Plasma Levels of Nitric Oxide Metabolites Are Markedly Reduced in Normotensive Men With Electrocardiographically Determined Left Ventricular Hypertrophy

Fumihiko Kamezaki, Masato Tsutsui, Masao Takahashi, Shinjo Sonoda, Tatsuhiko Kubo, Yoshiisa Fujino, Tetsuo Adachi, Haruhiko Abe, Masaaki Takeuchi, Toshihiko Mayumi, Yutaka Otsuji

Abstract—Recent studies have revealed that electrocardiographically determined left ventricular hypertrophy (ECG-LVH) is a risk factor for cardiovascular death not only in hypertensive patients but also in normotensive subjects. However, the underlying mechanisms remain to be elucidated. In this study, we tested our hypothesis that normotensive subjects with ECG-LVH have reduced nitric oxide production. A total of 840 Japanese male workers were enrolled, and 579 eligible subjects were studied. ECG-LVH was assessed according to the Sokolow–Lyon voltage criteria and the Cornell voltage–duration product. The median level of plasma NOx (nitrite plus nitrate), a marker of systemic nitric oxide production, was markedly lower in the normotensive subjects with ECG-LVH (n=73) than in those without (n=506), and the clinical characteristics were significantly different between the 2 groups (each \( P<0.05 \)). Importantly, a one-to-one propensity score matching analysis showed similar markedly lower median plasma NOx level in the normotensive subjects with ECG-LVH compared with that observed in the matched normotensive subjects without ECG-LVH (\( P<0.05 \)). Furthermore, the tertiles of the plasma NOx levels were inversely correlated with the prevalence and severity of ECG-LVH (both \( P<0.05 \)). The lower plasma NOx levels were associated with significantly higher plasma 8-isoprostane levels, a marker of systemic lipid peroxidation (\( P<0.05 \)). These results provide the first evidence that normotensive subjects with ECG-LVH exhibit defective nitric oxide production, along with increased oxidative stress. Our findings may thus explain, at least in part, a potential mechanism underlying the increased risk of cardiovascular death in normotensive individuals with ECG-LVH. *(Hypertension. 2014;64:516-522.)* • Online Data Supplement

Key Words: electrocardiography ■ hypertrophy, left ventricular ■ nitric oxide ■ oxidative stress

Cardiovascular disease is the leading cause of death in many countries around the world. Previous studies have reported that, among patients with hypertension, left ventricular hypertrophy (LVH) is one of the most important risk factors for cardiovascular disease, including angina pectoris, myocardial infarction, heart failure, cerebrovascular accidents, and sudden death.1–3 LVH would reflect target organ damage caused by hypertension. Notably, although the sensitivity of electrocardiography for detecting anatomic LVH is controversial, recent studies have revealed that, even among normotensive subjects, electrocardiographically determined LVH (ECG-LVH) is a risk factor for cardiovascular death.4,5 A large cohort study reported that, in a total of 6688 healthy participants selected randomly from community residents who were free of previous cardiovascular disease and free of the use of antihypertensive agents, ECG-LVH predicted future cardiovascular death, irrespective of the presence or the absence of hypertension.6 In addition, another large cohort study indicated that, in a total of 10755 participants including those with a history of previous cardiovascular disease and those taking antihypertensive agents, ECG-LVH predicted future stroke in normotensive subjects.5 Thus, the presence of ECG-LVH serves as an independent predictor of cardiovascular death, irrespective of blood pressure (BP) levels. However, the underlying mechanisms for the increased risk of cardiovascular death observed in normotensive subjects with ECG-LVH remain to be elucidated.

Nitric oxide (NO) is synthesized by 3 distinct NO synthase (NOS) isoforms, including neuronal, inducible, and endothelial ones, maintaining the cardiovascular function and structure.6 We previously demonstrated that mice lacking all 3
NOS isoforms spontaneously develop multiple cardiovascular disorders, including LVH, heart and renal failure, metabolic syndrome, acute myocardial infarction, and sudden cardiac death.6–8 These results suggest that the NO/NOS system plays a critical role in the prevention of cardiovascular disease. In the clinical setting, NO production in the human body is evaluated by assessing the plasma levels of NOx (nitrite [NO₂⁻] plus nitrate [NO₃⁻]), stable metabolites of NO.9 Based on this background, in this study, we tested our hypothesis that normotensive individuals with ECG-LVH exhibit reduced NO production by measuring the plasma NOx levels in 579 normotensive Japanese male workers.

