Genomic Association/Linkage of Sodium Lithium Countertransport in CEPH Pedigrees

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Abstract—Little is known about genetic determinants explaining variation in the erythocyte sodium-lithium countertransport (SLC), an intermediate phenotype of essential hypertension. We characterized the SLC in immortalized lymphoblasts and showed that its behavior is similar to that of erythrocyte SLC. We then performed association and linkage analyses of the SLC in immortalized lymphoblasts from 5 large pedigrees from the Center d’Etude du Polymorphisme Humain (CEPH) genomics repository. The results of these analyses showed that a number of genomic regions harboring genes involved in glutathione metabolism might explain variations in SLC activity. These findings support evidence that thiol groups play a central role in SLC activity. (Hypertension. 2002;40:619-628.)

Key Words: oxidative stress ■ ion transport ■ lymphocytes ■ glutathione ■ intermediate phenotype

The sodium-lithium countertransport (SLC) is an electroneutral transport system that exchanges Na⁺ (or Li⁺) for Li⁺ (or Na⁺) in the presence of ouabain, amiloride, and furosemide (bumetanide). The activity of SLC is modified by thiol alkylating agents, such as maleimide, which increases the affinity of the SLC to Li⁺. Although the kinetics of the SLC are well characterized, the nature of the transporter and the genes contributing to its expression are unknown. Evidently, functional properties of the SLC and its gene(s) are different from those of the ubiquitous Na⁺/H⁺-antitrop (NHE-1). Thus, a search for genes that explain variation in SLC activity is in order. The discovery of these genes may have clinical relevance because the SLC is one of the most studied intermediate phenotypes of essential hypertension. The National Library of Medicine lists >500 peer-reviewed papers that have explored the epidemiology and physiology of the SLC in the context of essential hypertension and related cardiovascular diseases. The activity of the SLC, as expressed in erythrocytes in vivo, has been shown to correlate with a host of cardiovascular risk factors that relate to essential hypertension, including insulin resistance and dyslipidemia. In addition, the activity of the SLC is lower in blacks than in whites (for review, see Aviv and Lasker ) and is lower in white women than in white men. Importantly, the activity of the SLC reflects both genetic and environmental factors.

In the present study, we describe a novel strategy for dissecting the genetic basis of the SLC by shielding it from influences owing to different environmental conditions that exist in vivo, and by taking advantage of the vast amount of publicly available genotype data. We accomplished this by studying the SLC in Epstein-Barr virus (EBV)-immortalized lymphoblasts of large reference Center d’Etude du Polymorphisme Humain (CEPH) pedigrees. The CEPH collection provides a considerable advantage in that extensive genotype data are already available for some of the pedigrees in the CEPH repository (http://landru.cepheh.fr) and the companion Coriell Cell Repositories (http://locus.umdnj.edu/nigms/ceph/ceph.html). By screening cell lines from CEPH pedigrees for variations in the SLC, we were able to rapidly use this enormous amount of genotype data.

Materials and Methods

Accessing the CEPH Pedigrees

Lymphoblasts from the members of 5 CEPH pedigrees were obtained from the CEPH and the Coriell Cell Repositories. These repositories contain information on >12,000 DNA microsatellite, simple tandem repeat (STR), and other markers typed on large pedigrees. We therefore obtained genotype information on the members of 5 CEPH pedigrees who have been typed for 7746 autosomal markers, placed on a map developed by Broman et al. Culture of EBV-Transformed Lymphoblasts

Lymphoblast cell lines from the 5 CEPH pedigrees were cultured under identical conditions in RPMI 1640 medium containing 2 mmol/L L- glutamine, 100 U/mL penicillin, 100 μg/L streptomycin, and 15% heat-inactivated fetal bovine serum (FBS, Irvine Scientific). Cells used for experiments were derived from fresh cultures thawed...
from frozen stocks. Cells were passaged 2 or 3 times per week, and cell density was maintained between $0.5 \times 10^6$ and $1 \times 10^6$ cells/mL. Cell numbers were determined with a Coulter Counter ZM. Cells were routinely monitored for mycoplasma contamination with the Mycotrim Triphasic Culture System (Irvine Scientific). In preparation for experiments, $2 \times 10^7$ to $5 \times 10^7$ cells ($2.0 \times 10^6 /mL$) were suspended for 24 hours in fresh culture medium containing 0.5% FBS.

