in the heart from pregnant dogs.26,27 There was no change in nitrotyrosine. Because nitrate production did not change in coronary microvessels and eNOS or phosphorylated eNOS proteins were not changed; the production of NO limited the amount of nitrotyrosine. Because the interaction of NO and O2− to form peroxynitrite is diffusion limited, and the stoichiometry of NO and superoxide is 1:1, the amount of peroxynitrite formed is determined by the lowest concentration of either NO or superoxide. Although tyrosine nitration could originate from the scavenging of NO by superoxide, other processes may dominate (eg, the breakdown of nitrate proteins). Because nitrite levels were similar in both animal groups and the biological activity of NO was restored by antioxidants (tiron, tempol, SOD, and ascorbic acid), we conclude that NO production was not different between LS and NS fed dogs but rather that the scavenging by superoxide was increased and biological...
half of NO was reduced resulting in aberrant control of cardiac metabolism and coronary vasodilation.

Our study adds a new perspective on the effects of LS diet in the treatment of both hypertension and heart failure. The early studies by Alderman\(^\text{11–14}\) suggested an increased mortality of cardiac origin in the face of a reduced mean arterial pressure in patients with LS diet. They concluded that the mortality was attributable to other than hemodynamic causes, perhaps caused by the reduction in cardiac NO bioactivity. In the case of heart failure, where eNOS is downregulated, the reduction in blood volume resulting from the use of diuretics and salt restriction may further increase the already elevated renin-angiotensin levels, competing with the effect of the AT1 blocker or angiotensin-converting enzyme inhibitor, scavenging NO and having effects on cardiac oxygen consumption and contractile efficiency. Characteristically, oxygen consumption is elevated in the failing heart not only because of the increased hemodynamic load, including diastolic wall stress in dilated myopathies, but, we think, also because of a reduction in NO.\(^\text{43,44}\) In the conscious dog, during graded treadmill exercise, oxygen consumption is increased, independent of load, by 10% at each level of exercise during inhibition of NO synthesis.\(^\text{49,50}\) Thus, the effect is small but leads to a reduced cardiac efficiency when calculated as work/oxygen consumed. The same conclusion pertains to the failing heart where NO production is reduced and oxygen consumption is elevated. There is an error in the calculation of oxygen consumption, which assumes that 100% of the measured oxygen consumed is used to support the generation of ATP, although some of the oxygen consumed is wasted because it forms superoxide anion and not ATP. When NO is present and competing with oxygen for the binding site on cytochrome oxidase, the amount of oxygen consumed may fall, leading to an increase in intracellular oxygen to maintain the diffusion gradient or to saturate myoglobin in cardiac cells.\(^\text{40}\) Hemodynamic measurements identified no reduction in contractile indices and no change in wall thickness, therefore 2 weeks of LS diet did not result in overt pathology.

In summary, our studies have defined a potential mechanism, the reduction in NO bioactivity, which may contribute to the detrimental effects of LS in patients. A report of a recent clinical trial NHIANES III, by Cohen, Hailpern, and Alderman,\(^\text{51}\) suggests a “modest and mostly not statistically significant” correlation of LS diet and mortality and no direct correlation with elevated salt. The clinical relevance of our study still remains to be determined.

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Disclosures
None.

References


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**Surgical preparation:** Male mongrel dogs (n=10, 25-28kg) were sedated with Acepromazine maleate (1mg/kg i.m.), anesthetized with sodium pentobarbital (25mg/kg i.v.) and ventilated with room air. A thoracotomy was performed, and catheters (Tygon) were placed in the aorta, left atrium, and coronary sinus for measurement of pressure and blood sampling. A left ventricular (LV) pressure gauge (P 6.5, Konigsberg Instruments Inc), a Doppler flow transducer (Craig Hartley), and a pair of pacing electrodes were implanted as described previously (1-6). 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 (HR) was monitored, and mean arterial pressure (MAP), mean CBF and LV dP/dt were derived, as we described previously (1-6).

**Echocardiographic assessment:** LV chamber size and LV function were measured using an echocardiograph (Sequoia C256, Acuson Corporation).

**Measurement of Plasma Angiotensin II:** Plasma concentration of Angiotensin II was measured with peptide enzyme immunoassay kit (Peninsula Laboratories Inc.). Briefly, 5ml of blood samples from aorta were collected into pre-chilled syringe and transferred into a polypropylene tube containing EDTA (1ug/ml of blood) and aprotinin (500 KIU/ml of blood) at 0°C, centrifuged at 3000×g for 10min at 0°C, and plasma was immediately collected and frozen in liquid nitrogen.

**Furosemide and low sodium diet:** After performing a control experiment, we gave an intramuscular injection of 100mg Furosemide to dogs, and then we put them on a low sodium diet, (0.05% of sodium chloride) for two weeks. Additional experiments were performed and the dog sacrificed to harvest tissues.