**Methods**

**Study Subjects**

See the online-only Data Supplement for details. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki and was approved by the committee of the Nuclear Science Research Institute, Tokai Research and Development Center, Japan Atomic Energy Agency.

**Definition of ECG-LVH**

See the online-only Data Supplement for details.

**Plasma NOx Levels**

See the online-only Data Supplement for details.

**Plasma Extracellular Superoxide Dismutase and 8-Isoprostane Levels**

See the online-only Data Supplement for details.

**Statistical Analysis**

See the online-only Data Supplement for details. The JMP Statistical Discovery Software package for Windows version 8 was used for the statistical analysis (SAS Institute Inc, Cary, NC). Values of P<0.05 were considered to be statistically significant. In this study, one-to-one matching between the subjects with and without ECG-LVH was also performed based on the estimated propensity scores of age, waist circumference, and systolic and diastolic BP in each subject using the STATA version 11.0 software program (STATA Corp, College Station, TX).

**Results**

**Plasma NOx Levels Were Markedly More Reduced in the Normotensive Subjects With ECG-LVH Than in Those Without**

A total of 840 male subjects were enrolled in this study, and 579 subjects were eligible in line with the exclusion criteria described in the Methods in the online-only Data Supplement.

**Table 1. Clinical Characteristics of Propensity Score–Matched Controls Without ECG-LVH and Subjects With ECG-LVH**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Subjects Without ECG-LVH (n=73)</th>
<th>Subjects With ECG-LVH (n=73)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>48 (43–54)</td>
<td>48 (43–54)</td>
<td>0.83</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>21.6 (20.6–23.9)</td>
<td>22.6 (20.9–24.2)</td>
<td>0.21</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>78.8 (74.1–84.1)</td>
<td>80.8 (74.2–84.7)</td>
<td>0.45</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>221 (196–240)</td>
<td>221 (196–245)</td>
<td>0.95</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>86 (63–136)</td>
<td>85 (65–107)</td>
<td>0.36</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>66 (55–80)</td>
<td>70 (59–85)</td>
<td>0.35</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>131 (111–153)</td>
<td>132 (110–155)</td>
<td>0.98</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>126 (120–132)</td>
<td>126 (122–130)</td>
<td>0.94</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>74 (70–80)</td>
<td>74 (71–78)</td>
<td>0.59</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>52 (47–56)</td>
<td>52 (48–56)</td>
<td>0.99</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>62 (56–69)</td>
<td>61 (54–69)</td>
<td>0.61</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>87 (83–93)</td>
<td>89 (84–92)</td>
<td>0.21</td>
</tr>
<tr>
<td>Fasting insulin, μIU/mL</td>
<td>3.2 (2.1–4.5)</td>
<td>3.0 (2.3–4.3)</td>
<td>0.67</td>
</tr>
<tr>
<td>eGFR, ml/min per 1.73 m²</td>
<td>76.7 (69.4–84.1)</td>
<td>77.3 (72.6–84.3)</td>
<td>0.44</td>
</tr>
<tr>
<td>Uric acid, mg/dL</td>
<td>5.6 (5.0–6.2)</td>
<td>5.4 (4.7–6.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>26 (35.6)</td>
<td>16 (21.9)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data are expressed as median with interquartile range or number (%). P value was evaluated by the Mann–Whitney test or the χ² test. ECG-LVH indicates electrocardiographically determined left ventricular hypertrophy; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.
Among them, 506 subjects (87.4%) did not exhibit ECG-LVH and 73 subjects (12.6%) manifested ECG-LVH (Figure 1). The prevalence of ECG-LVH diagnosed according to the Sokolow–Lyon voltage criteria and the Cornell voltage–duration product criteria was 11.9% (n=69) and 1.2% (n=7), respectively. No subjects showed marked ST-segment depression or prominent inverted T waves. The median level of plasma NOx (μmol/L) was significantly and markedly lower in the normotensive subjects with ECG-LVH (22.5 [IQR, 15.0–41.0]; n=73) than in those without (34.1 [IQR, 22.4–59.4]; n=506; P <0.05). This result did not change even when the patients with ECG-LVH by the Cornell voltage–duration product only (n=4) were excluded from the study. However, between the 2 groups, there were significant differences in the clinical characteristics, including body mass index, waist circumference, plasma levels of triglycerides, high-density lipoprotein cholesterol, insulin, and uric acid, and systolic and diastolic BP. The C-statistic for goodness of fit was 0.680 in the propensity score model. Using this analytical method, the significant differences in the clinical characteristics between the 2 groups disappeared, and all factors of the clinical characteristics became nearly identical between the matched normotensive subjects without ECG-LVH and those with ECG-LVH (Table 1). Notably, after the propensity score matching, the difference in the median level of plasma NOx (μmol/L) between the 2 subject groups increased (22.5 [IQR, 15.0–41.0] in the normotensive subjects with ECG-LVH [n=73] versus 39.0 [IQR, 22.4–59.4] in the matched normotensive subjects without ECG-LVH [n=73]; Figure 2), and a significant difference between the 2 groups was noted, as observed before the propensity score matching (P <0.05).