**Measurements of SLC Activity**

Lymphoblasts ($5 \times 10^7$ cells) were centrifuged at 250g for 10 minutes and resuspended in 15 mL of room-temperature wash solution (WS) comprised of (in mmol/L) $75 \text{MgCl}_2$, 95 sucrose, 10 MOPS/Tris (pH 7.4), and 0.1 ouabain. The cell pellet was gently overlaid with WS and decanted, and cells were resuspended for 3 hours at 37°C with LiCl loading solution (LS) comprised of 150 mmol/L LiCl.
10 mmol/L MOPS/Tris (pH 7.4), and 10 mmol/L glucose. The pellet was washed 1 additional time in ice-cold WS, and cells were aliquotted to 0.5 mL microfuge tubes (2×10⁶ to 4×10⁸/100 μL) for measurements of Li⁺ efflux and cell number. Li⁺ efflux was measured in cells suspended for 0 to 15 minutes in efflux solution (ES) at 37°C containing (in mmol/ L) 10 MOPS/Tris (pH 7.4), 10 glucose, 0.1 ouabain, and either 150 NaCl (Na⁺-containing ES) or 75 Mgl₂ and 75 sucrose (Na⁺-free ES). At different time intervals, cells were rapidly pelleted in a microfuge, and the supernatant was collected.

Li⁺ was determined in diluted supernatant fractions with a Perkin-Elmer atomic absorption spectrophotometer using Perkin-Elmer standards for Li⁺ (made up in Na⁺-free or Na⁺-containing ES). Under the above conditions, Li⁺ efflux was apparently linear in both ESs for at least 15 minutes. We note that the Na⁺-dependent Li⁺ efflux was not affected by 1 mmol/L amiloride or 10 μmol/L bumetanide (data not shown). The slope of Li⁺ efflux in Na⁺-free ES was subtracted from that in Na⁺-containing ES; Li⁺ efflux was expressed in nmol Li⁺/L×(10⁶ cells×min)⁻¹ (see Figure 2 for an example). SLC phenotype measurements were performed in duplicate on 3 separate occasions separated by 10- to 14-day intervals. In addition, SLC activity was measured in separate sets of experiments examining thiol group alkylation with N-ethylmaleimide (NEM). This agent was added for the last 15 minutes of the LiCl loading period. All chemicals were from Sigma.

Statistical Methods

Two analytic strategies relating variation at the 7746 marker loci to the SLC phenotype were pursued. The first analytic strategy involved testing each marker individually for association with SLC by use of the quantitative transmission-disequilibrium test (TDT) outlined by Xiong et al. Both the allele-specific and the omnibus, or overall-locus effect, tests were pursued. These statistics follow a χ² distribution asymptotically, with degrees of freedom given by the number of alleles tested minus 1. To make the statistics comparable, given that the loci varied considerably with respect to the number of alleles that could be tested, we standardized each test statistic by subtracting the degrees of freedom and dividing by the square root of twice the degrees of freedom. The probabilistic or statistical significance of resulting test statistics was assessed via genome-wide and chromosome-specific permutation tests.

To implement these tests, SLC values were randomly assigned to the CEPH pedigree members, and marker-specific tests were recomputed. Test statistics were then tallied and used to estimate genome-wide and chromosome-specific test-statistic distributions under the null hypothesis of no marker-SLC association. Chromosome-specific critical values were estimated in addition to genome-wide critical values, because (1) there was an unequal number of markers on each chromosome; (2) nonuniform distances exist between the markers in the map; (3) genomic regions display wide variation in disequilibrium strength; and (4) a recent study by Huttley et al suggested that relatively large groups of STRs exhibit long-range linkage disequilibrium in certain parts of the genome. These facts suggest that the correlations between test statistics may be radically different for different chromosomes and, hence, require different critical values for interpreting the statistical significance of associations involving loci on them. These distributions were then used to assess the probability of obtaining test statistics as large as those observed with the actual data.