**Cardiac metabolites:** Blood samples from aorta and coronary sinus were collected into plastic syringes treated with heparin or EDTA. Lactate and glucose were measured using a blood gas analyzer (IL-682 CO-Oximeter, Gem Premier 3000). FFA analysis was performed on plasma from EDTA treated samples using a colorimetric assay (NEFA C kit from Wako), as we described previously (7-10).

**Effects of low sodium diet on activation of Bezold-Jarisch reflex by veratrine:** We measured the NO-dependent response of CBF to veratrine as described previously (1-3). Briefly, in conscious dogs, veratrine at a dose of 5 μg/kg was administered as a bolus injection (1mL) into the left atrium through the implanted catheter with HR held constant (150bpm) by electrical pacing to avoid the effects of veratrine-induced bradycardia on MAP and CBF. After 2 weeks feeding of LS, the same assessment was performed, and either apocynin or ascorbic acid was used to determine the role of the NADPH oxidase and superoxide anion. Apocynin, which was administered intravenously at a dose of 10mg/kg for 120min, was used as an inhibitor of NADPH oxidase activation. On another day, at least two days apart, ascorbic acid was administered intravenously at an initial dose of 2000mg, followed by a constant infusion at 25mg/min for 120min to scavenge superoxide as previously reported. After these experiments, we sacrificed dogs and excised hearts for the measurement of nitrite production, oxygen consumption, lucigenin chemiluminescence, Western blotting, and gene expression. Control hearts were harvested from similarly instrumented dogs (7,8).

**Nitrite production of coronary microvessels:** To measure the capacity of the heart to produce NO, nitrite production of coronary microvessels was measured as described
previously (4).

**Measurement of O\textsubscript{2} consumption in Cardiac Muscle:** Myocardial tissue was isolated from the LV free wall of hearts. MVO\textsubscript{2} was measured polarographically *in vitro* using a Clark-type oxygen electrode (YSI-5331, Yellow Springs Instruments, Yellow Springs, OH). Tissue respiration was calculated as the rate of decrease in oxygen concentration after the addition of muscle slices, assuming an initial oxygen concentration of 224 nmol/ml, and 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 the B\textsubscript{2} kinin receptor agonist BK were used. The superoxide scavenger tiron (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 (7).

**Lucigenin chemiluminescence:** The chemiluminescence elicited by O\textsubscript{2}\textsuperscript{-} in the presence of lucigenin (5μmol/L) was measured in cardiac muscle segments from the same dog that was used for measurements of MVO\textsubscript{2} as described previously. Muscle segments were incubated with 10\textsuperscript{-8} mol/L AngII 30minutes before measurements of chemiluminescence. In separate experiments, the effects of 10\textsuperscript{-8} mol/L AngII on O\textsubscript{2}\textsuperscript{-} production were also determined in the presence of tiron (10\textsuperscript{-3}) or SOD (200 U/mL) (7).

**Western Blotting analysis:** The preparation of protein samples from myocardial tissues was as described previously (7-9). Using standard techniques for electrophoresis (150 V) and western blotting, proteins were separated out by molecular weight and transferred to a PVDF membrane (Amersham Pharmacia Biotech). Antibodies to Mn SOD (SOD-2) or gp91, p67 (BD Transduction; 1:1000 dilution), or p22\textsuperscript{phox} p47, (Santa Cruz Biotechnology; 1:2000 dilution), SOD1 (Calbiochem, 1:5000), eNOS, iNOS, nNOS (Affinity 1:1000) and nitrotyrosine (Upstate 1:5000) were used.

**RNA isolation and microarray analysis:** Total cardiac RNA was extracted from the left ventricle from the control and LS groups (n=4) as described previously (6). RNA quality was assessed by electrophoresis using the Agilent Bioanalyzer 2100. RNA from each sample was used to generate a high fidelity cDNA using the labeling protocols for sample preparation recommended by Affymetrix. The microarray labeling, hybridization and analysis procedures were described previously in more detail (6). Each individual sample was subjected to gene expression analysis via the the GeneChip® Canine 2.0 Genome Array. All the hybridization data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO: http://www.ncbi.nlm.nih.gov/geo) with GEO Accession Numbers for series GSE17149.

**Data Analysis:** All data are presented as mean and SEM. In studies *in vivo*, the responses are measured at the peak after administration of veratrine. Statistical significance of differences was determined with Student’s t-test for each peak response, and differences between groups were determined with repeated-measures ANOVA. Changes are considered significant at a value of p<0.05. Statistical significance for changes in gene expression was performed in GeneTraffic using a t-test and with variance stabilization. Differences were considered statistically significant at a nominal significance of P≤0.05 and at least a 2.0 fold change in expression between control and dogs fed LS for two weeks. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.


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