Even when BP is within the normal range, the presence of prehypertension (BP, 120–139/80–89 mm Hg) has been reported to increase the risk of cardiovascular disease.5 Therefore, we stratified the subjects according to prehypertension. Among the subjects with prehypertension, a significant and marked reduction in the median level of plasma NOx in the subjects with ECG-LVH was still noted compared with that observed in the matched subjects without ECG-LVH (P <0.05; Table 2). Because the presence of visceral obesity has also been shown to be a risk factor for cardiovascular disease, we then stratified the subjects according to a waist circumference of ≥85 cm. Among the subjects with a waist circumference of ≥85 cm, a marked but insignificant reduction in the median level of plasma NOx in the subjects with ECG-LVH was observed (Table 2).

### As the Plasma NOx Levels Decreased, the Prevalence of ECG-LVH Increased

We next examined the relationship between the tertile of the plasma NOx level and the prevalence of ECG-LVH. The ranges of the first (n=48), second (n=49), and third (n=49) tertiles of the plasma NOx level (μmol/L) were <21.2, 21.2 to 41.8, and >41.8, respectively. As the plasma NOx levels decreased, the prevalence of ECG-LVH significantly increased (P <0.05): the prevalence of ECG-LVH in the first, second, and third tertiles of the plasma NOx level was 68.8%, 49.0%, and 32.7%, respectively (Figure 3).

We further studied the odds ratio for the presence of ECG-LVH in the tertiles of the plasma NOx levels. The unadjusted logistic analysis indicated that the odds ratio for the presence

### Table 2. Plasma NOx Levels in Propensity Score–Matched Controls Without ECG-LVH and Subjects With ECG-LVH Stratified by Blood Pressure and Waist Circumference Levels

<table>
<thead>
<tr>
<th>Stratification</th>
<th>Subjects Without ECG-LVH</th>
<th>Subjects With ECG-LVH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>NOx, μmol/L</td>
</tr>
<tr>
<td>Blood pressure &lt;120/80 mm Hg</td>
<td>15</td>
<td>47.0 (25.1–68.3)</td>
</tr>
<tr>
<td>Blood pressure ≥120/80 mm Hg</td>
<td>58</td>
<td>38.7 (22.1–55.9)</td>
</tr>
<tr>
<td>Waist circumference &lt;85 cm</td>
<td>58</td>
<td>38.7 (22.6–61.0)</td>
</tr>
<tr>
<td>Waist circumference ≥85 cm</td>
<td>15</td>
<td>45.2 (22.0–52.4)</td>
</tr>
</tbody>
</table>