The second analytic strategy involved multipoint linkage analyses. We performed these analyses using the SIBPAL module in the SAGE computer package. Statistical significance of linkage was determined using the W2 test statistic and the criteria for linkage established by Lander and Kruglyak, in which scores with P=2.2×10⁻³ were used as the minimum threshold values for a significant linkage.

Results

A summary of activities of the SLC in EBV-immortalized lymphoblasts from the members of 5 chosen CEPH families

![Figure 2](http://hyper.ahajournals.org/10.1161/hr9273)

**Figure 2.** The effect of NEM on SLC in lymphoblasts. A, Li⁺ efflux under basal conditions without NEM. B, Li⁺ efflux in Na⁺-containing ES; C, Li⁺ efflux in Na⁺-free ES. B, Li⁺ efflux after treatment with 1 mmol/L NEM for 15 minutes. C, Summary of the rates of Na⁺-dependent Li⁺ efflux of 5 experiments without (control) or with NEM on SLC activity. Rates of Na⁺-dependent Li⁺ efflux were obtained by subtracting the slope of Li⁺ efflux in Na⁺-free ES from that of Na⁺-containing ES. Differences between control (2.03±0.43 nmol Li⁺/L×(10⁶ cells×min)⁻¹) and NEM (5.11±0.82 nmol Li⁺/L×(10⁶ cells×min)⁻¹) are significant at P=0.009.
is presented in Figure 1. The relatively narrow SEs of 3 measurements, performed at 10- to 14-day intervals, indicate that the SLC activity is stably expressed in these cells. We also found that NEM considerably enhanced SLC activity (Figure 2). Based on studies in cultured skin fibroblasts, we note the Li loading in 150 mmol/L LiCl was probably insufficient to raise the internal Li concentration to saturate the internal sites. The effect of NEM on SLC activity was therefore most likely exerted through reduction in the Michaelis-Menten constant (Km) (ie, increased affinity to Li).

Figure 3 depicts standardized TDT statistic values for associations (solid red line, left axis) and linkage (dashed black line, right axis) with SLC per locus location for 22 chromosomes. For the association analysis, standardized test statistic values of 2.95 and 6.29 were estimated from permutation studies to represent genome-wide critical values for type 1 error rates of 0.05 and 0.01, respectively; chromosome-specific critical values at 0.01 significance are indicated by the blue dashed lines (Figure 3). With these critical values in mind, the plots in Figure 3 suggest that a number of genomic regions are likely to harbor alleles that influence SLC activity.

It is noteworthy that for the association analysis, there is considerable build-up and decay in test-statistic values sur-
rounding markers producing the largest test statistic values. This is undoubtedly owing to the ability of the TDT construction to detect linkage, linkage disequilibrium, and direct association effects, even in the face of overt genetic stratification between the families studied.26 The results of the multipoint linkage analysis supports this claim, because the "contour" of the test statistics from the linkage results generally follows the contour of the quantitative TDT test. One notable exception occurs on chromosome 16, in which a statistically significant linkage was observed, although the TDT analysis did not reveal association. This result is probably owing to the unique properties of multipoint analysis, through which linkage from marker information is derived over an entire genomic region. Given an insufficient density of markers in a region for linkage disequilibrium–induced associations to emerge, traditional multipoint analysis can detect linkages in those regions that omnibus TDT association tests cannot.

Table 1 provides an overview of the results, listing the number of markers studied. The markers show the greatest evidence for association with the SLC, and chromosome-specific 1% critical values derived from chromosome-specific permutation studies. For chromosomes with no markers producing a test statistic significant at the genome-wide 1% level, the marker producing the largest test statistic is listed.

In addition to computing overall or omnibus tests of associations for all loci, we tested each allele at each locus individually. 

### Table 1. Chromosome-Specific Markers Showing the Greatest Evidence of Association With SLC

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Markers, n*</th>
<th>Peak Marker(s)†</th>
<th>Location, cM</th>
<th>Test Statistic</th>
<th>CSCV</th>
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<td>D22S927</td>
<td>48.193</td>
<td>12.037</td>
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*No. of markers studied from the map developed by Broman et al.17 †Peak Markers: STRs with test statistics greater than the chromosome-specific critical value (CSCV), assuming a type 1 error rate of 0.05 (see text). Genome-wide critical values are 2.95 and 6.19 for type 1 error rates of 0.05 and 0.01, respectively (see text).
locus). The upper panel of Figure 4 displays a plot of the test statistics for the allele giving the greatest evidence for association for each locus against locus location on chromosome 14. The lower panel of Figure 4 displays a plot of the fraction of informative meioses for each locus against locus location for chromosome 14. Similar plots were made for the other chromosomes (data not shown). Chromosome 14 was randomly chosen to showcase the results.

To further assess the statistical significance of the test statistic results, we pursued the graphical device described by Schweder and Spjotvoll. Figure 5 provides a plot of the observed probability values for the standardized omnibus statistic subtracted from 1 (i.e., 1 − p) against the number of the 1−probability values less than that value. If all null hypotheses are true (i.e., each locus does not harbor alleles associated with SLC values), this plot should reveal a straight line. The inflection of the curve at 1−probability values near 1.0 in Figure 5 suggests that some null hypotheses are not true, and there is therefore statistical evidence for association of alleles at these loci with SLC values. The length of the small line segment jutting away from the otherwise straight diagonal line is an estimate of the fraction of null hypotheses that should be rejected. Thus, based on Figure 5, ≈10% of the test statistics should lead to a rejection of the null hypothesis. This relatively large percentage of null hypotheses that should be rejected on a purely statistical basis probably reflects linkage and linkage disequilibrium effects of test statistics computed

Figure 4. Top, A plot of the evidence for association with SLC per locus location for the allele showing the most evidence for association at each locus. Bottom, A plot of the fraction of allele transmissions from parents to offspring that were informative for the transmission-disequilibrium tests for each locus.

Figure 5. A plot of the observed probability values subtracted from 1 (i.e., 1 − p) for each locus against the number of test statistics producing 1−probability values less than a specified 1−probability value. This type of plot was introduced by Schweder and Spjotvoll (see text).
at loci in the vicinity of loci exhibiting the largest test statistic values.

As a check on how compelling the associations are for those loci producing very large TDT values, we examined the actual SLC activity values of individuals transmitted and not transmitted particular alleles at different loci. As examples, Figure 6 plots SLC activity values for those transmitted a particular allele at marker locus D12S165 (top panel) and at STR locus D6S436 (bottom panel). The plots were created such that the order of the values in the T and U columns correspond to the values of the members of the 5 CEPH families. Thus, the values in first column under ‘T’ correspond to SLC values for individuals transmitted the allele in family 1, and the values in the first column under ‘U’ correspond to SLC values for individuals not transmitted the allele in family 1. Note that the fourth family was not informative for locus D6S436, and hence, no values are shown.

![Figure 6](http://hyper.ahajournals.org/file/6251201365.png)

Figure 6. Scatter plots of the SLC measures for individuals transmitted (T) and not transmitted (U) a specific allele at STR locus D12S165 (top) and at STR locus D6S436 (bottom). The plots were created such that the order of the values in the T and U columns correspond to the values of the members of the 5 CEPH families. Thus, the values in first column under ‘T’ correspond to SLC values for individuals transmitted the allele in family 1, and the values in the first column under ‘U’ correspond to SLC values for individuals not transmitted the allele in family 1. Note that the fourth family was not informative for locus D6S436, and hence, no values are shown.

The results of the association and linkage analyses may provide mechanistic explanation for variation in SLC among human beings. Thiols group(s) regulate the activity of the SLC by lowering the Km for Li⁺. Indeed, NEM increased the affinity of the transporter to Li⁺, ie, increasing the affinity of the transporter to Li⁺. Increased activities of the transporter were observed in lymphoblasts from CEPH pedigrees, pointing to loci that harbor genes involved in thiol metabolism, may be relevant in that it suggests a genetic basis for variation in SLC activity among individuals. In addition, the linkage analysis identified a region of chromosome 16 that harbors a gene encoding a protein similar to a protein similar to skeletal-type tropomyosin. Recently, Watkins et al proposed that a tropomyosin isoform in troponyosin isoform in erythrocytes is responsible for mediating abnormal thiol reactivity of SLC in a subset of patients with a family history of essential hypertension.