*All data are expressed as number or median with interquartile range. *P value was evaluated by the Mann–Whitney test. ECG-LVH indicates electrocardiographically determined left ventricular hypertrophy; NOx, nitrate plus nitrate. *P <0.05 vs matched subjects without ECG-LVH.
of ECG-LVH was 4.54 (95% confidence interval, 1.97–10.93) for the first tertile and 1.98 (95% confidence interval, 0.88–4.55) for the second tertile when compared with the third tertile (P<0.05; Figure 4A). In the 579 eligible subjects, a multivariate-adjusted logistic regression analysis adjusted for age, waist circumference, systolic and diastolic BP, and smoking status demonstrated that the odds ratio for the presence of ECG-LVH was 3.50 (95% confidence interval, 1.86–6.87; P<0.05) for the first tertile (<24.8 μmol/L; n=193) and 0.93 (95% confidence interval, 0.43–1.99; P=0.84) for the second tertile (24.8–44.9 μmol/L; n=193) when compared with the third tertile (>44.9 μmol/L; n=193; Figure 4B).

As the Plasma NOx Levels Decreased, the Severity of ECG-LVH Increased

We then explored the correlation between the tertile of the plasma NOx level and the extent of ECG-LVH. As the plasma NOx levels decreased, the severity of ECG-LVH evaluated according to the Sokolow–Lyon voltage significantly increased (P<0.05): the median values of the Sokolow–Lyon voltage (millimeters) in the first, second, and third tertiles of the plasma NOx level were 40 (IQR, 31–44), 36 (IQR, 28–42), and 30 (IQR, 23–41), respectively (Figure 5A). The extent of ECG-LVH assessed according to the Cornell voltage–duration product also tended to be inversely correlated with the plasma NOx level: the median values of the Cornell voltage–duration product (mm×ms) in the first, second, and third tertiles of the plasma NOx level were 1440 (IQR, 1040–1740), 1280 (IQR, 920–1600), and 1120 (IQR, 900–1440), respectively (P=0.06; Figure 5B).

**Plasma 8-Isoprostane Levels Were Higher in the Normotensive Subjects With ECG-LVH Than in Those Without**

It has been reported that NO deficiency is linked to oxidative stress and that oxidative stress participates in the pathogenesis of cardiovascular disease. Therefore, we studied the kinetics of the antioxidant and oxidant systems. There were no significant differences in the median level of plasma extra-cellular superoxide dismutase (ng/mL), a major cardiovascular antioxidant enzyme, between the matched normotensive subjects without ECG-LVH (48.6 [IQR, 43.2–55.4]) and those with ECG-LVH (50.3 [IQR, 42.2–57.7]). On the contrary, the median level of plasma 8-isoprostane (pg/mL), a marker of lipid peroxidation, was significantly and markedly higher in the normotensive subjects with ECG-LVH than in those without (45.4 [IQR, 18.2–72.9] versus 25.9 [IQR, 14.0–54.3]; P<0.05; Figure 6). As in the case of the Mann–Whitney test, the analysis of covariance with fasting glucose, waist circumference, and smoking status as covariates also showed that the median level of plasma 8-isoprostane was significantly higher in the subjects with ECG-LVH than in those without. There were no significant differences in the median number of leucocytes (per microliter) (5000 [IQR, 4400–5950] and 5300 [IQR, 4350–6450]), the median level of high-sensitive C-reactive protein (mg/L; 0.30 [IQR, 0.10–0.75] and 0.30 [IQR, 0.10–0.80]), or the median level of homeostasis model assessment of insulin resistance (0.70 [IQR, 0.47–0.95] and 0.64 [IQR, 0.50–0.97]) between the subjects with and without ECG-LVH.

Finally, we followed up cardiovascular death and newly diagnosed hypertension in the normotensive subjects with and without ECG-LVH. We were able to follow up 122 subjects of the 146 subjects (63 subjects without ECG-LVH and 59 with ECG-LVH). The follow-up time for the subjects was 45

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**Figure 3.** Association between the tertile of the plasma nitrate plus nitrate (NOx) level and the prevalence of electrocardiographically determined left ventricular hypertrophy (ECG-LVH). The ranges of the first (n=48), second (n=49), and third (n=49) tertiles of the plasma NOx level (μmol/L) were <21.2, 21.2 to 41.8, and >41.8, respectively. The P values were determined according to the χ² test.