It must be noted that pedigree 102 is of Venezuelan origin, in contrast to the Utah origin of the other 4 pedigrees. It is possible that genetic determinants of the SLC may differ across racial groups—a phenomenon that may confound the observations made in the present study. One must also carefully consider the matters of the effect of immortalization on SLC parameters and the considerable number of significant associations with the SLC that were identified in our study. Immortalization and the in vitro state may modify characteristics of the SLC. The insensitivity of the Na⁺-...

Table 2 offers a list of candidate genes near the markers showing the greatest evidence of association with the SLC using public domain databases and resources (ie, National Center for Biotechnology Information [NCBI] Web pages, http://www.ncbi.nlm.nih.gov). The results reveal loci associated with the SLC near STRs on different chromosomes that contain genes engaged in glutathione metabolism. These in-

include γ-Glu-Cys synthetase, several forms of glutathione S-tranferases (GSTs), and thioreductase-dependent peroxide reductase. Other regions near STRs showing evidence for association include a region of chromosome 4 that is syntenic with a region on baboon chromosome 5, demonstrating strong linkage with the SLC in the baboon, and the angiotensinogen locus. For angiotensinogen, the chromosome 1 location (ie, 2.55 cM from marker D1S1670) corresponds to a peak association test statistic of 54.7 at location 245.05 cM. In addition, associations with the SLC were observed for loci containing the regulatory unit of protein phosphatase 2, protein tyrosine kinase, calmodulin, and 3-phosphoinositide-dependent protein kinase (data not shown).

For the region on chromosome 16, showing a significant linkage without a pronounced association signal, there exist other candidate genes. These include cholesterol ester transfer protein, solute carrier family 12 Na/Cl transporter (SLC12A3), and a protein similar to cytoskeletal-type tropomyosin (TM30-NM; LOC 93404). In addition, a linkage study using systolic blood pressure as a phenotype for low concordant, hypertensive sib pairs identified a region on chromosome 16 (D16S3936) having a LOD-score >2.0. This region surrounds the marker showing peak linkage (marker GATA22F09) at 71.77 cM.

Discussion

The results of the association and linkage analyses may provide mechanistic explanation for variation in SLC among human beings. Thiols group(s) regulate the activity of the SLC by lowering the Km for Li⁺. Indeed, NEM increased the activity of the SLC in EBV-immortalized lymphoblasts. Because we did not perform kinetic analysis, we are not certain whether this effect was mediated via lowering the Km or raising the Vmax. We doubt, however, that our lithium loading procedure was sufficient to saturate the internal sites of the transporter. The association analysis in lymphoblasts from CEPH pedigrees, pointing to loci that harbor genes involved in thiol metabolism, may be relevant in that it suggests a genetic basis for variation in SLC activity among individuals. In addition, the linkage analysis identified a region of chromosome 16 that harbors a gene encoding a protein similar to skeletal-type tropomyosin. Recently, Watkins et al proposed that a tropomyosin isoform in erythrocytes is responsible for mediating abnormal thiol reactivity of SLC in a subset of patients with a family history of essential hypertension.

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dependent Li⁺ transport in immortalized lymphoblasts to ouabain, amiloride, and bumetanide and its modification by NEM suggest, however, that mechanistically, the SLC in these cells is similar to the SLC in erythrocytes. Because immortalization and growth conditions were the same for all the lymphoblastic cell lines we examined, variations in SLC activity among the cell lines could only arise from genetic factors intrinsic to these cells. With regard to these genetic factors, the SLC has been characterized as a phenotype of essential hypertension—a complex genetic trait. It is only reasonable to assume therefore that multiple genetic determinants are involved in the regulation of the SLC. The relatively large number of association of autosomal markers with the SLC may reflect this premise and the fact that our analysis has used an enormous number of these markers. No doubt, a subset of these associations may be artifactual, but one cannot ignore the fact that some of these associations point to loci on different chromosomes harboring genes that show common mechanisms involved not only in the biology of the SLC but also in the pathobiology of essential hypertension.