**Figure 4.** A, Unadjusted odd ratios for the presence of electrocardiographically determined left ventricular hypertrophy (ECG-LVH) in each tertile of the plasma nitrate plus nitrate (NOx) level in the 146 subjects. The ranges of the first (n=48), second (n=49), and third (n=49) tertiles of the plasma NOx levels (μmol/L) were <21.2, 21.2 to 41.8, and >41.8, respectively. B, Multivariate-adjusted odd ratios for the presence of ECG-LVH in each tertile of the plasma NOx level in the 579 eligible subjects. The ranges of the first (n=193), second (n=193), and third (n=193) tertiles of the plasma NOx levels (μmol/L) were <24.8, 24.8 to 44.9, and >44.9, respectively. CI indicates confidence interval. *P<0.05 vs the third tertile (reference) according to a logistic regression analysis.
months. All the subjects were alive, and 6 subjects without ECG-LVH and 5 subjects with ECG-LVH were newly diagnosed with hypertension (defined as ≥140/90 mm Hg).

**Discussion**

This is the first study to examine NO production in normotensive subjects with and without ECG-LVH. The major novel findings of this study were as follows: (1) the plasma NOx levels were markedly more reduced in the normotensive men with ECG-LVH than in those without; (2) the plasma NOx levels were inversely associated with the prevalence and severity of ECG-LVH; and (3) the plasma 8-isoprostane levels were significantly higher in the normotensive men with ECG-LVH than in those without. These findings suggest that reduced NO production is present in normotensive individuals with ECG-LVH in association with increased oxidative stress.

**Plasma NOx Level Is a Useful Marker of Systemic NO Production**

The half-life of NO is short (3–6 seconds), and NO is quickly degraded to stable metabolites of NOx by an oxidative reaction. The usefulness of the plasma NOx levels as a marker of systemic NO production has been demonstrated by the following lines of evidence. First, the plasma NOx levels are elevated by treatment with l-arginine, a precursor of NO, in rats and by overexpression of the endothelial NOS gene in mice. Second, the plasma NOx levels are attenuated by treatment with a NOS inhibitor in rats. Finally, the plasma NOx levels are decreased in accordance with the number of disrupted NOS genes in the order of single, double, and triple NOS−/− mice, and the plasma NOx levels in triple NOS−/− mice are nearly zero (∼3% of those observed in wild-type mice).

**Propensity Score Analysis**

In this study, we first compared the plasma NOx levels in all eligible normotensive subjects with and without ECG-LVH. However, the clinical characteristics of the 2 subject groups differed significantly. Therefore, we then performed a one-to-one propensity score matching analysis. A propensity score analysis is a statistical technique used to minimize bias because of confounding factors that can be recognized in statistical outcomes obtained from simple comparisons of data between study groups. Propensity score analyses are widely used in clinical observational studies. By applying this analytical method, the clinical characteristics of the 2 groups were matched completely.

**Reduced Plasma NOx Levels in the Normotensive Subjects With ECG-LVH**

The plasma NOx levels were markedly lower in the normotensive subjects with ECG-LVH than in the propensity score–matched normotensive subjects without ECG-LVH. The important point to make here is that stratifying by the presence of prehypertension or obesity does not change the differences in NOx levels between the subjects with and without ECG-LVH. In the present study, reduced plasma NOx levels were linked to the prevalence and severity of ECG-LVH in the normotensive subjects. It has been reported that NO attenuates cardiac myocyte hypertrophy in response to the growth-promoting effect of norepinephrine in rats and that NO deficiency induces cardiac myocyte hypertrophy in mice. Thus, it is possible that the reduced plasma NOx levels may be a potential cause of the presence and severity of ECG-LVH in the normotensive subjects.
Recent studies have revealed that ECG-LVH is at a higher risk of cardiovascular death not only in hypertensive patients but also in normotensive subjects.\(^4^,\(^5\)\) However, the underlying mechanisms remain to be clarified. We previously demonstrated that mice in which all 3 NOS genes are completely disrupted exhibit LVH, heart and renal failure, metabolic syndrome, acute myocardial infarction, and sudden cardiac death.\(^6^,\(^7\)\) These findings indicate that NO deficiency causes spontaneous development of a variety of cardiovascular disorders. NO deficiency activates the renin–angiotensin system and induces myocyte hypertrophy, renal pathological remodeling, abnormalities of lipid and glucose metabolism, vasoconstriction, and arteriosclerosis/atherosclerosis via the angiotensin II type 1 receptor pathway.\(^6^,\(^8\)\) It is thus conceivable that reduced NO production is involved in the increased risk of cardiovascular death observed in normotensive subjects with ECG-LVH.

**Increased Plasma 8-Isoprostane Levels in the Normotensive Subjects With ECG-LVH**

We previously reported that genetic deletion of all NOS isoforms in mice leads to oxidative stress, as evidenced by increases in the plasma 8-isoprostane and malondialdehyde levels.\(^7\) Therefore, we examined the redox state of the subjects. Extracellular superoxide dismutase is predominantly expressed in vascular endothelial cells\(^20\) and plays a major role in the cardiovascular antioxidant system.\(^21\) The plasma extracellular superoxide dismutase protein levels did not differ between the matched normotensive subjects without ECG-LVH and those with ECG-LVH. On the contrary, a prominent increase in the plasma 8-isoprostane levels, a marker of systemic lipid peroxidation, was noted in the normotensive subjects with ECG-LVH compared with that observed in the matched normotensive subjects without ECG-LVH. Oxidative stress, as well as NO deficiency, contributes to the pathogenesis of cardiovascular disease.\(^10^–\(^12\)\) The development of vascular diseases is initiated by endothelial cell dysfunction leading to expression of adhesion molecules for inflammatory cells. An oxidizing environment caused by the migration of inflammatory cells and vascular inflammation is critical for the progression of vascular diseases. The accumulating inflammatory cells produce abundant reactive oxygen species (ie, H\(_2\)O\(_2\), O\(_2^=\), and OH) and secrete inflammatory cytokines/chemokines and growth factors that contribute to endothelial cell dysfunction and vascular smooth muscle cell proliferation. It is thus possible that increased oxidative stress is also involved in the increased risk of cardiovascular death observed in normotensive subjects with ECG-LVH. Angiotensin II type 1 receptor blockers, angiotensin-converting enzyme inhibitors, and statins have an antioxidative effect. Although we excluded the patients taking those drugs from the present study, such patients might show less ECG-LVH. While NO deficiency leads to oxidative stress, oxidative stress (ie, superoxide) conversely diminishes the NO levels. It is unclear whether oxidative stress is the result or the cause of NO deficiency in normotensive subjects with ECG-LVH.

**Study Limitations**

There are several limitations linked to this study. First, with regard to the sensitivity of detecting LVH, ECG is inferior to echocardiography.\(^22\) However, compared with echocardiography, ECG is more low-cost, available, and reproducible with high specificity and is widely used in screening for LVH.\(^23\) Therefore, we used ECG to diagnose LVH in this study. Second, it remains to be clarified whether reduced levels of plasma NOx as well as increased levels of plasma 8-isoprostane predict future cardiovascular events in this study population. Finally, in this study, we studied normotensive middle-aged male Japanese workers who were not taking medications for hypertension, hypercholesterolemia, type 2 diabetes mellitus, or cardiovascular disease. It remains to be elucidated whether the outcomes of this study are relevant to the general population.