**Perspectives**

Enzymes involved in the metabolism of glutathione are important for the defense against oxidative stress and contribute to maintenance of cellular redox state. Variations in the activities of these enzymes may thus explain the sexual dimorphisms and race-related differences in the activity of the erythrocyte SLC. The lower SLC activity in erythrocytes in women than in men and in blacks than in whites may be explained by gender- and race-related levels of oxidative stress in these cells, as plasma hydrogen peroxide production is lower in women than in men and in blacks than in whites. Recent studies suggest that reactive oxygen species are centrally involved in the pathophysiology of hypertension in laboratory animals and in human beings. Of interest are findings that glutathione levels are reduced in vascular smooth muscle cells of spontaneously hypertensive rats and that glutathione deple-

### TABLE 2. Candidate Genes and Representative Markers Showing Greatest Evidence of Association With SLC Activity

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<thead>
<tr>
<th>Chromosome/Candidate Gene</th>
<th>Symbol</th>
<th>Marker*</th>
<th>Location, cM</th>
<th>Distance, cM†</th>
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<td>Angiotensinogen AGT</td>
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<td>D4S400‡</td>
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<td>Glutathione S-transferase A4 GSTA4</td>
<td>SGC32108</td>
<td>73.9–77.6</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase A2 GSTA2</td>
<td>SIS6914</td>
<td>77.6–78.2</td>
<td>4.47</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase A3 GSTA3</td>
<td>Sts-T98291</td>
<td>78.2–78.8</td>
<td>5.07</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase A4 GSTA4</td>
<td>SIE132039</td>
<td>78.2–78.8</td>
<td>5.07</td>
</tr>
<tr>
<td>14</td>
<td>Glutathione S-transferase zeta 1 GSTZ1</td>
<td>Bda66e04</td>
<td>75.3–76.4</td>
<td>7.76</td>
</tr>
<tr>
<td>22a</td>
<td>Glutathione S-transferase theta 1 GSTT1</td>
<td>Sts-R08187‖</td>
<td>16.7–22.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase theta 2 GSTT2</td>
<td>Sts-R08187</td>
<td>16.7–22.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Thioreductase-dep. peroxide reductase SP-22</td>
<td>A002022</td>
<td>33.4–39.5</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SGC31879</td>
<td>31.0–33.4</td>
</tr>
</tbody>
</table>

Chromosomes are for the candidate gene and corresponding gene symbol. The chromosome 6 locations correspond to a peak association test statistic of 15.7 at location 73.13 cM; the chromosome 14 locations correspond to a peak of 17.3 at 84.16 cM; the chromosome 22a locations correspond to a peak of 10.67 at 18.1 cM; and the chromosome 22b locations correspond to a peak of 9.70 at 41.42 cM.

*GB4 database designation for representative marker (note that some candidate genes have multiple markers, which are not shown).

†Distance of the candidate gene marker to peak marker found in the association analysis.

‡The linkage region obtained by Kammerer et al for baboon chromosome 5 is bounded by D4S414 and D4S413 and contains markers D4S2365 and D4S400. Their location on human chromosome 4 is given, and the distances to the markers showing a peak association TDT values are indicated.

‖GSTT1 and GSTT2 genes are localized to 22q11.2 and are separated by ~50 kb. Because GSTT2 is designated by the Sts-R08187 marker, Sts-R08187 is considered an approximate designation for GSTT1.
tion increases oxidative stress and causes severe hypertension in normal rats. Moreover, low serum levels of glutathione entail an increase in coronary heart disease risk in humans. Thus, association and linkage findings of SLC in immortalized lymphoblasts with genes engaged in glutathione metabolism may not be incidental, because SLC activity in erythrocytes correlates with a host of risk factors for coronary heart diseases that include not only essential hypertension but also insulin resistance and dyslipidemia. Further, a recent study has found an increase in reactive oxygen species production in immortalized lymphoblasts from patients with essential hypertension, in line with our observations.

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