**Perspectives**

In summary, we were able to demonstrate that the plasma NOx levels are markedly lower in normotensive men with ECG-LVH, associated with higher plasma 8-isoprostane levels. These results provide the first evidence that normotensive individuals with ECG-LVH exhibit defective NO production, along with increased oxidative stress. Our findings may thus explain, at least in part, a potential mechanism underlying the increased risk of cardiovascular death and the increased presence and severity of ECG-LVH in normotensive individuals with ECG-LVH. In addition, our findings may support the applicability of ECG-LVH in diagnosing and treating cardiovascular disease. It remains unknown whether correcting abnormal plasma NOx or 8-isoprostane levels with aggressive management (eg, lifestyle changes such as smoking cessation, increased physical activity, and dietary modification, and pharmacological intervention) prevents future cardiovascular deaths in normotensive subjects with ECG-LVH. In addition to the regulatory roles in signaling molecules, increasing lines of evidence suggest that the different genetic stability and cross-talk regulation among the epigenetic factors, such as DNA methylation, histone post-translational modifications, and microRNA alterations, may be important to the development of LVH. The cross-talk interactions between NO/oxidative stress and the development of LVH remain to be determined. Further studies are needed to clarify these points.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- This study provides the first evidence that plasma levels of nitric oxide metabolites were markedly more reduced in normotensive men with electrocardiographically determined left ventricular hypertrophy, associated with higher plasma 8-isoprostane levels.

**What Is Relevant?**

- Our findings may explain, at least in part, a potential mechanism underlying the increased risk of cardiovascular death observed in normotensive individuals with electrocardiographically determined left ventricular hypertrophy.
Plasma Levels of Nitric Oxide Metabolites Are Markedly Reduced in Normotensive Men With Electrocardiographically Determined Left Ventricular Hypertrophy

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Short title: NO Deficiency in Normotensives with ECG-LVH

Number: Tables 1, References 3

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Expanded Methods

Study Subjects

Nine-hundred and sixty-nine male workers (40-65 years of age) underwent health checkups in June 2010. Among them, 840 subjects (86.7%) provided their written informed consent to participate in this study. We excluded subjects with hypertension (≥140/90 mmHg), medications for hypertension, hypercholesterolemia, type 2 diabetes, or cardiovascular disease because such factors can affect the plasma NOx levels. We also excluded subjects with complete bundle branch block or heart rhythms other than a sinus rhythm. A total of 579 subjects were ultimately included in the study. At the health checkup, demographic data and health information were collected using self-administered questionnaires. Smoking habits were classified as either current or not. All participants were subjected to a physical examination to assess height, weight, waist circumference, and blood pressure (BP). Waist circumference was measured at the umbilicus using flexible anthropometric tape. BP was evaluated by trained technicians using a standard mercury sphygmomanometer after resting for ≥5 minutes in a sitting position. Pulse pressure was calculated as systolic BP minus diastolic BP. Venous blood samples were collected from each subject after fasting overnight (>12 hours). Serum levels of triglycerides (enzymatic method; Determiner C-TG, Kyowa Medex Co., Ltd., Tokyo, Japan) and HDL cholesterol (direct method; Determiner L HDL-C, Kyowa Medex Co., Ltd., Tokyo, Japan) and plasma levels of glucose (hexokinase method; Quick auto II GLU-HK, Shino-Test Co., Kanagawa, Japan) were measured using automated measurement devices. Plasma levels of insulin were measured using chemiluminescent immunoassay (ARCITECT Insulin, Abbott Japan Co., Ltd., Tokyo, Japan). Homeostasis model assessment of insulin resistance and estimated glomerular filtration rate were calculated using the following equations: (fasting insulin [μIU/mL] × fasting glucose [mg/dL])/405 and 194 × [creatinine (mg/dL)]^{1.094} × [age (years)]^{0.287}.

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki and was approved by the committee of the Nuclear Science Research Institute, Tokai Research and Development Center, Japan Atomic Energy Agency.

Definition of Electrocardiographically Determined Left Ventricular Hypertrophy

Standard 12-lead ECGs were recorded at a paper speed of 25 mm/s and 1 mV/cm of standardization. The QRS duration and voltage of R waves in leads I, II, III, aV_L (RaV_L), aV_F, V_5 (RV_5), and V_6 (RV_6) and the voltage of S waves in leads V_1 (SV_1) and V_3 (SV_3) were measured manually by two experienced cardiologists who were blinded to the information of the health checkup. Left ventricular hypertrophy was defined according to the following two criteria with reference to previous studies. a Sokolow-Lyon voltage (SV_1+RV_5 or RV_6) >38 mm and a Cornell voltage-duration product (or Cornell voltage) (RaV_L+SV_3)×QRS duration >2,440 mm×ms. Subjects meeting either or both of these criteria were considered to have electrocardiographically determined left ventricular hypertrophy.

Plasma NOx Levels

To minimize the influence of foods and beverages on the plasma NOx concentrations, we instructed the subjects to fast overnight (>12 hours) and collected venous blood in the morning under fasting conditions in all subjects studied. Vacuum tubes with sodium EDTA
were used to obtain the plasma. The blood samples were centrifuged at 3,000 rpm at 4°C for 15 minutes, and the supernatants were stored at –80°C until measurement. The plasma NOx levels were assessed by the Griess method, as we previously reported.¹

**Plasma Extracellular Superoxide Dismutase and 8-Isoprostane Levels**
The plasma levels of extracellular superoxide dismutase were measured using a two-step enzyme-linked immunosorbent (ELISA) assay, as we previously reported.³ The lower limit of detection was 50 pg/mL and the working range was up to 50 ng/mL. This ELISA system showed no cross-reactivity with other SOD isoforms. The plasma levels of 8-isoprostane were analyzed with an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

**Statistical Analysis**
Continuous data are expressed as the median with interquartile range because the values were not normally distributed. Categorical data are presented as absolute values and percentages. Continuous variables were compared using the Mann-Whitney or Kruskal-Wallis test, and categorical variables were compared using the chi-square test. The subjects were categorized according to the tertile of the plasma NOx level, and a multivariate logistic regression analysis was conducted in an analysis of the prevalence of electrocardiographically determined left ventricular hypertrophy (ECG-LVH) in each NOx tertile after adjusting for age, waist circumference, systolic and diastolic BP and smoking status. Values of \( P < 0.05 \) were considered to be statistically significant. The JMP Statistical Discovery Software package for Windows version 8 was used for the statistical analysis (SAS Institute Inc., Cary, NC, USA).

Various clinical factors, including age, obesity, hyperlipidemia, elevated BP and insulin resistance, can influence the plasma NOx levels. Therefore, in order to match these factors in the subject groups, we performed a propensity score analysis. One-to-one matching between the subjects with and without ECG-LVH was carried out based on the estimated propensity scores of age, waist circumference, and systolic and diastolic BP in each subject using the STATA version 11.0 software program (STATA Corp., College Station, TX, USA). The C-statistic for evaluating the goodness of fit was calculated.
References


<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Subjects Without ECG-LVH (n=506)</th>
<th>Subjects With ECG-LVH (n=73)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>48 (44-54)</td>
<td>48 (43-54)</td>
<td>0.72</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.3 (21.6-24.9)</td>
<td>22.6 (20.9-24.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>82.7 (78.4-87.5)</td>
<td>80.8 (74.2-84.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>216 (194-237)</td>
<td>221 (196-245)</td>
<td>0.40</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>96 (72-144)</td>
<td>85 (65-107)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>62 (53-75)</td>
<td>70 (59-85)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>130 (112-152)</td>
<td>132 (110-155)</td>
<td>0.86</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>122 (118-128)</td>
<td>126 (122-130)</td>
<td>&lt;0.05</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>74 (68-76)</td>
<td>74 (71-78)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>50 (46-54)</td>
<td>52 (48-56)</td>
<td>0.13</td>
</tr>
<tr>
<td>Heart rate (beats/minute)</td>
<td>63 (57-70)</td>
<td>61 (54-69)</td>
<td>0.22</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>88 (84-93)</td>
<td>89 (84-92)</td>
<td>0.79</td>
</tr>
<tr>
<td>Fasting insulin (µIU/mL)</td>
<td>3.6 (2.6-5.0)</td>
<td>3.0 (2.3-4.3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>76.0 (67.9-84.1)</td>
<td>77.3 (72.6-84.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.7 (5.0-6.2)</td>
<td>5.4 (4.7-6.1)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>129 (25.5)</td>
<td>16 (21.9)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Data are expressed as median with interquartile range (IQR), or number (%). ECG-LVH, electrocardiographically determined left ventricular hypertrophy; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein. P value was evaluated by the Mann-Whitney test or the chi-square test.